Neuropharmacology 133 (2018) 242–253

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Impaired glucocorticoid-mediated HPA axis negative feedback induced by juvenile social isolation in male rats

Giorgia Boero [a, *, 1], Maria Giuseppina Pisu [b, 1], Francesca Biggio [a], Laura Muredda [c], Gianfranca Carta [c], Sebastiano Banni [c], Elena Paci [a], Paolo Follesa [a, d], Alessandra Concas [a, d], Patrizia Porcu [b], Mariangela Serra [a, d]

[a] Department of Life and Environment Sciences, Section of Neuroscience and Anthropology, University of Cagliari, Cagliari, Italy
[b] Neuroscience Institute, National Research Council of Italy (CNR), Cagliari, Italy
[c] Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy
[d] Center of Excellence for Neurobiology of Dependence, University of Cagliari, Cagliari, Italy

ARTICLE INFO

Article history:
Received 7 August 2017
Received in revised form
24 January 2018
Accepted 29 January 2018
Available online 31 January 2018

Keywords:
Social isolation
HPA axis
Corticosterone
Glucocorticoid receptors
Endocannabinoids
Rat

ABSTRACT

We previously demonstrated that socially isolated rats at weaning showed a significant decrease in corticosterone and adrenocorticotropic hormone (ACTH) levels, associated with an enhanced response to acute stressful stimuli. Here we shown that social isolation decreased levels of total corticosterone and of its carrier corticosteroid-binding globulin, but did not influence the availability of the free active fraction of corticosterone, both under basal conditions and after acute stress exposure. Under basal conditions, social isolation increased the abundance of glucocorticoid receptors, while it decreased that of mineralocorticoid receptors. After acute stress exposure, socially isolated rats showed long-lasting corticosterone, ACTH and corticotrophin releasing hormone responses. Moreover, while in the hippocampus and hypothalamus of group-housed rats glucocorticoid receptors expression increased with time and reached a peak when corticosterone levels returned to basal values, in socially isolated rats expression of glucocorticoid receptors did not change. Finally, social isolation also affected the hypothalamic endocannabinoid system: compared to group-housed rats, basal levels of anandamide and cannabinoid receptor type 1 were increased, while basal levels of 2-arachidonoylglycerol were decreased in socially isolated rats and did not change after acute stress exposure. The present results show that social isolation in male rats alters basal HPA axis activity and impairs glucocorticoid-mediated negative feedback after acute stress. Given that social isolation is considered an animal model of several neuropsychiatric disorders, such as generalized anxiety disorder, depression, post-traumatic stress disorder and schizophrenia, these data could contribute to better understand the alterations in HPA axis activity observed in these disorders.

1. Introduction

Alterations in steroidogenesis and dysregulation of hypothalamic-pituitary-adrenal (HPA) axis function have been observed in several neuropsychiatric disorders (Heim and Nemeroff, 2001; Jacobson, 2014). Indeed, HPA axis hyperactivity may be implicated in melancholic depression (Gold and Chrousos, 2002), obsessive-compulsive disorder (Gustafsson et al., 2008), schizophrenia (Walker et al., 2008), and autism spectrum disorders (Jansen et al., 2006). In contrast, HPA axis activity seems to be blunted in post-traumatic stress disorder (PTSD) (Yehuda and Seckl, 2011) and atypical depression (Gold and Chrousos, 2002). Social isolation rearing, which involves isolation of the animals with no handling, starting at weaning and for an extended period, mimics several neuropsychiatric disorders. Thus, rats deprived of social contact with other rats at a young age experience a form of prolonged stress that leads to a plethora of behavioural, physiological, functional and molecular changes (Fone and Porkess, 2008; Pibiri et al., 2008; Marsden et al., 2011).

We have previously demonstrated that juvenile social isolation

* Corresponding author. Department of Life and Environment Sciences, Section of Neuroscience and Anthropology, University of Cagliari, Cagliari 09100, Italy.
E-mail address: giorgia.boero@gmail.com (G. Boero).
[1] Drs. Boero and Pisu contributed equally to this work.

https://doi.org/10.1016/j.neuropharm.2018.01.045
0028-3908/

decreased plasma concentrations of adrenocorticotropic hormone (ACTH) and corticosterone (CORT) (Serra et al., 2005; Pisu et al., 2016), and induced an enhanced response to acute stressful stimuli (Serra et al., 2000, 2003) in rats. It also increased the sensitivity of the pituitary to exogenous corticotrophin-releasing hormone (CRH), and it impaired HPA negative feedback tested with the dexamethasone suppression test (Serra et al., 2005). The general aim of this paper is to further characterize the hyperresponsiveness to acute stress, and to investigate the mechanisms involved in negative feedback regulation of the HPA axis in socially isolated (SI) rats.

The main effector of the stress response is represented by CRH release from hypothalamus, the most important secretagogue of ACTH, which stimulates the synthesis and secretion of corticosteroids from the adrenal glands (Tasker and Herman, 2011). Elevated CRH concentrations are found in patients affected by depression and anxiety disorders (Hauger et al., 2009); similarly, elevated CRH induces depression- and anxiety-like behaviour in animals (Bale and Vale, 2004). Likewise, ACTH and CORT are also dysregulated in several mood disorders. We thus examined the time course of foot-shock stress-induced changes in hypothalamic CRH, as well as plasma ACTH and CORT in SI rats. We specifically focused on variations in total and free CORT. In the systemic circulation CORT is coupled to different carriers, mainly corticosteroid binding globulin (CBG) (Hammond, 1995), whose major role has been proposed to be the maintenance of the circulating CORT pool to be delivered to target tissues (Moisan et al., 2014). In addition, CBG acts as a "buffer protein" to protect tissues from excess CORT, by sequestering it in an inactive complex (Perogamvros et al., 2012). Under stress conditions, only the free fraction of CORT binds to mineralocorticoid (MR) and glucocorticoid receptors (GR) in several brain targets; thus, changes in plasma CBG levels reflect the available CORT. Previous studies indicated that both acute and chronic stress exposure decreases CBG levels, leading to a substantial increase in free CORT (Tinnikov, 1999). We thus measured the total and free fractions of CORT, as well as CBG levels, in SI rats under basal conditions and 30 min after acute foot-shock stress exposure.

Suppression of stress-induced activation of HPA axis is exerted by CORT through two types of negative feedback regulation: a "slow" action, mediated by classical delayed transcriptional effects of intracellular receptors, and a "rapid" non-genomic action, which involves membrane-associated MR (mMR) and GR (mGR) (Di et al., 2003). We sought to investigate the effect of social isolation on mMR and mGR expression in the hippocampus and hypothalamus under basal conditions; we also examined the temporal availability of mGR after foot-shock stress in these same areas from SI and group-housed (GH) rats. Moreover, we tested GR function using the non-selective antagonist mifepristone.

We further evaluated the endocannabinoid (eCB) system by measuring the expression of cannabinoid receptors type 1 (CB1R), as well as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) concentrations in the hypothalamus of SI rats. In fact, glucocorticoid-induced eCB regulation of excitatory synaptic inputs to paraventricular nucleus (PVN) neuroendocrine cells seems to be a predominant mechanism in this area: activation of GR on CRH neurons stimulates the synthesis and release of AEA and 2-AG, which act as retrograde messengers on CB1R localized in glutamatergic and GABAergic interneurons (Di et al., 2003; Tasker and Herman, 2011). Given that 2-AG plays a key role in the activation of HPA axis negative feedback to terminate the stress response (Tasker and Herman, 2011; Morena et al., 2016), we also measured 2-AG content 30 min after foot-shock stress exposure. Finally, we explored CB1R activity with the antagonist AM251.

2. Material and methods

2.1. Animals

Male Sprague-Dawley CD rats (Charles River, Calco, IT) at 25–30 days of age (postnatal day PND 25–30), immediately after weaning, were housed for 30 days either in groups of five per cage (59 cm × 38 cm × 20 cm) (group-housed or GH rats), or individually in smaller cages (42 cm × 26 cm × 15 cm) (isolated or SI rats). They were maintained under an artificial 12-h light, 12-h dark cycle (light on 08:00–20:00 h) at a constant temperature of 23 ± 2 °C and 65% humidity. Food and water were freely available ad libitum. SI animals were left undisturbed in their cages and received the minimal handling associated with fortnightly husbandry. All the experiments were performed after the isolation period (PND 55–60), in the morning between 08:30 h and noon (see Fig. 1 for the timeline of the experimental procedures). Adequate measures were taken to minimize pain or discomfort of animals whose care and handling throughout the experimental procedures were in accordance with the European Parliament and the Council Directive of 22 September 2010 (2010/63/UE), and were approved by the Italian Ministry of Health according to the Italian Legislative Decree no. 26 of 4 March 2014.

2.2. Acute foot-shock stress

Animals were exposed to acute foot-shock stress at the end of the isolation period (SI rats) or at 55–60 days of age (GH rats, controls). Foot-shock stress consisted of a series of electrical impulses delivered in individual boxes with floors made of brass rods, 2 cm apart. Shocks (0.2 mA for 500 ms) were delivered every second over a period of 5 min. Separate groups of rats were sacrificed at different time points thereafter.

2.3. Pharmacological treatments

Mifepristone (#M8046, Sigma-Aldrich, Milan, Italy) was dissolved in absolute ethanol (5% final volume) and propylene glycol (Glavas et al., 2006). Animals received a single subcutaneous injection of mifepristone (120 mg/2 ml/kg bw) or vehicle (propylene glycol/5% ethanol) 60 min prior to foot-shock stress exposure, and were sacrificed 30 min later. Non-shocked controls were sacrificed 90 min after treatment.

AM251 (#S2819, Selleck Chemicals, Munich, Germany) was dissolved in DMSO, Tween-80 and physiological saline (0.9%) in a 1:1:8 ratio, respectively (Newsom et al., 2012). Rats received a single intraperitoneal injection of AM251 (2 mg/1 ml/kg bw) or vehicle (DMSO/Tween-80/0.9% saline) 30 min prior to foot-shock stress exposure, and were sacrificed 30 min later. Non-shocked controls were sacrificed 60 min after drug or vehicle injection.

2.4. ELISA

All animals were sacrificed by decapitation. Blood was collected from the trunk into K3-EDTA tubes, centrifuged at 900×g for 10 min at 4 °C and frozen at −80 °C until use. The enzyme-linked immunosorbent assay (ELISA) was used to quantify plasma CORT (#RE52211; IBL International, Hamburg, Germany), CBG (#SEB226Hu; Cloud-Clone Corp., Huston, TX, USA), and ACTH (#EK-001-21; Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) levels. ELISA assays were performed according to the manufacturer's instructions using a 96-well plate pre-coated with polyclonal antibodies against an antigenic site on the CORT, CBG or ACTH molecules. The kits also provided a seven-point standard curve using two-fold serial dilutions. Each sample was run in duplicate.

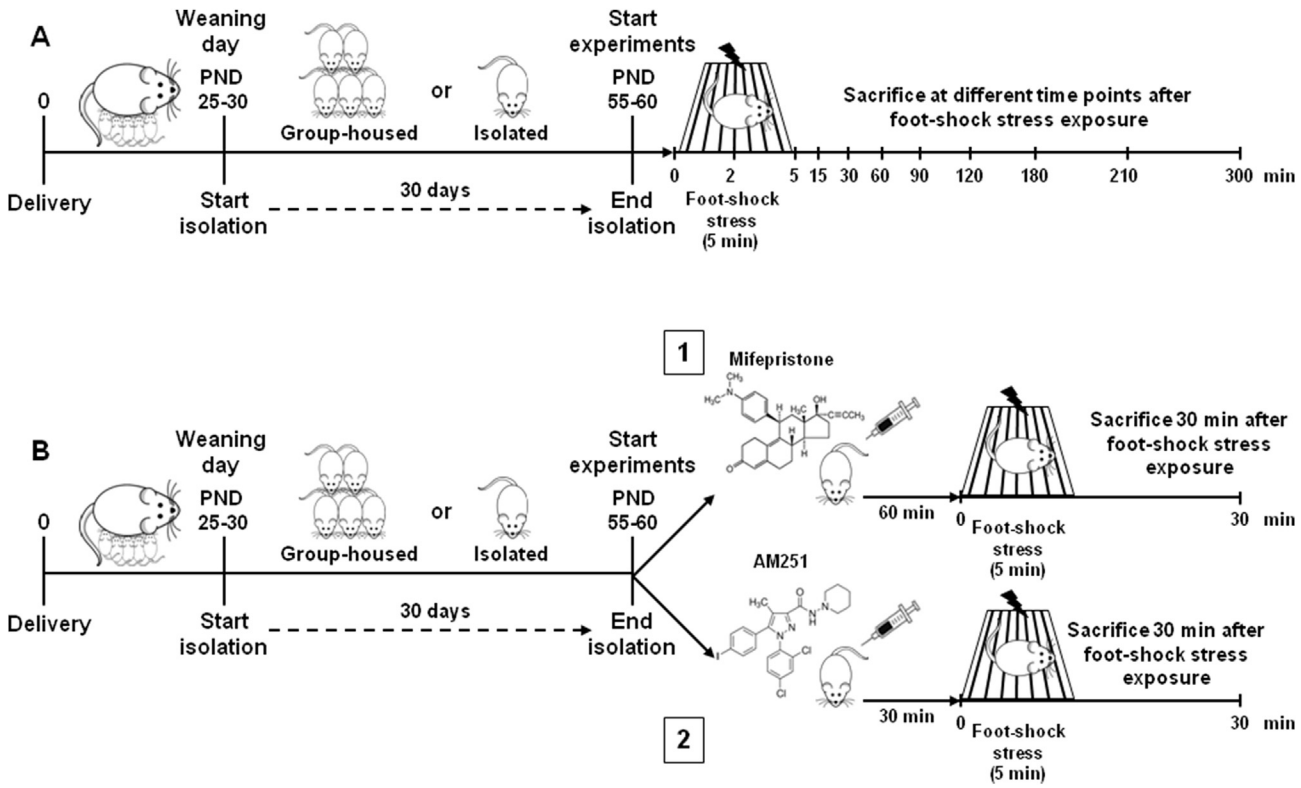

Fig. 1. Timeline of the experimental procedure. Panel A) Time course experiments. After weaning, animals were housed in group (5/6 per cage), or were isolated for 30 days. After this period, rats were exposed to foot-shock stress for 5 min, then sacrificed at the indicated time points for measurement of hormone levels and receptors abundance. Panel B) Pharmacological treatments. After the isolation or group-housing periods, rats were treated either with mifepristone (1), or with AM251 (2), were exposed to foot-shock stress 60 (1) or 30 (2) minutes later, and were sacrificed 30 min later to measure CORT levels.

CORT circulates bound to CBG (56–62 kDa) or albumin (65 kDa); thus, 300 μl of plasma was transferred to a Microcon™30 Centrifugal Filters (#MRCF0R030; Merck Millipore, Cork, Ireland), provided with a cellulose membrane that prevented passage of molecules larger than 30 kDa, and centrifuged (14,000 × g for 12 min at 25 °C) to obtain the free fraction of CORT.

2.5. Immunoblot assays

After decapitation, the brain was rapidly removed for dissection of the diencephalic region (hypothalamus), limbic region (hippocampus), and pituitary on frosted glass kept cold on crushed ice. The dissected tissue samples were immediately frozen with dry ice and stored at −80 °C until analysis. Total protein was extracted from hypothalamic and hippocampal tissue using a commercial kit to separate cytoplasmic, nuclear and membrane fractions (#BSP002; Membrane, Nuclear and Cytoplasmic Protein Extraction kit, Bio Basic Inc, Markham, Ontario, Canada); protein concentration in the extract was determined using a DC Protein Assay kit (Bio-Rad, Milan, Italy). The extract (40 μg of protein in 15 μl) was incubated for 10 min at 70 °C and then fractionated by SDS-polyacrylamide gel electrophoresis (NuPAGE Novex 4–12% Bis-Tris Midi Gel, Life Technologies, Monza, Italy). The separated proteins were transferred to a polyvinylidenedifluoride membrane (Immobilon-P; Millipore, Milan, Italy) with the use of a Criterion Blotter (Bio-Rad); the membrane was then incubated for 60 min at 25 °C with 5% non-fat dried milk in Tris-buffered saline containing 0.01% Tween-20, and then overnight at 4 °C with rabbit polyclonal antibodies to CRH (#sc-10718; Santa Cruz Biotechnology, Dallas, TX, USA), MR (#sc-11412; Santa Cruz Biotechnology), GR (#sc-1002; Santa Cruz Biotechnology) or CB1R (#209550; Calbiochem, Millipore). Antibody dilutions in 5% non-fat dried milk were 1:200 for CRH, MR and GR, and 1:500 for CB1R. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with mouse monoclonal antibodies (MAB-374; 1:1000 dilution; Millipore, Milan, Italy) as a loading control. The membrane was then washed with Tris-buffered saline containing 0.01% Tween-20 before incubation for 60 min at 25 °C with horseradish peroxidase–linked secondary antibodies to rabbit (#sc2004; Santa Cruz Biotechnology, 1:10000 dilution) or mouse (#sc2005; Santa Cruz Biotechnology, 1:5000 dilution) IgG in 5% non-fat dried milk. Immune complexes were detected with enhanced chemiluminescence reagents (GE Healthcare Biosciences). Optical density of the bands was determined with the use of an imaging system (Geliance 600; Perkin Elmer, Monza, Italy), and associated image acquisition (GeneSnap, Perkin Elmer) and analysis (GeneTools, Perkin Elmer) software. Values for CRH, MR, GR and CB1R were normalized by the corresponding GAPDH values.

2.6. Endocannabinoid analysis

Frozen hypothalamic tissues were homogenized and extracted in 50 mM chloroform/methanol/Tris-HCl, pH 7.5 (2:1:1, v/v) containing 2 μg of vitamin E and internal standards for AEA (200 ng) and 2-AG (300 ng), as [2H]8 AEA and [2H]5 2-AG deuterated isotopes (Cayman Chemical, Ann Arbor, MI, USA) (Di Marzo et al., 2001). Aliquots from the lipid-containing organic phase were dried down and reconstituted with 0.3 ml of 100% methanol. AEA and 2-AG were quantified by liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry [1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with MS Detector 6110 single quadrupole] using selected ion monitoring at

M+1 values for the two compounds and their deuterated homologs, as previously described (Piscitelli et al., 2011).

2.7. Statistical analysis

Quantitative data are presented as means ± SEM from 8 animals for each experimental group, except for the pharmacological treatments with mifepristone and AM251, where 10 rats per group were used. Differences in basal levels of each parameter between SI and GH rats were analyzed by unpaired Student's t-test. Three-way ANOVA was used to analyse CORT data, considering the housing condition (GH vs. SI), the plasma fraction (total vs. free) and the stress effects (shocked vs. non-shocked). Data from the time course experiments were analyzed by two-way ANOVA with housing condition (GH vs. SI) and time after stress exposure effects. Data from the pharmacology experiments were analyzed by three-way ANOVA with housing condition (GH vs. SI), drug treatment (drug vs. vehicle) and stress effect (shocked vs. non-shocked). Individual means have been compared with Newman–Keuls post hoc test; for each statistical analysis a value of $p < 0.05$ was considered statistically significant. Spearman's correlation was used to correlate hippocampal mGR abundance and total CORT in the time course experiments.

3. Results

3.1. Basal and stress-induced levels of plasma CORT and CBG in SI rats

Total and free CORT content was measured in SI and GH control rats. Two-way ANOVA revealed a significant effect of housing condition [$F(1,28) = 28.4$, $p = 0.000011$], a significant effect of CORT fraction [$F(1,28) = 99.2$, $p = 0.00001$], and a significant interaction between factors [$F(1,28) = 27.3$, $p = 0.000015$]. Social isolation resulted in a marked decrease in plasma total CORT concentration in male rats (98 ng/ml vs. 31 ng/ml, in GH and SI rats, respectively, −68%, $p < 0.01$, Fig. 2A). Plasma concentrations of free CORT, measured in the same samples, were significantly lower than those of total CORT in both GH and SI rats (1.85 ng/ml and 1.17 ng/ml, respectively, $p < 0.01$), and were not affected by social isolation (Fig. 2A). In agreement with the observed reduction in total CORT concentrations, social isolation decreased plasma CBG levels (566 nmol/L vs. 409 nmol/L, in GH and SI rats, respectively, −28%; [$t(14) = 5.949$, $p = 0.001$], Fig. 2B).

To evaluate CORT and CBG responses to acute stress, rats were exposed to foot-shock stress. Three-way ANOVA for CORT revealed a significant effect of housing condition [$F(1,56) = 4.28$, $p = 0.043$], a significant effect of CORT fraction [$F(1,56) = 75.25$, $p = 0.000001$], a significant effect of stress [$F(1,56) = 38.91$, $p = 0.000001$], a significant interaction between housing condition and CORT fraction [$F(1,56) = 4.27$, $p = 0.048$], a significant interaction between CORT fraction and stress [$F(1,56) = 42.62$, $p = 0.002$], but no interaction between the three factors (isolation, CORT fraction and stress) [$F(1,56) = 0.5921$, $p = 0.45$]. As expected (Serra et al., 2005), social isolation induced a greater increase in the total CORT response, measured 30 min from foot-shock stress exposure (from a basal value of 31 ng/ml to a stress value of 329 ng/ml), compared to GH rats (from a basal value of 98 ng/ml to a stress value of 246 ng/ml), +961% and +151%, in SI and GH rats, respectively, $p < 0.000002$ (Fig. 2C). In contrast, the free CORT response to foot-shock stress did not differ between GH and SI rats (Fig. 2C). Moreover, two-way ANOVA for CBG revealed a significant effect of housing condition [$F(1,28) = 21.8$, $p = 0.000016$], a significant effect of stress [$F(1,28) = 22.89$, $p = 0.00001$], and a significant interaction between factors [$F(1,28) = 15.88$, $p = 0.000018$]. Accordingly, CBG concentration after foot-shock stress exposure was decreased in GH rats (−40%, $p < 0.01$), while acute stress did not alter CBG levels in SI rats (Fig. 2D).

3.2. Time course of foot-shock stress-induced changes in total CORT levels in SI rats

Total CORT levels were measured at different time points after acute foot-shock stress exposure to evaluate the HPA axis activity. Two-way ANOVA revealed a significant effect of housing condition [$F(1,140) = 423.94$, $p = 0.001$], a significant effect of stress [$F(9,140) = 29.81$, $p = 0.001$], and a significant interaction between factors [$F(9,140) = 19.43$, $p = 0.001$]. In GH rats, total CORT levels (basal value: 109 ng/ml) in response to acute foot-shock stress peaked at 15 min (289 ng/ml, $p < 0.01$), were still elevated at 30 min (259 ng/ml, $p < 0.01$) and returned to baseline by 60 min (Fig. 3A). By contrast, in SI rats total CORT levels (basal value: 39 ng/ml) were dramatically elevated 5 min from stress exposure (258 ng/ml, $p < 0.01$), peaked at 60 min (471 ng/ml, $p < 0.01$) and remained significantly elevated up to 210 min from stress exposure (151 ng/ml, $p < 0.01$) (Fig. 3A).

3.3. Time course of foot-shock stress-induced changes in hypothalamic CRH concentrations in SI rats

We have previously demonstrated that social isolation increases pituitary sensitivity to exogenous CRH (Serra et al., 2005) and hypothalamic CRH content (Pisu et al., 2016). To test if the greater increase in total CORT concentrations and its prolonged response to acute stress in SI rats could be due to a different regulation in CRH content, we measured its changes across time following acute stress exposure. Two-way ANOVA revealed a significant effect of housing condition [$F(1,112) = 381.17$, $p = 0.00001$], a significant effect of stress [$F(7,112) = 33.46$, $p = 0.00001$], and a significant interaction between factors [$F(7,112) = 38.59$, $p = 0.00001$]. Acute foot-shock stress increased hypothalamic CRH to a greater extent in SI rats compared to GH rats. Moreover, while in GH rats CRH content peaked at 2 min (+90%, $p < 0.05$) and returned to baseline at 5 min, in SI rats its levels rose at 2 min (+102%, $p < 0.05$), peaked at 30 min (+474%, $p < 0.01$), remained elevated at 90 min (+163%, $p < 0.01$), and returned to baseline at 300 min (Fig. 3B).

3.4. Time course of foot-shock stress-induced changes in plasma ACTH concentrations in SI rats

The greater release in CRH and total CORT induced by acute foot-shock stress in SI rats is accompanied by a greater and prolonged increase in plasma ACTH concentrations. Two-way ANOVA revealed a significant effect of housing condition [$F(1,84) = 1097.7$, $p = 0.000001$], a significant effect of stress [$F(5,84) = 408.5$, $p = 0.000001$], and a significant interaction between factors [$F(5,84) = 286.4$, $p = 0.00001$]. In GH rats (basal value: 4.5 ng/ml), plasma ACTH levels peaked 2 min after acute foot-shock stress exposure (7.4 ng/ml, $p < 0.01$), then decreased and returned to baseline levels 15 min after stress (Fig. 3C). In SI rats (basal value: 3.1 ng/ml), ACTH levels were markedly elevated by acute foot-shock stress; they also peaked at 2 min (27.4 ng/ml, $p < 0.01$), but remained significantly elevated at 5 and 15 min (14.0 ng/ml and 12.8 ng/ml, respectively, $p < 0.01$), and slowly returned to baseline 300 min from stress exposure (Fig. 3C).

3.5. Effect of social isolation on basal and stress-induced expression of hippocampal mMR and mGR

The greater CRH response to acute stress suggests that social

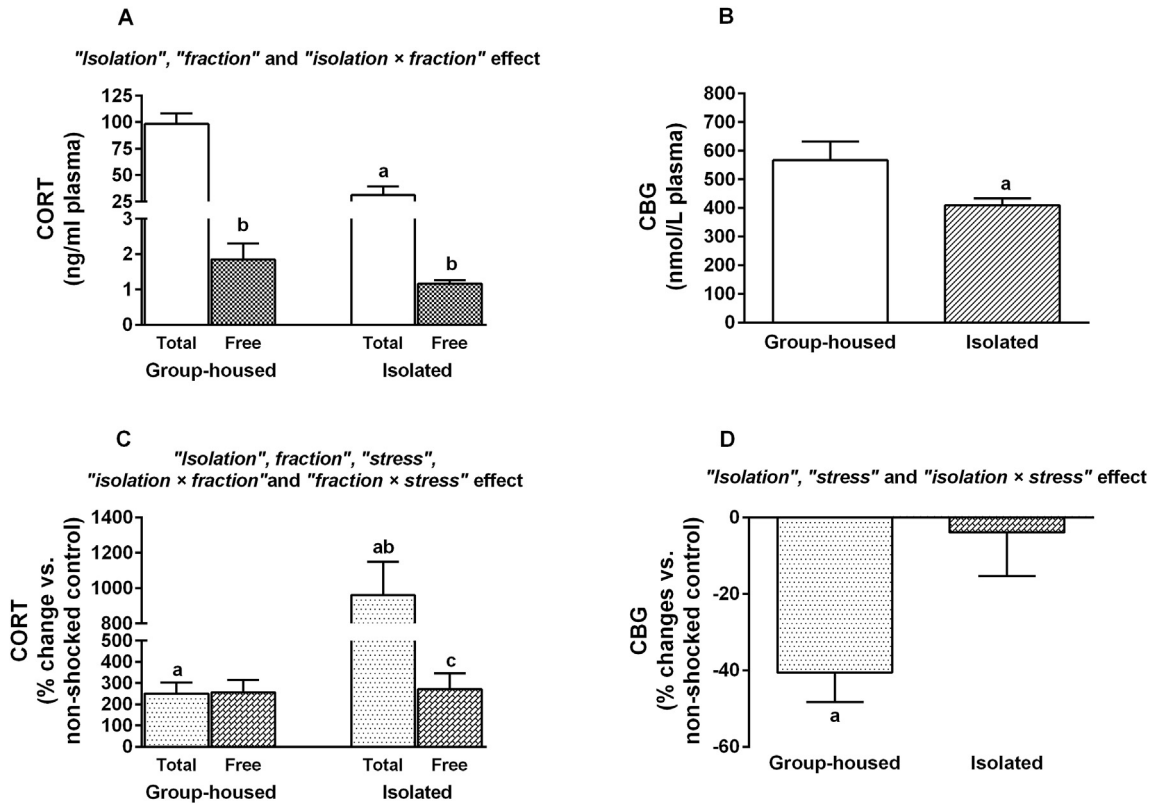

Fig. 2. Basal and acute stress-induced changes in plasma CORT and CBG concentrations in socially isolated rats. Panel A) Effect of social isolation on total and free plasma CORT levels. Data are expressed in ng/ml and were analyzed by two-way ANOVA (housing × CORT fraction conditions) followed by Newman-Keuls post-hoc test. [a]p<0.01 vs. the respective group-housed rats; [b]p < 0.01 vs. the respective total CORT fraction. Panel B) Effect of social isolation on plasma CBG levels. Data are expressed in nmol/ml and were analyzed by unpaired Student's t-test. [a]p<0.01 vs. group-housed rats. Panel C) Effect of foot-shock stress exposure on total and free plasma CORT levels. Data are expressed as the stress-induced percent change in the respective CORT fraction vs. the corresponding basal levels in non-shocked controls. Basal values for CORT are: GH Total = 98 ng/ml; GH Free = 1.85 ng/ml; SI Total = 31 ng/ml; SI Free = 1.17 ng/ml. Data were analyzed by three-way ANOVA (housing × stress conditions x CORT fraction) followed by Newman-Keuls post-hoc test. [a]p<0.01 vs. the respective non-shocked controls; [b]p < 0.01 vs. total CORT in group-housed rats; [c]p < 0.01 vs. the respective total CORT in socially isolated rats. Panel D) Effect of foot-shock stress exposure on CBG levels. Data are expressed as the stress-induced percent change vs. the corresponding basal levels in non-shocked controls. Basal CBG levels are: GH = 566 nmol/l; SI = 409 nmol/l. Data were analyzed by two-way ANOVA (housing × stress conditions) followed by Newman-Keuls post-hoc test. [a]p<0.01 vs. the respective non-shocked control.

isolation might alter the glucocorticoid-mediated regulation of CRH release. We thus measured the expression of mGR and mMR in hippocampal membrane preparations. Immunoreactive bands to GR and MR were obtained at ~95 kDa and ~102 kDa, respectively, consistent with the molecular weight of the corresponding antigens. Under basal conditions, social isolation induced a significant increase in hippocampal mGR abundance (+85%, [t(14) = 3.941, p < 0.0004], Fig. 4A), and a significant decrease in mMR expression (−41%, [t(14) = 2.805, p < 0.05], Fig. 4B). Given the key role played by GR in the response to acute stress, we evaluated the abundance of hippocampal mGR in SI and GH rats exposed to acute foot-shock stress. Two-way ANOVA revealed a significant effect of housing condition [F(1,98) = 14.072, p = 0.001], no significant effect of stress [F(6,98) = 1.098, p = 0.37], but a significant interaction between factors [F(6,98) = 3.69, p = 0.004]. In fact, acute foot-shock stress exposure in GH animals slowly increased mGR expression across time, which reached a peak 90 min after stress exposure (+129%, p < 0.01), and returned to baseline by 300 min (Fig. 4C). At variance, in SI rats mGR expression did not change at any of the time points examined (Fig. 4C). Interesting, in GH, but not SI animals, we found a negative correlation between stress-induced changes in mGR abundance and total CORT concentrations across time (rs = −0.497, n = 48, p < 0.01; 95% CI ranging from −0.6947 to −0.2298) (Fig. 4D).

3.6. Effect of social isolation on basal and stress-induced expression of hypothalamic mGR

Hypothalamic GR are localized on CRH neurons and exert an indirect control on CRH release through the endocannabinoid system. To test if the greater CRH release induced by acute stress exposure in SI rats might be due to an impairment in this circuit, we first evaluated mGR expression in this area. Immunoreactive bands to GR were obtained at ~95 kDa, consistent with the molecular weight of the corresponding antigen. Similar to the changes observed in hippocampus, social isolation induced a significant increase in basal mGR abundance in hypothalamus (+96%, [t(14) = 2.185, p = 0.04]; Fig. 5A). Following acute stress exposure, two-way ANOVA revealed a significant effect of housing condition [F(1,98) = 6.59, p = 0.01], no significant effect of stress [F(6,98) = 1.21, p = 0.31], and no significant interaction between factors [F(6,98) = 0.23, p = 0.95]. In GH animals mGR levels increased with time, reaching a peak 90 min after stress exposure (+89%, p < 0.05; Fig. 5B), similar to the changes observed in the hippocampus of the same rats. By contrast, in SI rats, acute foot-shock stress failed to modify hypothalamic mGR expression at any of the time points examined (Fig. 5B).

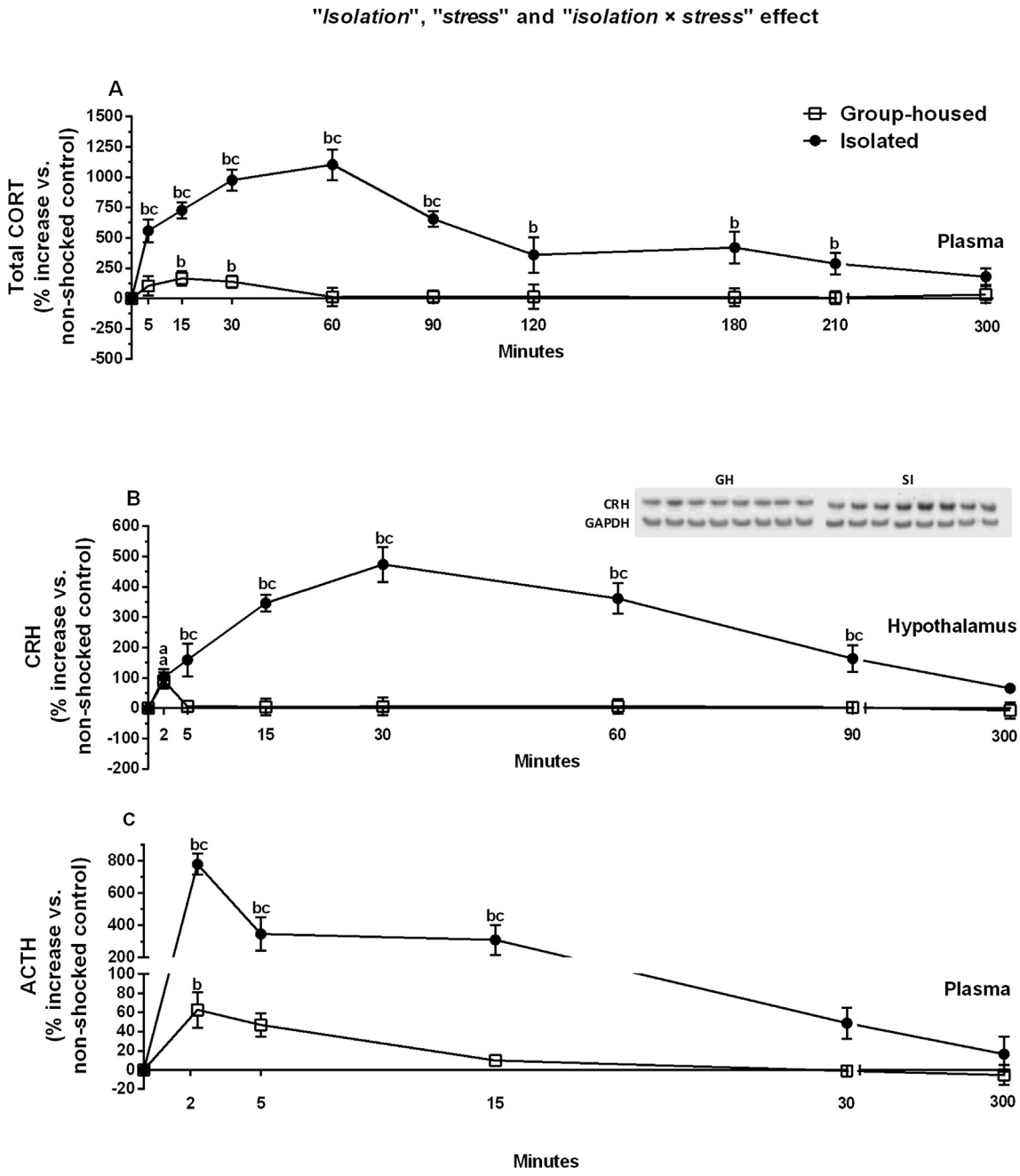

Fig. 3. Time course of foot-shock stress-induced changes on CORT, CRH and ACTH levels in socially isolated rats. Group-housed and socially isolated rats were subjected to acute foot-shock stress and were sacrificed at the indicated time points after stress exposure for measurement of plasma CORT levels (Panel A), hypothalamic CRH content (Panel B), and plasma ACTH levels (Panel C). Data are expressed as percent change vs. the corresponding basal levels in non-shocked controls, and are the mean ± SEM of values from 8 rats per group per time point. Basal values are: CORT, GH = 109 ng/ml, SI = 39 ng/ml; ACTH, GH = 4.54 ng/ml, SI = 3.14 ng/ml. Two-way ANOVA (housing × stress conditions), followed by Newman-Keuls post-hoc test. [a]p<0.05, [b]p < 0.01 vs. the respective non-shocked control; [c]p < 0.01 vs. the respective time point in group-housed rats.

3.7. Effect of acute mifepristone administration on stress-induced plasma CORT levels

Given that social isolation increased hippocampal and hypothalamic GR expression, we hypothesized that an impairment in GR function might occur in SI animals. Thus, we used mifepristone, a non-selective GR antagonist, to evaluate stress-induced plasma CORT concentrations in a GR-blocked condition. Three-way ANOVA revealed a significant effect of housing condition [$F(1,72) = 361.44$, $p = 0.0001$], a significant effect of drug treatment [$F(1,72) = 31.33$, $p = 0.0001$], a significant effect of stress [$F(1,72) = 4018.03$, $p = 0.0001$], a significant interaction between housing condition x drug treatment [$F(1,72) = 5.613$, $p = 0.021$], a significant interaction between housing condition x stress [$F(1,72) = 573.04$, $p = 0.0001$], a significant interaction between drug treatment x stress [$F(1,72) = 15.566$, $p = 0.0002$], and a significant interaction between the three factors [$F(1,72) = 43.225$, $p = 0.0001$]. Under non stress conditions, mifepristone slightly increased basal CORT levels in GH rats (+18%, $p < 0.05$, Fig. 6). Further, as expected (van Haarst et al., 1997), mifepristone treatment resulted in an enhanced CORT

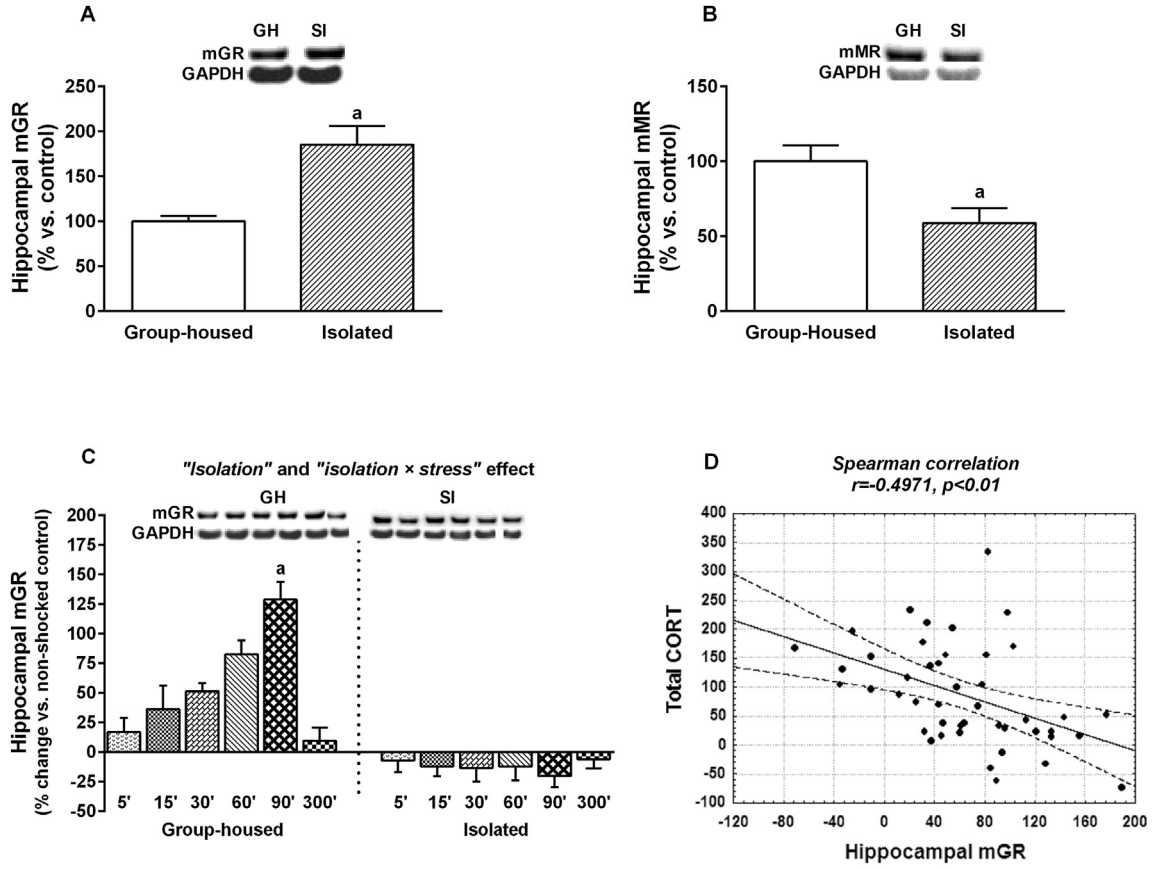

Fig. 4. Effect of social isolation on basal and stress-induced hippocampal mMR and mGR in male rats. Panel A) Basal expression of hippocampal mGR. Densitometric quantitation of mGR was normalized by the corresponding amount of GAPDH; data were expressed as percentage vs. group-housed rats, and were analyzed by unpaired Student's t-test. [a]p<0.01 vs. group-housed rats. Panel B) Basal expression of hippocampal mMR. Densitometric quantitation of mMR was normalized by the corresponding amount of GAPDH; data were expressed as percentage vs. group-housed rats, and were analyzed by unpaired Student's t-test. [a]p<0.05 vs. group-housed rats. Panel C) Time course of foot-shock stress-induced changes in hippocampal mGR expression. Densitometric quantitation of mGR was normalized by the corresponding amount of GAPDH; data were expressed as percent change vs. the respective non-shocked control, and were analyzed by two-way ANOVA followed by Newman-Keuls post-hoc test. [a]p<0.05 vs. the respective non-shocked control. Panel D) Spearman correlation between foot-shock stress-induced changes in plasma CORT and hippocampal mGR levels in group-housed rats. $r(48) = -0.4971$, $p < 0.01$.

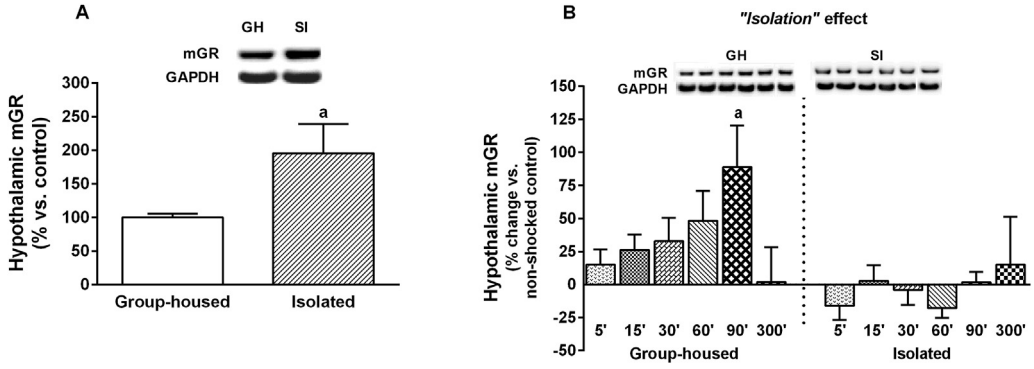

Fig. 5. Effect of social isolation on basal and stress-induced hypothalamic mGR in male rats. Panel A) Basal expression of hypothalamic mGR. Densitometric quantitation of mGR was normalized by the corresponding amount of GAPDH; data were expressed as percentage vs. group-housed rats, and were analyzed by unpaired Student's t-test. [a]p<0.05 vs. group-housed rats. Panel B) Time course of foot-shock stress-induced changes in hypothalamic mGR expression. Densitometric quantitation of mGR was normalized by the corresponding amount of GAPDH; data were expressed as percent change vs. the respective non-shocked control, and were analyzed by two-way ANOVA followed by Newman-Keuls post-hoc test. [a]p<0.05 vs. the respective non-shocked control.

response to stress in GH rats (mifepristone + foot-shock vs. vehicle + foot-shock rats, +578%, $p < 0.01$; Fig. 6). In contrast, in SI rats mifepristone did not affect basal and stress-induced CORT concentrations, compared to respective vehicle-treated SI rats (Fig. 6).

3.8. Effect of social isolation and acute foot-shock stress on the endocannabinoid system in the hypothalamus

CORT, by binding to GR localized on CRH neurons, induces the release of eCBs that, through a retrograde mechanism, act on

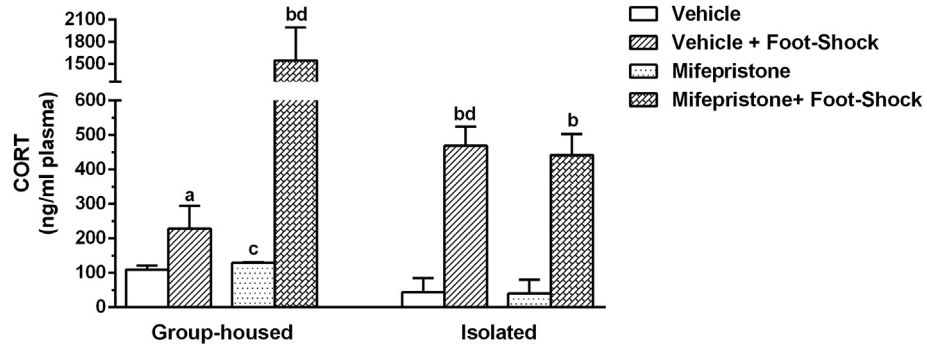

Fig. 6. Effect of mifepristone treatment on the CORT response to stress. Group-housed and socially isolated rats were subcutaneously injected with mifepristone or vehicle 60 min before exposure to foot-shock stress, and were sacrificed 30 min thereafter. Non-shocked controls were sacrificed 90 min after injection. Data are mean ± SEM of values from 10 rats per group and are expressed as ng/ml of plasma. Data were analyzed by three-way ANOVA (housing × drug treatment × stress conditions) followed by Newman-Keuls post-hoc test. [a]p<0.05, [b]p < 0.01 vs. the respective non-shocked controls; [c]p < 0.05 vs. group-housed vehicle-treated rats; [d]p < 0.01 vs. group-housed vehicle-treated + foot-shock rats.

glutamatergic neurons to reduce CRH release (Tasker and Herman, 2011). To test if the greater CRH increase observed in SI rats following acute foot-shock stress may be due to alterations in this microcircuit, we measured AEA, 2-AG and CB1R levels in the hypothalamus. Under basal conditions, social isolation induced a significant increase in AEA concentrations (+77%, p < 0.05; Fig. 7A), while it reduced 2-AG levels (−56%, p < 0.01; Fig. 7B). Immunoreactive bands to CB1R were obtained at ~60 kDa, consistent with the size of the corresponding antigens. Social isolation significantly increased the abundance of basal hypothalamic CB1R (+118%, [t(14) = 5.554, p = 0.001], Fig. 7C) compared to GH controls.

Given that compelling evidence indicates that 2-AG mediates the termination of the stress response through activation of the HPA negative feedback (Tasker and Herman, 2011; Morena et al., 2016), we measured hypothalamic 2-AG levels after acute stress in order to clarify if social isolation-induced changes in eCBs

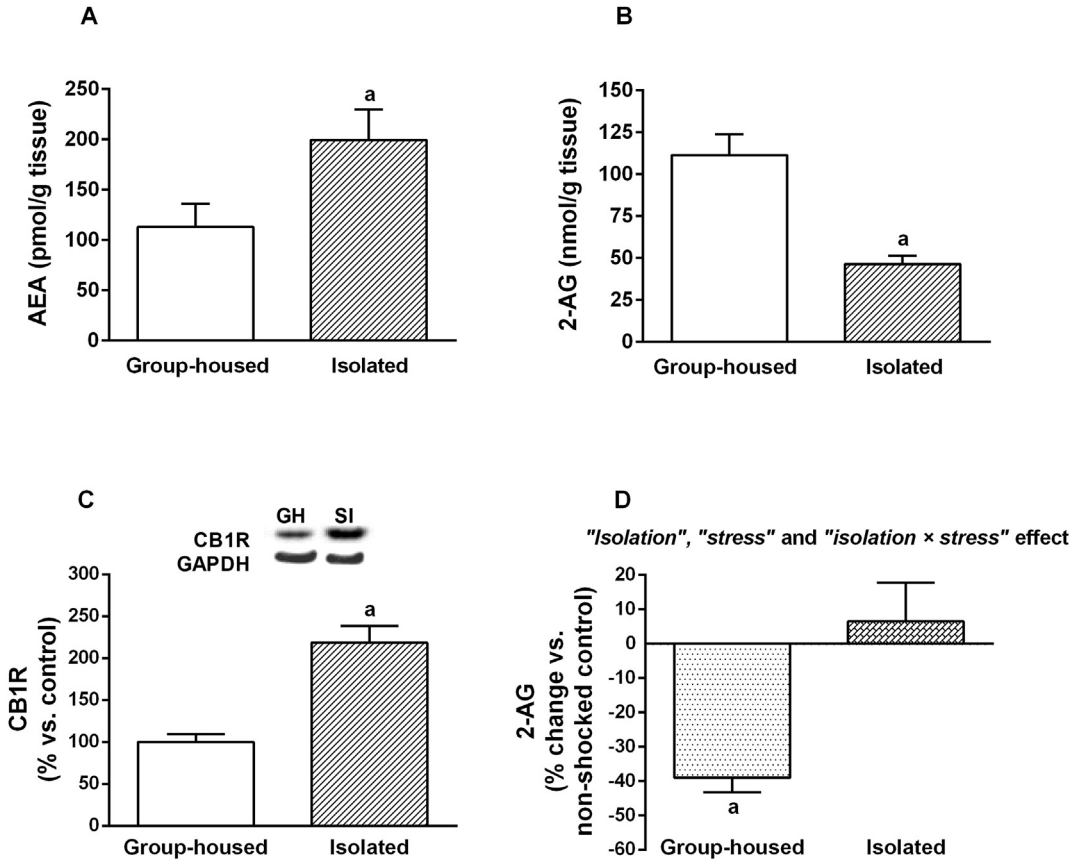

Fig. 7. Effect of social isolation and foot-shock stress on the endocannabinoid system in the hypothalamus. Panel A) Effect of social isolation on AEA concentrations. Data are expressed as pmol/g tissue and were analyzed by unpaired Student's t-test. [a]p<0.05 vs. group-housed (GH) rats. Panel B) Effect of social isolation on 2-AG concentrations. Data are expressed as nmol/g tissue and were analyzed by unpaired Student's t-test. [a]p<0.01 vs. group-housed rats. Panel C) Basal expression of hypothalamic CB1R. Densitometric quantitation of CB1R was normalized by the corresponding amount of GAPDH; data were expressed as percentage vs. group-housed rats, and were analyzed by unpaired Student's t-test. [a]p<0.01 vs. group-housed rats. Panel D) Effect of foot-shock stress exposure on 2-AG levels. Data are expressed as stress-induced percent change vs. the corresponding non-shocked control, and were analyzed by two-way ANOVA (housing × stress conditions) followed by Newman-Keuls post-hoc test. [a]p<0.05 vs. the respective non-shocked control.

activity are involved in HPA axis negative feedback dysregulation in these animals. Two-way ANOVA revealed a significant effect of housing condition [$F(1,28) = 30.39$, $p = 0.0001$], a significant effect of stress [$F=(1,28) = 7.08$, $p = 0.01$], and a significant interaction between factors [$F(1,28) = 9.33$, $p = 0.004$]. 30 min after foot-shock stress exposure, 2-AG levels were significantly decreased in GH rats (−39% vs. the respective non-shocked control, $p < 0.05$), but no changes were observed in SI animals (Fig. 7D).

3.9. Effect of acute administration of AM251 on basal and stress-induced plasma CORT levels

To clarify the eCB system functionality, we blocked CB1R with the antagonist AM251, and evaluated the CORT response under basal conditions and after acute stress exposure. Three-way ANOVA revealed a significant effect of housing condition [$F(1,72) = 35.66$, $p = 0.0001$], a significant effect of drug treatment [$F(1,72) = 2027.1$, $p = 0.0001$], a significant effect of stress [$F(1,72) = 10436.08$, $p = 0.0001$], a significant interaction between housing condition x drug treatment [$F(1,72) = 1133.03$, $p = 0.0001$], a significant interaction between housing condition x stress [$F(1,72) = 163.09$, $p = 0.0001$], a significant interaction between drug treatment x stress [$F(1,72) = 1347.71$, $p = 0.0001$], and a significant interaction between the three factors [$F(1,72) = 739.14$, $p = 0.001$]. As expected (Newsom et al., 2012), in GH rats AM251 increased basal CORT levels (+64%, $p < 0.01$), as well as the stress-induced CORT response (AM251 + foot-shock vs. vehicle + foot-shock rats +293%, $p < 0.01$; Fig. 8). By contrast, administration of AM251 did not affect basal and stress-induced CORT concentrations in SI rats (Fig. 8).

4. Discussion

The present study showed that social isolation of male rats from weaning, without additional stressors, or after acute stress, did not change the pool size of CORT available for accessing to target tissue. However, SI rats showed a delayed shut-off of the response to acute stress, indicative of reduced feedback efficacy; they also showed an altered mMR and mGR expression in the hypothalamus and hippocampus. Moreover, social isolation induced a dysregulation of eCB signalling in the hypothalamus, which is responsible for the direct feedback inhibition of the HPA axis by glucocorticoids.

Basal total CORT levels 30 days after social isolation were significantly decreased, as previously demonstrated (Pisu et al., 2016), a finding consistent with the decrease in plasma concentrations of ACTH, previously reported in SI animals (Serra et al., 2005). These results are in agreement with other studies showing the inability of continuously stressed rats to maintain elevated plasma ACTH and CORT levels (Rivier and Vale, 1987; Allen et al., 2011). Juvenile social isolation represents an animal model of neuropsychiatric disorders (Fone and Porkess, 2008). Accordingly, low basal cortisol levels were found in patients affected by atypical depression (Gold and Chrousos, 2002), PTSD (Yehuda and Seckl, 2011), and in suicide attempters (Keilp et al., 2016). Moreover, in SI rats we also observed a reduction in basal levels of CBG, consistent with studies showing that deficiency of this glycoprotein, the main carrier of circulating corticosteroids (Hammond, 1995), is paralleled by a reduction in total circulating CORT (Richard et al., 2010; Moisan et al., 2014). Given that CBG concentrations may affect levels of free CORT available to enter tissues (Mendel, 1989), the decrease in circulating CBG levels in SI rats would represent an adaptive change to ensure the maintenance of free hormone required for biological functions. Similar mechanisms have previously been described; for instance, the decrease in CBG content after acute stress, observed in GH rats, is a physiological response necessary to increase the free CORT fraction able to bind its receptor, in order to exert metabolic effects and to trigger the HPA negative fast feedback (Fleshner et al., 1995; Deak et al., 1999; Tinnikov, 1999). Hence, in GH animals, the decrease in CBG levels following foot-shock stress is crucial in promoting CORT access to and activation of GR; therefore, total CORT epitomizes the amount of hormone available. At variance, in SI animals, CBG levels were unchanged 30 min after foot-shock stress, resulting in free CORT concentrations similar to those found in GH animals in spite of the huge amount of total CORT. These results suggest that the regulation of available CORT levels after stress is preserved following social isolation; therefore, one could expect an efficient HPA axis negative feedback to turn off acute stress responses in SI animals. However, the time course of total CORT concentrations after foot-shock stress exposure revealed that SI rats showed a long-lasting response. CORT levels reached a peak 60 min after stress exposure, and remained elevated up to 210 min in SI rats, while in GH animals CORT returned to basal values 60 min after foot-shock stress. Likewise, in SI rats acute foot-shock stress exposure induced a greater and prolonged increase in CRH and ACTH levels over time, indicating that regulation of the HPA axis is altered by social isolation.

While MR sustain basal HPA axis activity and are mostly involved in setting the threshold for stress responsiveness (de Kloet and Reul, 1987), the extinction of the stress response is mediated, in part, by the negative feedback loop by way of GR (de Kloet et al.,

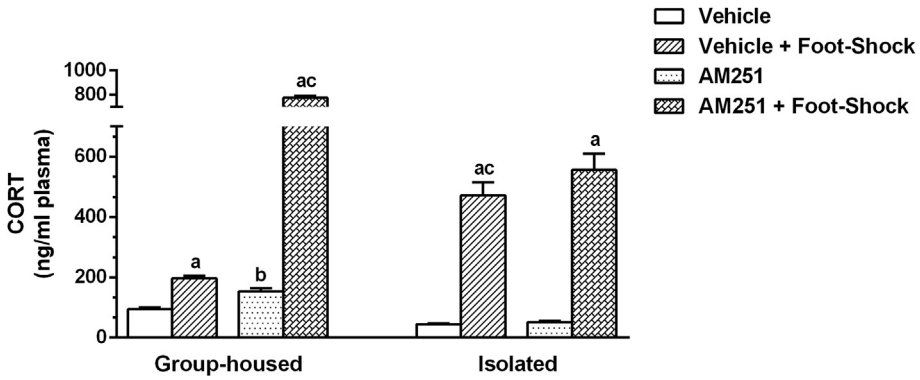

Fig. 8. Effect of AM251 treatment on the CORT response to stress. Group-housed and socially isolated rats were intraperitoneally injected with AM251 or vehicle 30 min before exposure to foot-shock stress, and were sacrificed 30 min thereafter. Non-shocked controls were sacrificed 60 min after injection. Data are mean ± SEM of values from 10 rats per group and are expressed as ng/ml of plasma. Data were analyzed by three-way ANOVA (housing × drug treatment × stress conditions) followed by Newman-Keuls post-hoc test. [a]$p<0.01$ vs. the respective non-shocked controls; [b]$p < 0.01$ vs. group-housed vehicle-treated rats; [c]$p < 0.01$ vs. group-housed vehicle-treated + foot-shock rats.

1998; Herman et al., 2012). It has been postulated that a disruption in the balance between central MR and GR leads to a dysregulation of the stress system (de Kloet et al., 2005). Social isolation induced a downregulation of hippocampal mMR, and an upregulation of hippocampal and hypothalamic mGR; given the role of hippocampal MR in controlling the inhibitory tone along the HPA axis (de Kloet and Reul, 1987), the decrease in its expression may account for the reduced resilience to a novel acute stress observed in SI rats. Accordingly, it has been shown that overexpression of MR confers resilience to the effects of chronic stress on hippocampus-dependent function and structural plasticity (Kanatsou et al., 2015). The reduced expression of hippocampal mMR, and the increased expression of mGR in both hippocampus and hypothalamus, are in agreement with a previous observation by Orlowsky and coll. (2014) that demonstrated similar changes in MR and GR mRNA levels after chronic restraint stress. Moreover, adult social isolation leads to increased hippocampal GR measured in nuclear fractions (Djordjevic et al., 2009). The significance of the observed overexpression in GR mRNA has been linked to the stress-induced hippocampal neuronal loss (Orlovsky et al., 2014). In humans, an increase in GR expression is associated to a significant reduction in the levels of DNA methylation at the promoter of the gene that encodes GR (NR3C1) in blood samples from male combat veterans that developed PTSD (Yehuda et al., 2015); interestingly, a decrease in basal cortisol was also found in these individuals (Yehuda et al., 2015), and SI mice have been proposed as an animal model of this disease (Pibiri et al., 2008).

A wealth of research suggests a role for GR in the fast feedback mechanisms (de Kloet et al., 1998; Herman et al., 2012, 2016); however, most of the current research on stress-modulated HPA axis activity has focused on the consequent changes in cytoplasmic/nuclear GR signalling. Our results in GH animals show that mGR expression increased with time following acute foot-shock stress exposure, resulting significantly elevated 90 min thereafter. It is interesting to observe that in these animals the pattern of mGR expression following acute stress was negatively correlated with total CORT levels; mGR expression reached a peak when CORT levels are returning to baseline, suggesting that an increased availability of mGR is required to reduce CORT concentrations and to terminate the stress response (de Kloet et al., 1998). At variance, we found no change in hippocampal mGR expression in SI rats at any of the time points examined, suggesting that social isolation results in a temporal unavailability of mGR that causes a delay in the activation of the HPA axis negative feedback. A similar scenario has been observed in the hypothalamus. In this area, a critical interplay between mGR and nuclear GR has been hypothesized, with nuclear GR that would be responsible for transducing the rapid steroid response at the membrane (Nahar et al., 2015). These authors suggest that the rapid glucocorticoid effects mediated by mGR depend on the functional expression of nuclear GR. Thus, the evidence that in GH animals blocking of nuclear GR with mifepristone resulted in an increased CORT response to acute stress, while mifepristone had no effect in SI rats, suggests that nuclear GR function is also reduced in these animals. However, acute stress in SI rats increases CORT levels to values a little below the ones induced by mifepristone in stressed GH rats, raising the possibility that the CORT increase 30 min from acute stress may have reached a ceiling effect in SI rats.

Given that the rapid glucocorticoid-induced retrograde eCBs inhibition of glutamatergic synapses is dependent on both membrane and nuclear GR function, we postulated that impairment in this mechanism may occur in SI rats. Accordingly, these animals showed a decrease in basal hypothalamic 2-AG concentrations and an increase in both AEA levels and CB1R expression in the same area. These results are in agreement with the study by Robinson and coll. (2010) showing an increase in CB1R mRNA levels and alterations in the levels of the enzymes responsible for the synthesis and degradation of AEA and 2-AG in hypothalamic nuclei of SI rats. Specifically, they found an increase in the enzymes responsible for the synthesis of AEA (NAPE-PLD), a decrease in DAGLα, responsible for the synthesis of 2-AG, and an increase in MAGL, the enzyme that degrades this endocannabinoid (Robinson et al., 2010). The increase in the abundance of hypothalamic CB1R was unpredicted since blockade of these receptors results in a strong potentiation of CORT release in response to acute stress (Morena et al., 2016), and SI rats are hyperresponsive to foot-shock stress. Nevertheless, the function of these receptors seems to be decreased as demonstrated by the evidence that blocking CB1R with AM251 increased HPA axis responsiveness to acute stress in GH but not in SI rats. Interestingly, increased CB1R expression was also found in a number of brain regions in schizophrenic patients (Newell et al., 2006), who also show higher circulating levels of AEA (De Marchi et al., 2003); our data demonstrated that these changes are mirrored in SI rats, thus endorsing the face validity of social isolation as an animal model of schizophrenia (Fone and Porkess, 2008). The decrease in hypothalamic 2-AG levels. observed in SI rats, is in contrast with previous studies that reported an increase in the same area in mice subjected to repeated social stress (Dubreucq et al., 2012), but it is in agreement with the increased MAGL mRNA in the ventrolateral hypothalamic nucleus of SI rats (Robinson et al., 2010). Moreover, given that SI rats manifest an anxiety-like profile in several anxiometric tests (Serra et al., 2000; Fone and Porkess, 2008), the decrease in 2-AG levels found in these animals is consistent with the evidence that chronic MAGL inhibition prevented chronic stress-induced anxiety-like behaviour (Sumislawski et al., 2011). Foot-shock stress did not change 2-AG content in the hypothalamus of SI rats, suggesting that social isolation alters eCB-mediated signalling in the hypothalamus, likely inducing an impairment in glutamatergic and GABAergic inputs that control CRH release (Tasker and Herman, 2011; present data), and hence contributing to the impairment of the fast feedback inhibition of the HPA axis. By contrast, in GH animals, acute stress significantly decreased 2-AG levels; this result is in agreement with previous studies that showed a reduction in hypothalamic 2-AG after acute restraint stress in mice (Patel et al., 2004), suggesting that the mechanism leading to stress-induced changes in 2-AG levels may be common to foot-shock and restraint stressors. However, other studies have shown either an increase or no change in 2-AG content following different stressful stimuli (Morena et al., 2016). This variability may be a consequence of the different stressors and/or the different time points for tissue collection after the final stress.

5. Conclusions

In conclusion, our results indicate that social isolation alters basal HPA axis activity and impairs the glucocorticoid-mediated negative feedback after acute stress. Given that social isolation is considered an animal model of several psychiatric disorders such as generalized anxiety disorders, depression, PTSD and schizophrenia (Parker and Morinan, 1986; Karim and Arslan, 2000; Weiss et al., 2004; Skelly et al., 2015), these data could contribute to better understand the alterations in HPA axis activity observed in these disorders.

Contributors

BG performed part of the biochemical experiments and of the statistical analysis, and wrote the first draft of the manuscript. PMG performed part of the biochemical experiments and of the statistical analysis and edited the revision of the manuscript. BF

performed part of the immunoblot analysis. ML and CG performed the endocannabinoids analysis. BS supervised the endocannabinoids analysis. PE assisted with animal treatment. FP and CA contributed to the interpretation of the data. PP assisted with interpretation of the data and provided input on subsequent drafts of the manuscript. SM designed the study and supervised the experiments and the manuscript writing. All authors contributed to and have approved the final manuscript.

Role of the funding source

The study was funded by a research grant from Faculty Resources Grant, University of Cagliari to SM and a grant from the "Enrico ed Enrica Sovena Foundation" awarder to BG. None of the funding sources had any role in study design, analyses, and interpretation of results.

Conflicts of interest

All authors declare that they have no conflict of interest.

References

Allen, C.D., Lee, S., Koob, G.F., Rivier, C., 2011. Immediate and prolonged effects of alcohol exposure on the activity of the hypothalamic-pituitary-adrenal axis in adult and adolescent rats. Brain Behav. Immun. 25 (S1), 50–60.
Bale, T.L., Vale, W.W., 2004. CRF and CRF receptors: role in stress responsivity and other behaviors. Annu. Rev. Pharmacol. Toxicol. 44, 525–557.
de Kloet, E.R., Joëls, M., Holsboer, F., 2005. Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. 6 (6), 463–475.
de Kloet, E.R., Vreugdenhil, E., Oitzl, M.S., Joëls, M., 1998. Brain corticosteroid receptor balance in health and disease. Endocr. Rev. 19 (3), 269–301.
de Kloet, E.R., Reul, J.M., 1987. Feedback action and tonic influence of corticosteroids on brain function: a concept arising from the heterogeneity of brain receptor systems. Psychoneuroendocrinology 12 (2), 83–105.
De Marchi, N., De Petrocellis, L., Orlando, P., Daniele, F., Fezza, F., Di Marzo, V., 2003. Endocannabinoid signalling in the blood of patients with schizophrenia. Lipids Health Dis. 19 (2), 5.
Deak, T., Nguyen, K.T., Cotter, C.S., Fleshner, M., Watkins, L.R., Maier, S.F., Spencer, R.L., 1999. Long-term changes in mineralocorticoid and glucocorticoid receptor occupancy following exposure to an acute stressor. Brain Res. 847 (2), 211–220.
Di Marzo, V., Goparaju, S.K., Wang, L., Liu, J., Bátkai, S., Járai, Z., Fezza, F., Miura, G.I., Palmiter, R.D., Sugiura, T., Kunos, G., 2001. Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature 410 (6830), 822–825.
Di, S., Malcher-Lopes, R., Halmos, K.C., Tasker, J.G., 2003. Nongenomic glucocorticoid inhibition via endocannabinoid release in the hypothalamus: a fast feedback mechanism. J. Neurosci. 23 (12), 4850–4857.
Djordjevic, A., Adzic, M., Djordjevic, J., Radojcic, M.B., 2009. Chronic social isolation is related to both upregulation of plasticity genes and initiation of proapoptotic signaling in Wistar rat hippocampus. J. Neural. Transm. (Vienna) 116 (12), 1579–1589.
Dubreucq, S., Matias, I., Cardinal, P., Häring, M., Lutz, B., Marsicano, G., Chaouloff, F., 2012. Genetic dissection of the role of cannabinoid type-1 receptors in the emotional consequences of repeated social stress in mice. Neuropsychopharmacology 37 (8), 1885–1900.
Fleshner, M., Deak, T., Spencer, R.L., Laudenslager, M.L., Watkins, L.R., Maier, S.F., 1995. A long-term increase in basal levels of corticosterone and a decrease in corticosteroid-binding globulin after acute stressor exposure. Endocrinology 136 (12), 5336–5342.
Fone, K.C., Porkess, M.V., 2008. Behavioural and neurochemical effects of post-weaning social isolation in rodents-relevance to developmental neuropsychiatric disorders. Neurosci. Biobehav. Rev. 32 (6), 1087–1102.
Glavas, M.M., Yu, W.K., Weinberg, J., 2006. Effects of mineralocorticoid and glucocorticoid receptor blockade on hypothalamic-pituitary-adrenal function in female rats prenatally exposed to ethanol. Alcohol Clin. Exp. Res. 30 (11), 1916–1924.
Gold, P.W., Chrousos, G.P., 2002. Organization of the stress system and its dysregulation in melancholic and atypical depression: high vs low CRH/NE states. Mol. Psychiatr. 7 (3), 254–275.
Gustafsson, P.E., Gustafsson, P.A., Ivarsson, T., Nelson, N., 2008. Diurnal cortisol levels and cortisol response in youths with obsessive-compulsive disorder. Neuropsychobiology 57 (1–2), 14–21.
Hammond, G.L., 1995. Potential functions of plasma steroid-binding proteins. Trends Endocrinol. Metabol. 6 (9–10), 298–304.
Hauger, R.L., Risbrough, V., Oakley, R.H., Olivares-Reyes, J.A., Dautzenberg, F.M., 2009. Role of CRF receptor signaling in stress vulnerability, anxiety, and depression. Ann. NY Acad. Sci. 1179, 120–143.
Heim, C., Nemeroff, C.B., 2001. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol. Psychiatr. 49 (12), 1023–1039.
Herman, J.P., McKlveen, J.M., Solomon, M.B., Carvalho-Netto, E., Myers, B., 2012. Neural regulation of the stress response: glucocorticoid feedback mechanisms. Braz. J. Med. Biol. Res. 45, 292–298.
Herman, J.P., McKlveen, J.M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., Scheimann, J., Myers, B., 2016. Regulation of the hypothalamic-pituitary-adrenocortical stress response. Comp. Physiol. 6, 603–621.
Jacobson, L., 2014. Hypothalamic-pituitary-adrenocortical axis: neuropsychiatric aspects. Comp. Physiol. 4 (2), 715–738.
Jansen, L.M., Gispen-de Wied, C.C., Wiegant, V.M., Westenberg, H.G., Lahuis, B.E., van Engeland, H., 2006. Autonomic and neuroendocrine responses to a psychosocial stressor in adults with autistic spectrum disorder. J. Autism Dev. Disord. 36 (7), 891–899.
Kanatsou, S., Fearey, B.C., Kuil, L.E., Lucassen, P.J., Harris, A.P., Seckl, J.R., Krugers, H., Joels, M., 2015. Overexpression of mineralocorticoid receptors partially prevents chronic stress-induced reductions in hippocampal memory and structural plasticity. PLoS One 10 (11), e0142012.
Karim, A., Arslan, M.I., 2000. Isolation modifies the behavioural response in rats. Bangladesh Med. Res. Counc. Bull. 26 (1), 27–32.
Keilp, J.G., Stanley, B.H., Beers, S.R., Melhem, N.M., Burke, A.K., Cooper, T.B., Oquendo, M.A., Brent, D.A., John Mann, J., 2016. Further evidence of low baseline cortisol levels in suicide attempters. J. Affect. Disord. 190, 187–192.
Marsden, C.A., King, M.V., Fone, K.C., 2011. Influence of social isolation in the rat on serotonergic function and memory–relevance to models of schizophrenia and the role of 5-HT$_6$ receptors. Neuropharmacology 61 (3), 400–407.
Mendel, C.M., 1989. The free hormone hypothesis: a physiologically based mathematical model. Endocr. Rev. 10 (3), 232–274.
Moisan, M.P., Minni, A.M., Dominguez, G., Helbling, J.C., Foury, A., Henkous, N., Dorey, R., Béracochéa, D., 2014. Role of corticosteroid binding globulin in the fast actions of glucocorticoids on the brain. Steroids 81, 109–115.
Morena, M., Patel, S., Bains, J.S., Hill, M.N., 2016. Neurobiological interactions between stress and the endocannabinoid system. Neuropsychopharmacology 41 (1), 80–102.
Nahar, J., Haam, J., Chen, C., Jiang, Z., Glatzer, N.R., Muglia, L.J., Dohanich, G.P., Herman, J.P., Tasker, J.G., 2015. Rapid nongenomic glucocorticoid actions in male mouse hypothalamic neuroendocrine cells are dependent on the nuclear glucocorticoid receptor. Endocrinology 156 (8), 2831–2842.
Newell, K.A., Deng, C., Huang, X.F., 2006. Increased cannabinoid receptor density in the posterior cingulate cortex in schizophrenia. Exp. Brain Res. 172 (4), 556–560.
Newsom, R.J., Osterlund, C., Masini, C.V., Day, H.E., Spencer, R.L., Campeau, S., 2012. Cannabinoid receptor type 1 antagonism significantly modulates basal and loud noise induced neural and hypothalamic-pituitary-adrenal axis responses in male Sprague-Dawley rats. Neuroscience 204, 64–73.
Orlovsky, M.A., Dosenko, V.E., Spiga, F., Skibo, G.G., Lightman, S.L., 2014. Hippocampus remodeling by chronic stress accompanied by GR, proteasome and caspase-3 overexpression. Brain Res. 1593, 83–94.
Parker, V., Morinan, A., 1986. The socially-isolated rat as a model for anxiety. Neuropharmacology 25, 663–664.
Patel, S., Roelke, C.T., Rademacher, D.J., Cullinan, W.E., Hillard, C.J., 2004. Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. Endocrinology 145 (12), 5431–5438.
Perogamvros, I., Ray, D.W., Trainer, P.J., 2012. Regulation of cortisol bioavailability–effects on hormone measurement and action. Nat. Rev. Endocrinol. 8 (12), 717–727.
Pibiri, F., Nelson, M., Guidotti, A., Costa, E., Pinna, G., 2008. Decreased corticolimbic allopregnanolone expression during social isolation enhances contextual fear: a model relevant for posttraumatic stress disorder. Proc. Natl. Acad. Sci. USA 105 (14), 5567–5572.
Piscitelli, F., Carta, G., Bisogno, T., Murru, E., Cordeddu, L., Berge, K., Tandy, S., Cohn, J.S., Griinari, M., Banni, S., Di Marzo, V., 2011. Effect of dietary krill oil supplementation on the endocannabinoidome of metabolically relevant tissues from high-fat-fed mice. Nutr. Metab. (Lond) 8 (1), 51.
Pisu, M.G., Garau, A., Boero, G., Biggio, F., Pibiri, V., Dore, R., Locci, V., Paci, E., Porcu, P., Serra, M., 2016. Sex differences in the outcome of juvenile social isolation on HPA axis function in rats. Neuroscience 320, 172–182.
Richard, E.M., Helbling, J.C., Tridon, C., Desmedt, A., Minni, A.M., Cador, M., Pourtau, L., Konsman, J.P., Mormède, P., Moisan, M.P., 2010. Plasma transcortin influences endocrine and behavioral stress responses in mice. Endocrinology 151 (2), 649–659.
Rivier, C., Vale, W., 1987. Diminished responsiveness of the hypothalamic-pituitary-adrenal axis of the rat during exposure to prolonged stress: a pituitary-mediated mechanism. Endocrinology 121 (4), 1320–1328.
Robinson, S.A., Loiacono, R.E., Christopoulos, A., Sexton, P.M., Malone, D.T., 2010. The effect of social isolation on rat brain expression of genes associated with endocannabinoid signaling. Brain Res. 1343, 153–167.
Serra, M., Pisu, M.G., Floris, I., Biggio, G., 2005. Social isolation-induced changes in the hypothalamic-pituitary-adrenal axis in the rat. Stress 8 (4), 259–264.
Serra, M., Pisu, M.G., Floris, I., Cara, V., Purdy, R.H., Biggio, G., 2003. Social isolation-induced increase in the sensitivity of rats to the steroidogenic effect of ethanol. J. Neurochem. 85 (1), 257–263.
Serra, M., Pisu, M.G., Littera, M., Papi, G., Sanna, E., Tuveri, F., Usala, L., Purdy, R.H., Biggio, G., 2000. Social isolation-induced decreases in both the abundance of

neuroactive steroids and GABA(A) receptor function in rat brain. J. Neurochem. 75 (2), 732–740.
Skelly, M.J., Chappell, A.E., Carter, E., Weiner, J.L., 2015. Adolescent social isolation increases anxiety-like behavior and ethanol intake and impairs fear extinction in adulthood: possible role of disrupted noradrenergic signaling. Neuropharmacology 97, 149–159.
Sumislawski, J.J., Ramikie, T.S., Patel, S., 2011. Reversible gating of endocannabinoid plasticity in the amygdala by chronic stress: a potential role for monoacylglycerol lipase inhibition in the prevention of stress-induced behavioral adaptation. Neuropsychopharmacology 36 (13), 2750–2761.
Tasker, J.G., Herman, J.P., 2011. Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic-pituitary-adrenal axis. Stress 14 (4), 398–406.
Tinnikov, A.A., 1999. Responses of serum corticosterone and corticosteroid-binding globulin to acute and prolonged stress in the rat. Endocrine 11 (2), 145–150.
van Haarst, A.D., Oitzl, M.S., de Kloet, E.R., 1997. Facilitation of feedback inhibition through blockade of glucocorticoid receptors in the hippocampus. Neurochem. Res. 22 (11), 1323–1328.
Walker, E., Mittal, V., Tessner, K., 2008. Stress and the hypothalamic pituitary adrenal axis in the developmental course of schizophrenia. Annu. Rev. Clin. Psychol. 4, 189–216.
Weiss, I.C., Pryce, C.R., Jongen-Rêlo, A.L., Nanz-Bahr, N.I., Feldon, J., 2004. Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. Behav. Brain Res. 152 (2), 279–295.
Yehuda, R., Flory, J.D., Bierer, L.M., Henn-Haase, C., Lehrner, A., Desarnaud, F., Makotkine, I., Daskalakis, N.P., Marmar, C.R., Meaney, M.J., 2015. Lower methylation of glucocorticoid receptor gene promoter 1F in peripheral blood of veterans with posttraumatic stress disorder. Biol. Psychiatr. 77 (4), 356–364.
Yehuda, R., Seckl, J., 2011. Stress-related psychiatric disorders with low cortisol levels: a metabolic hypothesis. Endocrinology 152 (12), 4496–4503.