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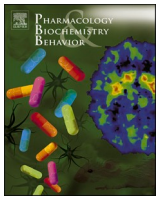

Acute stress induces different changes on the expression of CB1 receptors in the hippocampus of two lines of male rats differing in their response to stressors

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ABSTRACT

The stress-induced alterations in cognitive processes and psychiatric disorders can be accelerated when acute stressors challenge the hippocampal functions. To address this issue, we used Western Blot (WB) and immunohistochemistry assays to investigate the impact of acute forced swimming (FS) on the expression of the CB1 cannabinoid receptors (CB1R) in the hippocampus (HC) of the male outbred Roman High- (RHA) and Low-Avoidance (RLA) rat lines, one of the most validated genetic models for the study of behavior related to fear/anxiety and stress-induced depression.

The distinct responses to FS confirmed the different behavioral strategies displayed by the two phenotypes when exposed to stressors, with RLA and RHA rats displaying reactive vs. proactive coping, respectively. In control rats, the WB analysis showed lower hippocampal CB1R relative levels in RLA rats than in their RHA counterparts. After FS, RLA rats showed increased CB1R levels in the dorsal HC (dHC) vs. no change in the ventral HC (vHC), while RHA rats displayed no change in the dHC vs. a decrease in the vHC. In the tissue sections from dHC, FS elicited an increment over the control level of CB1R-like immunoreactivity (LI) in the CA1 and CA3 sectors of the Ammon's horn of RLA rats, while in RHA rats the density of CB1R-LI increased only in the CA1 sector. In tissue sections from the vHC, FS caused an increase over the control values of CB1R-LI only in the CA1 sector of RLA rats and a decrement of the CB1R-LI in the CA1 sector and dentate gyrus of control RHA rats.

This study shows for the first time that, in baseline conditions, the CB1Rs are present in the dHC and the vHC of the Roman rat lines with a different distribution along the septo-temporal extension of the HC and that the FS induces rapid and distinct changes in the hippocampal expression of CB1R of RLA vs. RLA rats, in keeping with the view that endocannabinoid signaling may contribute to the molecular mechanisms that regulate the different responses of the dHC vs. the vHC to aversive situations in male Roman rats. Our results also provide evidence supporting the involvement of CB1R in the molecular underpinnings of the susceptibility of RLA rats and the resistance of RHA rats to stress-induced depression-like behavior.

1. Introduction

Major depressive disorder (MDD), or depression, is a common heritable neuropsychiatric syndrome characterized by the persistence of negative thoughts and emotions that disrupt mood, cognition, motivation, and behavior.

The etiopathogenesis of depression is poorly understood due to the diversity of symptoms shown by depressed patients and the individual

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differences in vulnerability to risk factors. A depressive episode may be triggered or worsened by acute or chronic stressful life events, and stress can affect neuronal integrity and viability in specific brain structures (Duman and Monteggia, 2006; Gold and Chrousos, 2002). Hence, many preclinical models of depression are based on stress-inducing behavioral paradigms that are used to study alterations of the brain structure and function. Among these models, one of the most studied are the Roman low- (RLA) and high-avoidance (RHA) rats, which are psychogenetically selected and bred for poor vs. rapid acquisition of two-way active avoidance, respectively (Broadhurst and Bignami, 1965; Fernández-Teruel et al., 2023). The selection procedure has generated two divergent phenotypes that display reactive (RLA) vs. proactive (RHA) coping styles when exposed to stressors (Giorgi et al., 2003, 2019). Remarkably, the higher fearfulness/anxiety of RLA rats compared to their RHA counterparts is also related to their susceptibility to display a stress-induced depression-like phenotype that is normalized by chronic antidepressant treatment (Piras et al., 2010, 2014). Notably, the susceptibility to depression-like behavior depends on the abnormal expression of genes, among which the most studied are those encoding for the trophic factors, that may cause dysfunctions of the mechanisms underlying the neural plasticity (Duman et al., 1999; Castrén, 2005; Nestler et al., 2002).

Preclinical and clinical evidence further indicates that, besides activating the HPA axis and the sympathetic tonus, acute stress also plays a role in memory (Henckens et al., 2009) and activates signaling molecular cascades that influence the hippocampus (HC), a region involved in learning and memory consolidation, spatial navigation, and expression of emotion-related behaviors (Sannino et al., 2016; Henckens et al., 2009).

In a previous study, aimed at assessing the relationship between the dynamic rapid changes in the expression of BDNF protein in the dorsal and ventral subdivisions of the HC and exposure to an intense acute stressor (Serra et al., 2018), we found that forced swimming (FS) elicits opposite changes in BDNF levels in the vHC and dHC of depression-susceptible RLA rats but not in depression-resistant RHA rats.

Molecular signaling pathways involving endocannabinoids (eCBs) like anandamide (AEA) also play a role in depression (Hill and Gorzalka, 2005; Koethe et al., 2007; Patel and Hillard, 2009; Huang et al., 2016; Gallego-Landin et al., 2021). Thus, eCBs contribute to the fine-tuning of synaptic neurotransmission via interaction with the cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R) (Patel and Hillard, 2009; Gallego-Landin et al., 2021) and alterations in eCB signaling have been reported in patients with depression (Hill and Gorzalka, 2005; Koethe et al., 2007; Huang et al., 2016). Moreover, polymorphisms of the genes encoding CB1R and CB2R have been associated with major depressive disorder (Monteleone et al., 2010; Minocci et al., 2011] and a single allele variation of the CB1R gene has been reported to confer the risk of antidepressant treatment resistance (Domschke et al., 2008). The eCBs also increase exercise-induced neuroplasticity in the HC of rats (Hill et al., 2008). Accordingly, it was recently shown that, in the mouse HC, a synthetic full agonist of the CB1R and CB2R blocked, in a dose-dependentl manner, the acute despair response to FS stress and that this effect was attenuated by pretreatment with a CB1R antagonist (Fang and Wang, 2023). Furthermore, it has recently been shown that acute intense exercise induces in humans a persistent increase of plasmatic AEA levels that positively correlates with serum BDNF concentrations, suggesting the existence of a link through which the CB1R-mediated signaling may influence exercise-induced neuroplasticity and the antidepressant effect of BDNF (Heyman et al., 2012). Together, the above findings are consistent with a possible interacting role between eCB and BDNF signaling in the HC in the expression of stress-induced depression-like behaviors and highlight the intrinsic differences in connectivity and functional role of the HC along its septo-temporal axis (Tanti and Belzung, 2013; Maras et al., 2014; Floriou-Servou et al., 2018). The present study was therefore aimed at assessing the presence of differences in the levels of CB1Rs in the HC of RLA vs. RHA rats in baseline conditions and examining whether the CB1R is involved in the molecular underpinnings of the susceptibility of RLA rats vs. the resistance of RHA rats to display stress-induced depression-like behaviors following exposure to FS. We expected to confirm the different behavioral performances of each line exposed to FS, with RLA rats exhibiting longer-lasting immobility and spending significantly less time displaying active behaviors, like swimming and climbing, than RHA rats. Furthermore, we hypothesized that FS may rapidly affect in a line-dependent fashion the levels of CB1R protein in the HC, perhaps with differences between the dHC and the vHC, and expected to find possible correlations between line- and hippocampal subregion-dependent changes in the relative levels of CB1Rs and the behavioral scores elicited by the stressor. To test the above hypotheses, we recorded the behavioral activity and performed western blot (WB) and immunohistochemical assays to investigate the impact of a single exposure to FS on the expression of CB1Rs in the dorsal and ventral hippocampal subdivisions of RLA and RHA rats.

2. Material and methods

2.1. Animals

Male outbred Roman rats ($N = 28$ for each line), obtained from the colony established in 1998, at the University of Cagliari, Italy (Giorgi et al., 2005), were used throughout and were four months old (weight = 400–450 g) at the beginning of the experiments. Animals were housed in groups of four per cage and maintained under temperature- and humidity-controlled environmental conditions (23 °C ± 1 °C and 60 % ± 10 %, respectively), with a 12 h light-dark cycle (lights on at 8:00 a.m.). Standard laboratory food and water were available ad libitum. To avoid stressful stimuli resulting from manipulation, a single attendant carried out the maintenance activities in the animal house, and bedding in the home cages was not changed on the two days preceding the test. All procedures were performed according to the guidelines and protocols of the European Union (Directive 2010/63/EU). The experimental protocol was approved by the Committee for Animal Experimentation of the Universidad Autónoma de Barcelona and was authorized by the Ministerio de Ciencia e Innovación (authorization No. PID2020-114697GB-I00, 01/01/2021). Every effort was made to minimize animal pain and discomfort and to reduce the number of experimental subjects.

2.2. FS and behavioral measurements

RHA and RLA rats were randomly assigned to the control or FS groups and processed in parallel according to a schedule counterbalanced for animal line and treatment. All animals ($N = 28$ for each line) were naïve at the beginning of the experiments and were used only once. Rats in the FS groups ($N = 14$ for each line) were singly moved from the animal house to a sound-attenuated, dimly illuminated test room whereas controls ($N = 14$ for each line) were kept in their home cages in the animal house until sacrifice. All testing was performed between 10:00 a.m. and 6:00 p.m. and consisted of a 15-min forced swim session as described previously (Piras et al., 2010; Serra et al., 2018, 2022). All the behaviors, summarized in Table 1, were quantified by a single well-trained observer blind to the rat line. A time-sampling technique was used to record the predominant behavior in each 15-s time period.

2.3. Sampling

Forty-five min after the end of the FS session, the animals used for WBs were killed by decapitation; in contrast, the animals used for immunohistochemical assays were deeply anesthetized with chloral hydrate (500 mg/kg, i.p., 2 ml/kg) and transcardially perfused with ice-cold PBS (Phosphate Buffered Saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.3) and 4 % paraformaldehyde.

Table 1
Behavioral measures recorded during the Forced Swim (FS) session.

Behavioral measure	Definition
Immobility	Floating passively in the water without struggling and doing only those movements necessary to keep the head above water
Immobility latency	The time from the beginning of the FS until the first immobility episode
Swimming	Showing moderate active motions around in the cylinder more than necessary to keep the head above water
Climbing	Making active vigorous movements with forepaws in and out of the water, usually directed against the walls
Diving	Swim underwater looking for a way out of the cylinder.
Boli	Number of excreted fecal boli

Immediately after sacrifice, the brains were rapidly removed from the skull and processed for either WB or immunohistochemistry (Fig. 1). For WB assays ($N = 32$, i.e. 8 rats in each experimental group), brains were cooled in dry ice for 15 s, placed in a brain matrix, and cut into 2 mm thick coronal slices using the stereotaxic coordinates of the rat brain atlas of Paxinos and Watson [1998] as a reference. The AP coordinates (from bregma) were approximately −3.30 mm and − 6.04 mm for the dHC and vHC, respectively. Bilateral punches (diameter 2.5 mm) of the dHC and vHC were taken as described by Palkovits (1983). For each rat, tissue punches from both hemispheres were pooled and rapidly frozen at −80 °C. On the day of the assay, the tissue punches were homogenized in distilled water containing 2 % sodium dodecylsulfate (SDS) (300 μl/100 mg of tissue) and a cocktail of protease inhibitors (cOmpleteTM, Mini Protease Inhibitor Cocktail Tablets, Cat# 11697498001, Roche, Basel, Switzerland). For immunohistochemistry ($N = 24$, i.e. 6 rats in each experimental group), brains were postfixed by immersion in freshly prepared 4 % phosphate-buffered paraformaldehyde, pH 7.3, for 4–6 h at 4 °C, and then rinsed until they sank in 0.1 M phosphate buffer (PB), pH 7.3, containing 20 % sucrose.

2.4. Western blot

Proteins from hippocampal tissue homogenates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE), using precast polyacrylamide gradient gel (NuPAGE 4–12 % Bis-Tris Gel Midi, Cat# NP0321, Novex by Life Technologies, Carlsbad, CA, USA), in the XCell4 Sure LockTM Midi-Cell chamber (Life Technologies). Internal mw standards (Precision Plus Protein Western C Standards, Cat# 161–0376, Bio-Rad, Hercules, CA, USA) were run in parallel. Immunostaining of protein blots was performed as previously described (Serra et al., 2017, 2018, 2022). The primary antibodies were: a rabbit polyclonal antibody directed against CB1R protein (Synaptic System, Göttingen, Germany) and a mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MAB374, RRID: AB_2107445, EMD Millipore, Darmstadt, Germany), both diluted 1:1000, in 20 mM Tris base and 137 mM sodium chloride (TBS) containing 5 % milk powder and 0.02 % sodium azide. Incubations with primary antiserum were conducted for one night at 4 °C. After rinsing in TBS/T, blots were incubated at room temperature, for 60 min, with peroxidase-conjugated goat anti-rabbit serum (Cat#9169, RRID: AB_258434, Sigma Aldrich, St Louis, MO, USA) and goat anti-mouse serum (AP124P, RRID: AB_90456, Millipore, Darmstadt, Germany), respectively, both diluted 1:10,000, as the secondary antiserum. To control for non-specific staining, blots were stripped and incubated with the relevant secondary antiserum. To check for antibody specificity and cross-reactivity, the anti-CB1R antibody was challenged with an excess of the antigen peptide (ACROByosystems, Newark, DE, USA). After rinsing in TBS/T, protein bands were developed using the Super-SignalTM WestPico PLUS Chemiluminescent Substrate (Cat# 34580, Thermo Scientific, Rockford, IL, USA) and visualized using the Image-Quant LAS-4000 (GE Healthcare, Little Chalfont, UK). The approximate molecular weight (mw) and relative optical density (O.D.) of the labeled protein bands were evaluated by a blinded examiner. The ratio of the intensity of the CB1R-immunostained bands to the intensity of the GAPDH-positive ones was used to compare the relative expression levels of these proteins in both rat lines. The O.D. was quantified by the Image Studio Lite Software (RRID: SCR_014211, Li-Cor).

2.5. Immunohistochemistry

Coronal brain sections from RLA and RHA rats were examined in pairs placed on the same slide. Semiconsecutive cryostat sections (14 μm thick) were collected on chrome alum-gelatin coated slides and processed by the avidin-biotin-peroxidase complex (ABC) immunohistochemical technique as previously described (Serra et al., 2017, 2018, 2022). The primary antibody (i.e., rabbit polyclonal antibody against the CB1R) was the same as the one used for WB and was diluted 1:500. A biotin-conjugated goat anti-rabbit serum (BA-1000, RRID: AB_2313606, Vector, Burlingame, CA, USA), diluted 1:400, was used as the secondary

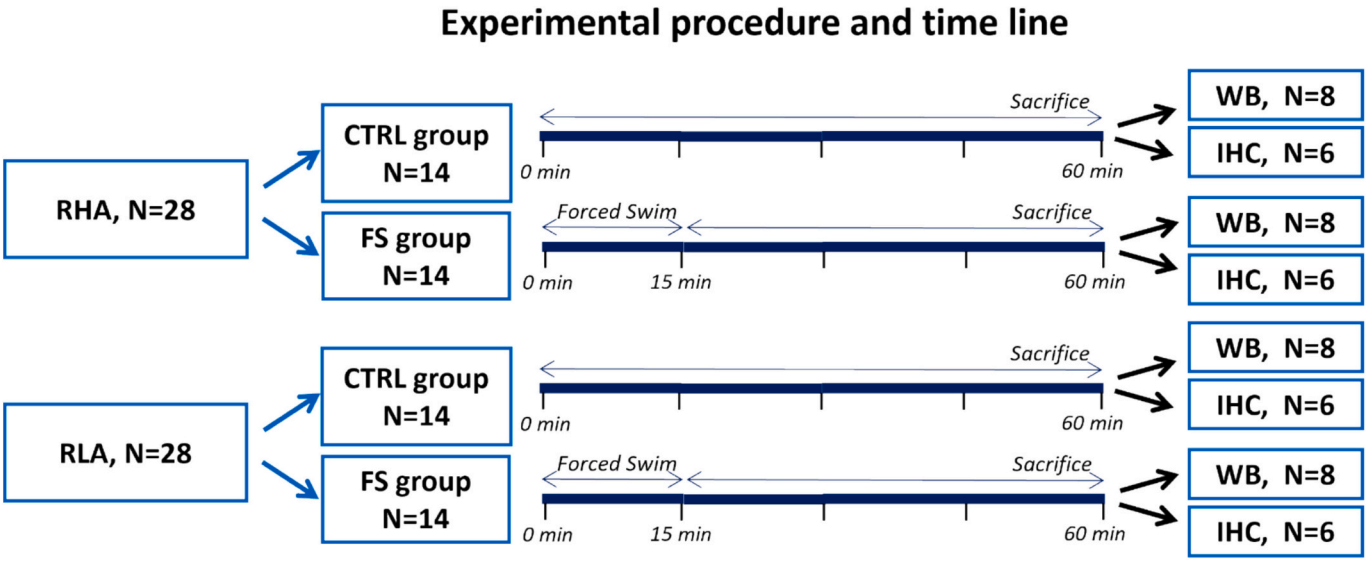

Fig. 1. Schematic representation of the experimental procedure and groups. CTRL, control; FS, forced swimming; IHC, immunohistochemistry; N, number of animals; RHA, Roman high avoidance; RLA, Roman Low Avoidance; WB, western blot.

antiserum. The reaction product was revealed with the ABC (Cat#G011-61, BioSpa Div. Milan, Italy), diluted 1:250, followed by incubation with a solution of 0.1 M PB, pH 7.3, containing 0.05% 3,3′-diaminobenzidine (Sigma Aldrich, St Louis, MO, USA), 0.04% nickel ammonium sulfate and 0.01% hydrogen peroxide. All antisera and the ABC were diluted in phosphate-buffered saline (PBS), containing 0.2% Triton X-100 (PBS/T). Incubation with primary antibodies was carried out overnight at 4 °C. Incubations with the secondary antiserum and ABC lasted 60 min and were performed at room temperature. Negative control preparations were obtained by incubating tissue sections in parallel with either PBS/T alone, or in one of the following ways: (i) with the relevant primary antiserum pre-absorbed with an excess of the corresponding peptide antigen (ACROByosystems, Newark, DE, USA), or (ii) by substituting the corresponding primary antiserum with normal goat serum. Slides were observed with an Olympus BX61 microscope and digital images were captured with a Leica DFC450C camera.

2.6. Image densitometry

For the quantitative evaluation of the CB1R immunohistochemical labeling representative 10× magnification microscopic fields were taken from twelve coronal sections of six animals for each condition. The sections corresponded to the AP coordinates used to obtain the tissue samples used for the WB assays and were blindly analyzed with ImageJ (http://rsb.info.nih.gov/ij/; RRID: SCR_003070) to calculate the density of immunoreactivity per μm^2. To exclude the background staining, mean gray values from the unstained areas were subtracted from the gray values of the immunostained regions. The immunoreactivity density values measured in every hippocampal region of each animal were averaged and used for statistical evaluation.

2.7. Statistical analyses

Behavioral measurements were statistically evaluated using the Student's *t-test* for independent samples. WB and immunohistochemical data were statistically assessed by two-way ANOVA (see Table 2). Before performing both Student's *t*-tests and the ANOVAs, data sets of each experimental group were inspected for normal distribution of data and homogeneity of variances, with the Shapiro-Wilk's test and the Bartlett's test, respectively. When two-way ANOVAs revealed statistically significant interactions, the sources of significance were ascertained by pairwise *post-hoc* contrasts with the HSD Tukey's test. In all the other cases, pairwise comparisons were performed with the two-tailed t-test with Sidak's corrected alpha values. Spearman's test was used for the correlations between the behavioral measurements ($N = 14$ for each line in the FS groups; $N = 14$ for each line in the CTRL groups) and the CB1R molecular measurements ($N = 8$ for each experimental group in the WB assays; $N = 6$ for each experimental group in the immunohistochemical assays). All the statistical analyses were carried out with the PRISM, GraphPad 8 Software (San Diego, CA, USA) with the significance level set at $p < 0.05$.

3. Results

3.1. Behavioral measures during FS

The RHA and RLA rats displayed patently different behavioral performances when exposed for the first time to a 15-min session of FS (Fig. 2). Thus, compared to their RHA counterparts, RLA rats displayed a shorter immobility latency ($p = 0.0088$) while showing a longer cumulative immobility time ($p < 0.0001$). On the other hand, compared with their RLA counterparts, RHA rats spent significantly more time displaying active behaviors like climbing ($p = 0.0004$) and diving ($p = 0.0004$).

3.2. Western blot

The antibody against the CB1R protein recognized a protein band with a relative molecular weight (mw) of about 53 kDa (Fig. 3/Table 2), in agreement with the reported mw (Howlett et al., 1998). Assessment of the densitometric values of CB1R, in the tissue homogenates from the dHC, by a two-way ANOVA (between groups factors—rat line and FS), revealed a significant interaction line x FS but no significant effects of either line or FS alone (Table 2). Pairwise contrasts showed that the relative levels of the CB1R protein, in the control groups, were lower in RLA vs. RHA rats ($p = 0.0058$) (Fig. 3A). Further pairwise comparisons showed that, after FS, the relative level of the CB1R-like immunoreactivity (LI) of RLA rats was 137 % higher than that of the respective controls ($p < 0.0001$), whereas no significant changes were observed in RHA rats. Additional *post-hoc* contrasts revealed that, after FS, the relative level of the CB1R-LI was 50 % higher in RLA than RHA rats ($p = 0,0183$). After FS, no correlations between the behavioral performances and the CB1R-LI relative levels were found in the dHC of either RHA or RLA rats.

In the vHC, a two-way ANOVA revealed significant effects of line, FS, and the interaction line × FS (Table 2). Additional *post-hoc* contrasts showed that the relative levels of the CB1R-LI in the control groups were lower in RLA vs. RHA rats ($p < 0.0001$; Fig. 3B); after FS, the relative level of the CB1R-LI of RHA rats was 66 % lower than the respective control ($p < 0.0001$), while in RLA rats, the control level of CB1R-LI remained unchanged. Moreover, in the vHC, after FS, Spearman's test revealed a negative correlation between the diving activity and the CB1R-LI of RLA rats ($r = -0.7856$; $p = 0.0256$).

3.3. Immunohistochemistry

The CB1R-like immunoreactive structures (Figs. 4, 5, S1, S2) were unevenly distributed within the hippocampal formation and consisted of labeled neuronal slight filaments and dot-like elements, interpreted as nerve fibers and terminals, respectively, that were arranged as nerve fiber networks of variable thickness. Occasional CB1R-labeled perikarya were observed in the pyramidal layer. In the Ammon's horn (Figs. S1 and S2), meshworks of CB1R-like immunoreactive nerve fibers and dot-like terminals of different caliber outlined the pyramidal layer, where they were arranged as coarse elements between and around the unstained perikarya, and as tiny punctate-like elements homogenously distributed in the molecular layer of the CA1 (Figs. S1A—D; S2A—D), CA2 (Figs. S1E—H; S2E—H), and CA3 sectors (Figs. S1I–L; S2I—N). Sparse

Table 2
Statistical analysis by two-way ANOVAs performed on western blot data of the cannabinoid receptor 1 protein shown in Fig. 2.

ANOVA Main factors	Line		FS		Line × FS		
Brain area	F	p	F	p	F	p	d.f.
Dorsal Hippocampus	0.0004	n.s.	1.949	n.s.	**26.63**	**< 0.0001**	1, 28
Ventral Hippocampus	**25.45**	**< 0.0001**	**5.459**	**0.0269**	**33.68**	**< 0.0001**	1, 28

d.f.—degrees of freedom; F – variance; p – significance level; n.s.—not significant.

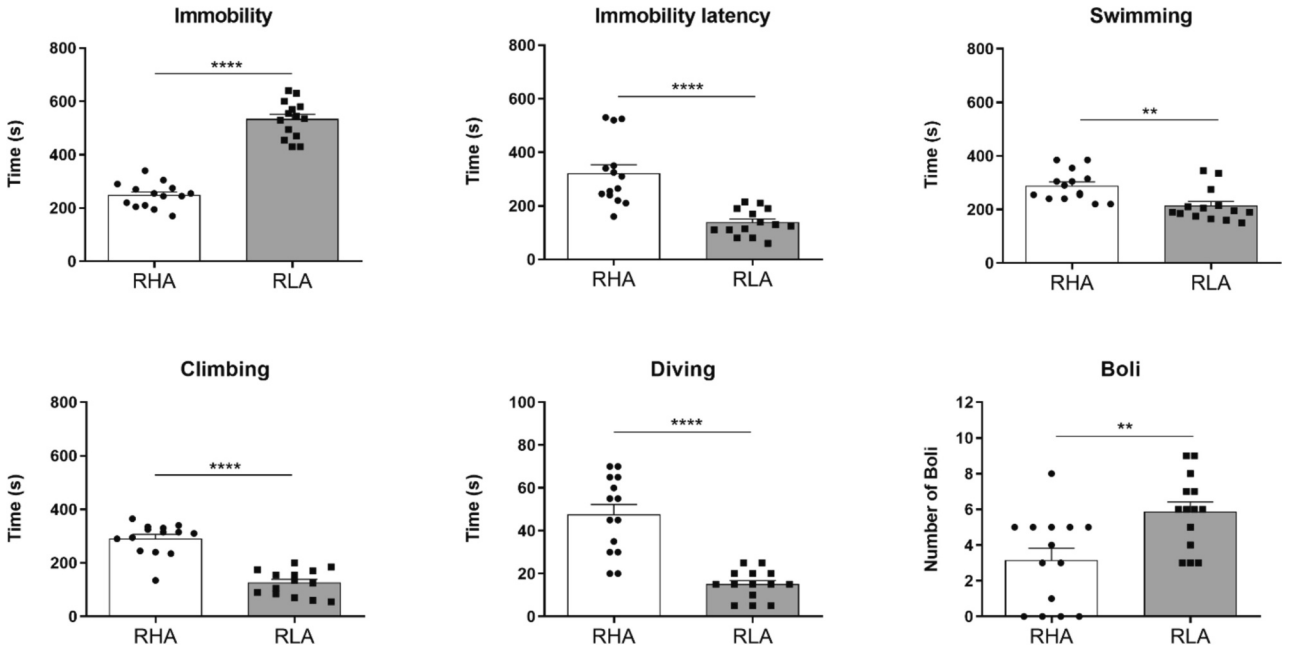

Fig. 2. Behavioral performance of the Roman High-Avoidance (RHA) and the Roman Low-Avoidance (RLA) rats, during the 15 min forced swimming session. The columns and bars represent the mean ± SEM ($N = 14$ for each line). ** $p < 0.01$, **** $p < 0.0001$ (Student's *t*-test for independent samples).

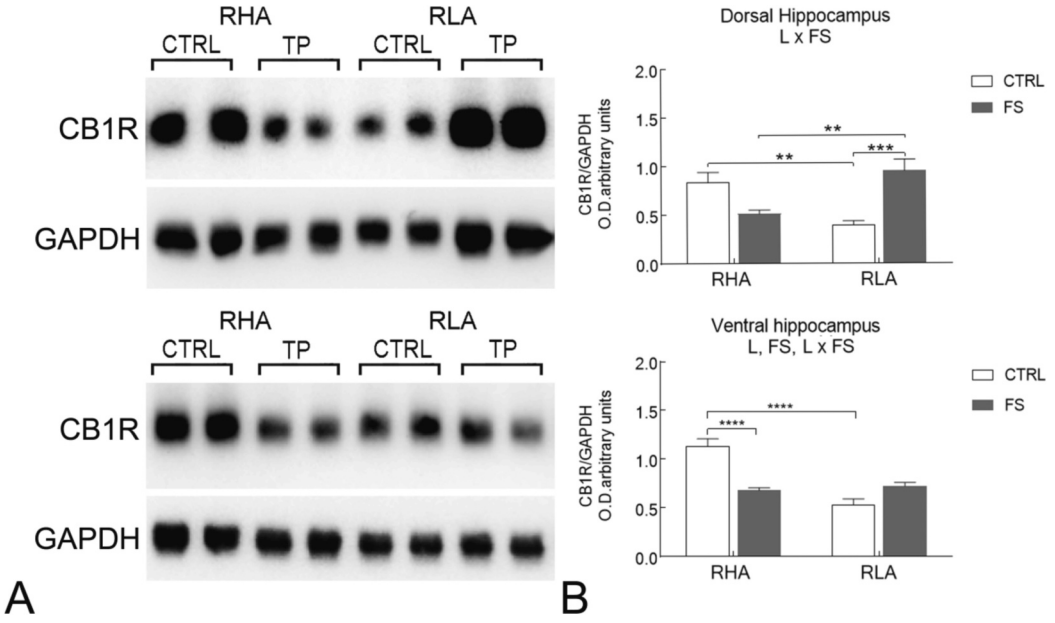

Fig. 3. Western blot analysis of the cannabinoid receptor 1 (CB1R) in the dorsal (A) and ventral hippocampus (B) of RHA and RLA rats, under baseline conditions (CTRL), and after acute forced swim stress (FS). (A): CB1R- and GAPDH-immunostained blots, showing representative samples from two rats for each condition; (B): Densitometric analysis of the CB1R/GAPDH band gray optical density (O.D.) ratios. Columns and bars denote the mean ± S.E.M. of eight rats in each experimental group. **: $p < 0.01$; ***: $p < 0.001$; **** $p < 0.0001$. (Tukey's post-hoc test for multiple comparisons).

CB1R-positive punctate-like elements were also present in the stratum lucidum, particularly in RLA rats, and the oriens layer (Figs. S1I—K; S2A—N). In the dentate gyrus (DG), the bulk of CB1R-LI was observed in the molecular layer (ML) where it contributes to outlining its tri-laminar appearance; specifically, in the dHC, labeled nerve fiber meshworks were particularly thick in its inner (IML) and outer (OML) bands, while, in the vHC, the highest density of CB1R-LI occurred in a narrow band in the middle molecular layer (MML) (Figs. S1M—P; S2I—N). In contrast, in the granule cell layer, and in the hilus CB1R-LI labeled a sparse to moderately dense nerve fiber meshwork (Figs. S1M—P; S2I—N). The densitometric analysis in the CA sectors of the HC and the DG (Figs. 4, 5) revealed significant differences in the CB1R-LI between the RHA and RLA lines and between the control and FS conditions. Thus, as shown in Table 3, in the dHC the two-way ANOVA revealed the effects of line in the DG, of FS in the CA1, CA2 and CA3 sectors, and in the DG, and a significant line × FS interaction in the CA3 sector. Moreover, pairwise contrasts showed that in the DG the control CB1R-LI was significantly higher (+30 %; $p = 0.0138$) in RLA vs. RHA rats. After FS, the CB1R-LI was, respectively, 49 % ($p = 0.0264$) and 52 % ($p = 0.0312$) higher than the control value in the CA1 of RHA and RLA rats, 73 % ($p = 0.0099$)

higher in the CA3 sector of RLA rats, and 47 % ($p = 0.0076$) and 44 % ($p = 0.0004$) lower than the respective controls in the DG of RHAs and RLAs (Fig. 4). In the dHC, after FS, Spearman's test revealed that in RHA rats, the CB1R-LI density in the CA1 ($r = -0.8407$; $p = 0.0222$) and CA3 ($r = -0.8117$; $p = 0.014$) sectors negatively correlated with the diving activity. Spearman's test also revealed a negative correlation between the density of CB1R-LI in the CA1 vs. the CA3 of RHA rats ($r = -0.3714$; $p = 0.033$).

In the vHC, a two-way ANOVA revealed a significant line × FS interaction in the CA1 sector, whereas in the DG there was an effect of FS and a significant line x FS interaction (Table 3). In addition, *post-hoc* contrasts showed that, in the CA1 sector and the DG, the control CB1R-LI was, respectively, 52 % ($p = 0.0396$) and − 41 % ($p = 0.0182$) lower in RLA vs. RHA rats; moreover, upon FS, the CB1R-LI was respectively 50 % ($p = 0.0476$) and 63 % ($p = 0.0003$) lower than the respective control values in the CA1 and DG of RHA rats. In contrast, in the CA1 of RLA rats, the CB1R-LI was 119 % higher ($p = 0.0209$) vs. the control value (Fig. 5). After FS, the CB1R-LI in the CA1 and DG was 112 % ($p = 0.0253$) and 105 % ($p = 0.0275$) higher in RLA vs. RHA rats, respectively. In the vHC, the CB1R-LI after FS in the DG of RHA rats positively correlated with the line-matched immobility latency; ($r = 0.8857$; $p = 0.033$). On the other hand, in RLA rats, the CB1R-LI density upon FS in the DG positively correlated with that in the CA3 sector ($r = 0{,}8857$; $p = 0.033$).

4. Discussion

The present report provides the first immunochemical characterization of the effects of an acute severe stressor on the expression of the CB1R in the dHC and vHC of the Roman rat lines. The main findings are as follows: i) in keeping with our previous studies (Piras et al., 2010, 2014), when exposed to FS, RLA rats display a reactive coping strategy characterized by long-lasting cumulative immobility and a low frequency of active behaviors like swimming, climbing and diving while RHA rats exhibit a proactive coping strategy characterized by intense active behaviors associated with infrequent immobility episodes; ii) FS elicits rapid dynamic changes of CB1R expression which are distinctly different in the dHC and vHC of RLA vs. RHA rats.

It must be underlined that RLA rats show innate distinctive behavior in the FS, since their propensity to adopt a passive strategy during the FS is a heritable trait, as it was consistently reproduced among the generations of Roman rats (Piras et al., 2014; Serra et al., 2018). The immobility behavior during FS is considered a marker of reactive coping and behavioral despair and is the most widely used to estimate the effectiveness of antidepressants. Accordingly, in Roman rats, the chronic treatments with antidepressants (desipramine, clomipramine, and fluoxetine), at doses that were ineffective when given subacutely, reduced FS-induced immobility and increased active behaviors, such as swimming and climbing, in RLA rats but not in their RHA counterparts (Piras et al., 2014). As for the neuronal systems involved, as an example, it has been shown in the rat hippocampus that, during immobility, location-specific neurons in the DG (together with subsets in the CA1, CA2, and CA3) constitute the morphological ground for the neural code indicating the current position of the animal (Kay et al., 2016).

Only recently, evidence in male mice has shown that a synthetic agonist of CB1R, injected at the dose of 50 mg, decreased immobility in the FS and produced antidepressant effects, indicating a direct involvement of CB1R activation in the immobility behavior (Fang and Wang, 2023). Further evidence in the literature showing a statistical correlation between FS-induced behavior and the immunochemical

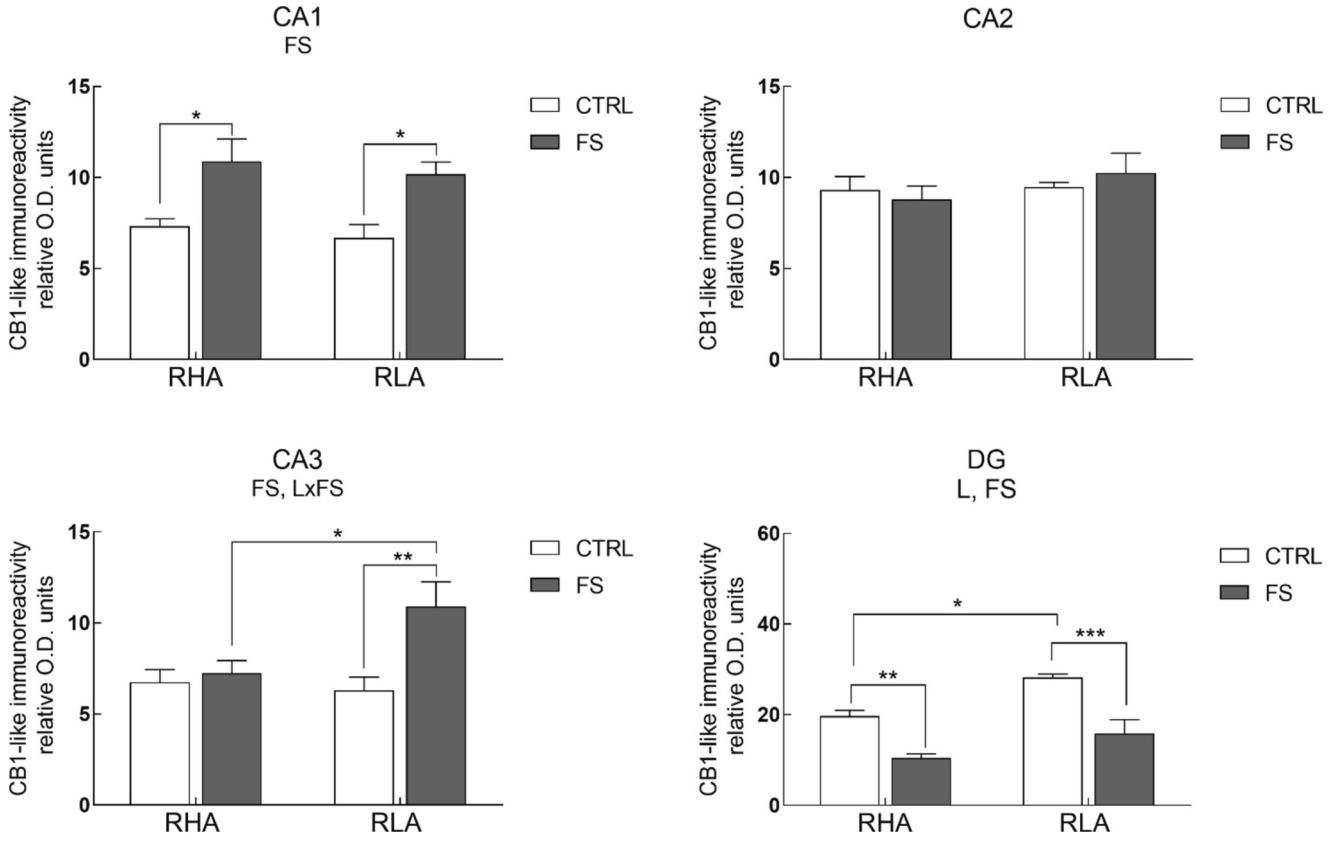

Fig. 4. Densitometric analysis of the cannabinoid receptor 1-like immunoreactivity in the CA1–CA3 sectors of the Ammon's horn, and in the dentate gyrus (DG) of the dorsal hippocampus (dHC) under baseline conditions (CTRL) and after forced swim (FS). Columns and bars denote the mean ± S.E.M. of six rats in each experimental group. Two different sections were analyzed for each rat. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ (Tukey's *post-hoc* test or Sidak's correction for multiple comparisons).

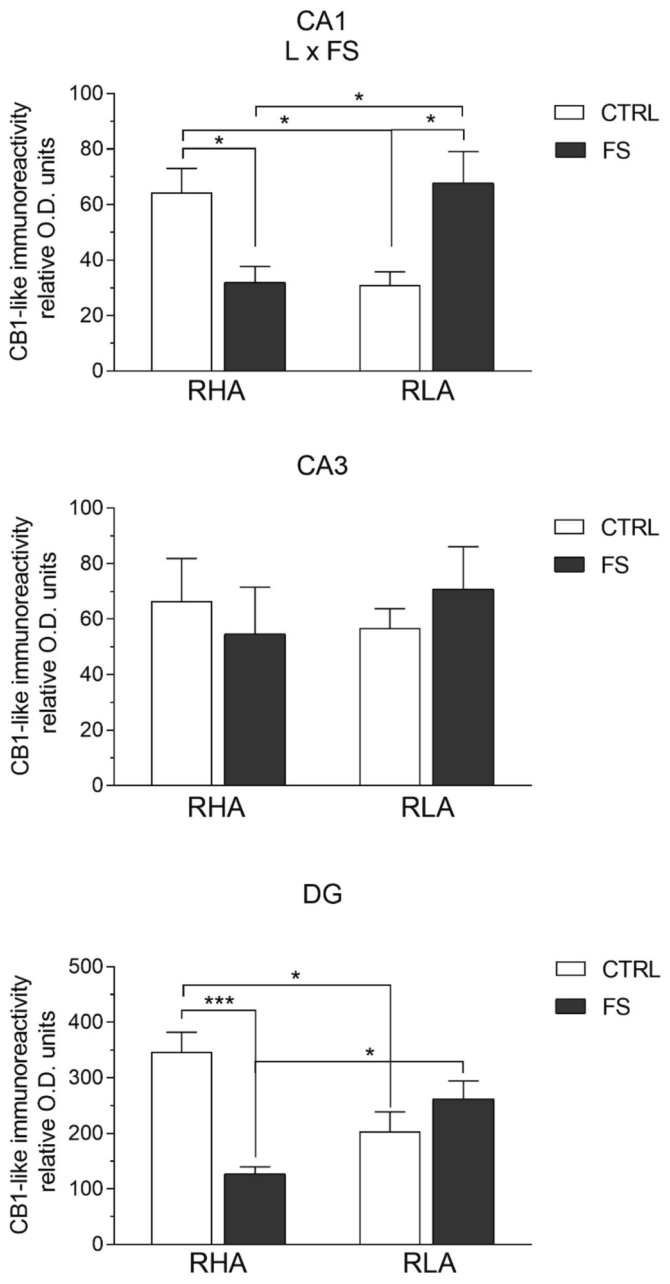

Fig. 5. Densitometric analysis of the cannabinoid receptor 1-like immunoreactivity in the CA1 and CA3 sectors of the Ammon's horn, and in the dentate gyrus (DG) of the ventral hippocampus (vHC) under baseline conditions (CTRL) and after forced swim (FS). Columns and bars denote the mean ± S.E.M. of six rats in each experimental group. Two different sections were analyzed for each rat. *: $p < 0.05$; ***: $p < 0.001$ (Tukey's *post-hoc* test or Sidak's correction for multiple comparisons).

expression of a given molecule in hippocampal homogenates was given by Borsoi et al. (2015) in a study about BDNF expression. Their results, however, cannot be directly compared with ours. Thus, in the Roman rats, BDNF is markedly affected by FS, which produces opposite changes in the vHC and dHC of RLA but not RHA rats (Serra et al., 2018). Instead, Borsoi et al. (2015), while reporting no FS-induced changes in the BDNF protein levels in the prefrontal cortex and HC (without distinction between dHC and vHC) of Wistar rats, also showed that immobility was correlated with the BDNF protein levels in the prefrontal cortex but not the hippocampal BDNF (Borsoi et al., 2015).

In the present investigation, we extended our observations of FS-induced behavioral performances to a wider range. Thus, in the absence of any pharmacological manipulation, we examined the possible correlations among the six behaviors recorded during the FS session and the semiquantitative evaluation of the hippocampal CB1R-LI. We found that only two of the examined behaviors correlated with the CB1R-LI. Such paucity of significant correlations argues against our expectation that CB1Rs may contribute to FS-induced behaviors in Roman rats, but also highlight the pleiotropic molecular control of the behavioral performances. Moreover, it was unexpected that the behavior of RLA rats correlated only with the CB1R-LI detected in tissue homogenates. In contrast, the semiquantitative values of CB1R-LI in the hippocampal subregions correlated only with behaviors displayed by the stress-resistant RHA rats. It is interesting, however, that correlations of CB1R-LI with the line-matched behavioral performances identify some hippocampal regions of RHA rats (i.e. the CA1 and CA3 sectors in the dHC, and the DG in the vHC) where the CB1Rs might be selectively involved in the regulation of the diving activity and immobility latency. The negative correlation between the diving activity and the CB1R-LI levels in tissue homogenates from the vHC of RLA rats is also difficult to interpret because no FS-induced changes were detected in the temporal pole of the HC (see ANOVA data). It appears relevant, however, that the diving activity during a single episode of FS has been reported to be positively correlated with serotonin release (Linthorst et al., 2002; Linthorst, 2005). Accordingly, RHA rats, which show a higher diving activity vs. their RLA counterparts, display a higher functional serotonergic tone than RLA rats (Giorgi et al., 2003). Moreover, the CB1R agonists are able to modulate the 5-HT transmission in the raphe and HC like antidepressants, such as selective 5-HT re-uptake inhibitors (Scarante et al., 2017; Bright and Akirav, 2022). Further studies are therefore warranted to establish whether the observed correlation between the diving behavior during FS and the CB1R-LI in the vHC of RLA rats (as measured in hippocampal tissue homogenates) and in CA1 and CA3 in the vHC of RHA rats (as measured in the hippocampal tissue sections) may also be linked to the serotonergic tone in these hippocampal subregions.

The different impact of FS on the immunoreactivity to CB1Rs along the septo-temporal extension of the HC suggests distinct roles for the CB1R-mediated signaling in the intrinsic and extrinsic hippocampal neuronal circuitries. Thus, given the role played by the dHC in spatial navigation and memory consolidation vs. the involvement of the vHC in emotion-related behaviors, it may be suggested that stressors can uphold opposite neuroplastic adjustments in the dHC and vHC. Importantly, the stress-induced adaptive responses of CB1R protein levels can be further amplified by the known interactions of the eCB system with the stress hormones, whose imbalance is involved in stress-induced depression in vulnerable individuals (de Kloet et al., 2007), and by the crosstalk between eCBs and BDNF (Khaspekov et al., 2004). Of note in this context, the functional characteristics of the hypothalamus-pineal-adrenal (HPA) axis of RLA rats are reminiscent of those of depressed subjects during a Major Depression Disorder (MDD) episode (Rybakowski and Twardowska, 1999). Thus, the exposure to stressful events causes a transient elevation of plasma corticosterone levels in both lines, with more pronounced amplitude and duration in RLA vs. RHA rats (Walker et al., 1989), and, in the dexamethasone/corticotropin-releasing hormone test, the increment of plasma corticosterone concentration is significantly higher in RLA vs. RHA rats (Steimer et al., 2007). Furthermore, under control conditions, protein levels of BDNF and its receptor trkB are lower in the HC of RLA vs. RHA rats (Serra et al., 2017) and FS elicits different line-dependent changes in BDNF and trkB protein levels in the vHC vs. dHC (Serra et al., 2018).

4.1. Effect of acute stress on CB1 receptor protein levels in the dorsal and ventral hippocampus

The results of WB assays, together with the correlation of the relative CB1R-LI protein levels with diving in the vHC of FS-exposed RLA rats, are consistent with the role of CB1Rs in the adaptive molecular processes

Table 3
Statistical analysis by two-way ANOVAs performed on data obtained from densitometric image analysis of tissue section distribution of the cannabinoid receptor 1-like immunoreactivity (LI) shown in Figs 4 and 5.

ANOVA Main factors	Line		FS		Line × FS		
Brain area	F	p	F	p	F	p	d.f.
Dorsal Hippocampus							
CA1	0.6908	n.s.	**18.79**	**0.0003**	0.0031	n.s.	1,20
CA2	1.107	n.s.	**0.0222**	**0.0045**	0.7062	n.s.	1,20
CA3	3.119	n.s.	**7.732**	**0.0115**	**5.026**	**0.0365**	1,20
DG	**36.88**	**< 0.0001**	**15.47**	**0.0008**	0.7721	n.s.	1,20
Ventral Hippocampus							
CA1	0.0761	n.s.	0.0225	n.s.	**18.22**	**0.0004**	1,20
CA3	0.8224	n.s.	0.0058	n.s.	0.8428	n.s.	1,20
DG	0.2751	n.s.	**15.05**	**0.0003**	**39.35**	**< 0.0001**	1,20

d.f.—degrees of freedom; F – variance; p – significance level; n.s.—not significant.

involved in the different plastic changes elicited by stress on the HC of the two lines. Our findings further add to the concept that stress modulates hippocampal plasticity in opposite directions, according to the different roles and connectivity along the hippocampal dorsoventral axis (Fanselow and Dong, 2010). Neuronal localization studies in the rodent HC show that the bulk of CB1Rs is present in neurons, about 75 % of them occurring in GABAergic neurons, the remaining 25 % in glutamatergic neurons, and in astrocytes (Marsicano and Lutz, 1999; Wilson and Nicoll, 2002; Steindel et al., 2013; Silva-Cruz et al., 2017). CB1R signaling inhibits the transmission of presynaptic nerve endings, and it is believed that CB1Rs play a role in keeping the balance between excitation and inhibition (Silva-Cruz et al., 2017; Di Franco et al., 2022), thus regulating hippocampal intrinsic firing. Accordingly, data obtained in the mouse HC in vivo have recently shown that inhibition of the monoacylglycerol lipase induces a CB1R-dependent suppression of inhibitory GABAergic synapses and long-term depression (LTD) of excitatory glutamatergic synapses (Wang et al., 2017).

Many of the CB1R-mediated mechanisms are based on the ability of the eCB system to stimulate the secretion of BDNF during neuronal stress challenges (Marsicano et al., 2003; Khaspekov et al., 2004). In keeping with the above data, we have shown that, in RLA rats, FS elicited an increment of BDNF protein levels in the dHC vs. a decrease in the vHC, suggesting that the adaptive neuronal plasticity in response to the acute stress might also be influenced by the endocannabinoid/BDNF crosstalk. Consistently, the density of BDNF-LI in the vHC of RHA rats also decreased after exposure to FS (Serra et al., 2018).

4.2. Effect of forced swim on the regional and subregional immunohistochemical distribution of CB1Rs in the dorsal and ventral hippocampus

The distribution of CB1R-LI in hippocampal coronal tissue sections showed distinct differences in CB1R-LI between the Roman lines, across the hippocampal regional fields, and septo-temporal extension in baseline conditions and upon FS. While the present data do not allow us to determine whether any effect reported in the rat groups exposed to FS depends on the different values of the respective control groups, the densitometric changes of CB1R-LI observed in the Ammon's horn are worth a comment. Thus, Spearman's test revealed hippocampal interregional correlations of the line-related CB1R-LI differences along the septo-temporal extension of the HC. Specifically, in the dHC, the correlation of the CB1R-LI density between the CA1 and the CA3 sector was found only in the RHA rats; in contrast, in the vHC, the correlation of the CB1R-LI in the DG with that in the CA3 sector was observed only in the RLA rats. Hence, it can be hypothesized that the CB1R may play a role in the modulation of the input-output activity of the CA3 pyramidal neurons in response to stressors.

Early studies have demonstrated differences between the hippocampal mossy fiber system of the Roman lines, with RLA rats exhibiting a much larger total mossy fiber terminal field in the CA3 (Schwegler and Lipp, 1981). On a morphological ground, the outcome of our immunohistochemical assays shows that the FS-induced increment of CB1R-LI in the CA1 and CA3 sectors of the dHC of RLA rats is due to the distinctive distribution of the labeled punctate-like elements –around the pyramidal cell bodies, in the ML, and in the stratum lucidum-, which may imply a role for CB1Rs in the presynaptic modulation of the FS-induced plastic events. In contrast, the exposure to FS failed to induce variations of the CB1R-LI density in the ventral extension of the CA3 sector.

On the other hand, it must be considered that the CB1Rs are expressed not only in glutamatergic neurons but also in GABAergic intrinsic neural cells. Thus, the augmented density of CB1R-LI punctate elements we observed in the CA3 sector of RLA rats in the dHC might also be associated with the inhibitory synaptic terminals originating from intrinsic GABAergic projections reaching their neuronal somata and dendritic processes of the pyramidal cells in the ML, thereby impacting their signaling activity. Notably, a proposed mechanism of the pharmacological action of the antidepressant ketamine is the disinhibition hypothesis, positing that ketamine's rapid effect relies on the inhibition of GABAergic interneurons in the prefrontal cortex and HC, which leads to downstream glutamatergic stimulation and synaptic plasticity and causes the release of BDNF (Borsellino et al., 2023). Accordingly, in a recent set of immunochemical observations, we showed that FS induces an increase of BDNF-LI levels in CA2 and CA3 subfields of the dHC of RLA rats, while in the vHC it induces a marked decrease in CA1 and CA3 subfields of both lines (Serra et al., 2018).

4.3. Impact of the acute stress on the density of CB1R-LI in the dentate gyrus of the dorsal and ventral hippocampus

In keeping with earlier studies (Monory et al., 2006; Marsicano et al., 2002; Yu et al., 2015), our present results show that, in the Roman rats, most CB1R-LI in the DG is distributed in the ML, with a characteristic distribution pattern of CB1R-LI differing along the hippocampal septo-temporal axis since the richest labeling was associated with the IML in the dHC and the MML in the vHC. In the mouse HC, different axonal projections of dHC and vHC hilar mossy cells, including their targeting of different molecular sublayers along the septo-temporal axis (Houser et al., 2021; Botterill et al., 2021), have been implicated in the functional differences of DG in the dHC and vHC (Botterill et al., 2021). Therefore, it could be suggested that, in the Roman rats, the origin of CB1R-positive nerve fibers in the IML in the dHC or the MML of the vHC, is at least partly representing the extension of the existing commissural/associational projections and contributes to the line-related differences in the distribution of the CB1R-LI and eCB signaling. Accordingly, we have previously shown that, in the DG of the dHC, the BDNF- and trkB-labeled

nerve fibers are localized in the IML (Serra et al., 2018). Further studies are warranted to confirm or exclude the possible crosstalk between the CB1R and the BDNF/trkB signaling.

5. Conclusions

The results of the present preliminary report, together with our earlier studies on the effects of FS on the expression of BDNF and its cognate receptor trkB in the HC, and the different responses of the HPA axis to stressors of RLA vs. RHA rats have set the stage for the use of these two lines to investigate the role of the functional interactions among eCB, BDNF and glucocorticoid signaling in the adaptive plastic responses to acute and chronic stressors. Such studies, using the RLA phenotype which is vulnerable to stress-induced depression-like behavior vs. the depression-resistant RHA phenotype, may further our understanding of the pathophysiology of depression and lead to the identification of novel targets for the treatment of mood disorders. The behavioral performance of Roman rats in response to FS-induced stress was previously characterized by subacute and chronic treatment with antidepressants (Piras et al., 2010, 2014). However, future studies with specific pharmacological manipulation are needed to test the interpretation of the FS-induced changes in CB1R-LI in the HC of Roman rats.

A limitation of this study is that it does not consider that sex-related differences in the sensitivity to stress and coping mechanisms are an important issue deserving attention and further investigation. Thus, epidemiologic studies indicate that clinical depression is about 50 % more common among women than men (World Health Organization, 2023). Moreover, it is well known that ovarian hormones and their neuroactive metabolites modulate behavioral responses that may be related to emotionality or anxiety, thus contributing to sex differences in stress-induced effects on the CNS (Palanza, 2001; Steimer and Driscoll, 2005; Oliveras et al., 2022). Regarding specifically the Roman rat lines, the few available publications focusing on females demonstrate that male and female subjects can respond either differently or even in opposite directions to novelty and stressors (Driscoll, 1986; Castanon et al., 1994; Escorihuela et al., 1997; Palanza, 2001; Oliveras et al., 2022). Thus, for example, while no sex-related differences were observed in active avoidance learning (Driscoll, 1986; Castanon et al., 1994), it has also been shown that females from both Roman lines tended to be more active and to defecate less than males in a novel environment (Escorihuela et al., 1997). Analysis of responses to a battery of novel/threatening tests and learned fear paradigms reveal a pattern of unidirectional sex effects with male Roman rats being more fearful than females (Aguilar et al., 2003). An important issue to be addressed is the evaluation of the stress effect on CB1R signaling in the HC of female RHA and RLA rats. Interestingly, the reciprocal interplay between the eCB system and gonadal hormones (androgens, estrogens, and progesterone) has been shown to regulate emotionality and sexual motivation in rats (Gorzalka and Dang, 2012). Moreover, men appear to show greater CB1R activity than women, but the CB1R activity of women increases with age (Van Laere et al., 2008).

In closing, the results of this study indicate that, in male Roman rats, a strong acute stressor elicits rapid changes in the expression of CB1Rs in the dHC and vHC in a line-dependent manner. However, the mechanisms underlying the distinct effects of FS on CB1R-mediated signaling in RLA vs. RHA rats are still poorly understood. Further studies are therefore warranted to shed light on the molecular mechanisms sustaining resilience vs. vulnerability to potentially dangerous adverse environmental conditions.

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CRediT authorship contribution statement

Maria Pina Serra: Methodology, Investigation, Formal Analysis, Data curation, Funding acquisition, Writing – review & editing. **Marianna Boi:** Methodology, Investigation, Formal analysis, Data curation. **Ylenia Lai:** Methodology, Investigation, Data curation. **Marcello Trucas:** Data curation. **Alberto Fernández-Teruel:** Writing – review & editing, Funding acquisition. **Maria Giuseppa Corda:** Writing – review & editing, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Osvaldo Giorgi:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization. **Marina Quartu:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

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Data availability

Data will be made available on request.

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