Research paper: Ouabain induces the extinction of contextual fear memory in rats subjected to chronic unpredictable stress

Jacqueline Alves Leite[1,2], Ana Maria Orellana[1], Diana Zukas Andreotti[1], Amanda Midori Matumoto[1], Larissa de Sá Lima[1], Vinicius Watanabe Nakao[1], Elisa Mitiko Kawamoto[1], Carolina Demarchi Munhoz[1] and Cristoforo Scavone[1].

[1]Departament of Pharmacology, Institute of Biomedical Sciences, University of São Paulo.

[2]Department of Pharmacology, Institute of Biological Sciences, Universidade Federal de Goiás, Goiânia, Brazil.

Address: Av. Prof. Lineu Prestes, 1524, ICB I – Cidade Universitária. ZIP: 05508-900, São Paulo, SP, Brazil.

Jacqueline Alves Leite: jacquelineleite@ufg.br
Ana Maria M. Orellana: amm.orellana@gmail.com
Amanda Matumoto: amanda.matumoto@gmail.com
Diana Zukas Andreotti: dzandreotti@gmail.com
Larissa de Sá Lima: limalarissausp@gmail.com
Vinicius Watanabe Nakao: nakaovw@gmail.com
Elisa Mitiko Kawamoto:elisamk@usp.br
Carolina Munhoz: cdmunhoz@usp.br
Cristoforo Scavone: criscavone@usp.br

The data that support the findings of this study are available from the corresponding author upon request.

Correspondence
Cristoforo Scavone, Department of Pharmacology,
Institute of Biomedical Sciences I, Room 338, Av.
Prof. Lineu Prestes, 1524, 05508-900, Cidade
Universitária, University of São Paulo, São Paulo,
SP, Brazil.
Email: cristoforo.scavone@gmail.com
Bullet points:

1. What is already known: Ouabain has been suggested to have anti-inflammatory effects, improving harmful parameters caused by stressful conditions.
2. What this study adds: For the first time, it was observed that OUA might improve the extinction of contextual fear memory.
3. Clinical significance: The findings brought by this study can be of great importance in post-traumatic stress disorder studies by helping to understand the possible molecular mechanisms involved in stress response and fear memory extinction.

45   **Abstract**

46   BACKGROUND AND PURPOSE

47   Ouabain (OUA) is an inhibitor of Na^+, K^+ -ATPase that has been identified as an

48   endogenous substance present in human plasma, and it has been shown to be associated

49   with the response to acute stress in both animals and humans. Chronic stress is a major

50   aggravating factor of psychiatric disorders, including depression and anxiety. The present

51   work investigates the effects of OUA intermittent administration during chronic

52   unpredictable stress (CUS) protocol in the rat's central nervous system (CNS).

53   EXPERIMENTAL APPROACH

54   Adult male Wistar rats were pretreated intraperitoneally with ouabain (1.8 μg/kg),

55   followed by CUS protocol for 14 days. The levels of serum corticosterone, ACTH, and

56   CRH serum were evaluated through ELISA and the expression of CRH, CRHR1, and

57   CRHR2 genes in the hypothalamus and hippocampus of the animals through RT-PCR.

58   Inflammatory parameters were also investigated, as well as the behavioral CUS effects

59   on memory, that were assayed through the object recognition task, contextual fear

60   conditioning, and memory extinction paradigms.

61   KEY RESULTS

62   The results suggest that intermittent OUA treatment reversed CUS-induced HPA axis

63   hyperactivity through the reduction of (i) glucocorticoids levels, (ii) CRH-CRHR1

64   expression, and by decreasing neuroinflammation with the reduction of iNOS activity,

65   without interfering with the expression of antioxidant enzymes. These changes in both

66   the hypothalamus and hippocampus may reflect in the rapid extinction of aversive

67   memory.

68   CONCLUSION AND IMPLICATIONS

69   The present data demonstrate, for the first time, the ability of OUA to modulate the HPA

70   axis as well as the disappearance of aversive memory in rats.

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72   **Keywords:** Ouabain, CUS, Fear Memory, CRHR, HPA, and iNOS.

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Introduction

Chronic stress is associated with the development of neuropsychiatric disorders, for instance, post-traumatic stress disorder (PTSD), or depression and anxiety-related disorders, where changes in hormones and neuropeptides related to stress ensue, such as in the production and release of corticotropin-releasing hormone (CRH) (Kasckow, Baker & Geracioti, 2001; Nemeroff & Vale, 2005). This neuropeptide is widely distributed in the CNS, and its expression is stimulated by neurotransmitters, such as serotonin and norepinephrine and by cytokines, as the interleukin (IL)-1 and -6 and the tumor necrosis factor (TNF) -α (Itoi et al., 1994; Tsigos & Chrousos, 2002; Turnbull & Rivier, 1999). CRH initiates the response of the hypothalamic-pituitary-adrenal axis (HPA) at the pituitary level and modulates the brain regions that regulate behavioral responses to stress. This CRH activity occurs through its G protein-coupled receptors (GPCRs), CRHR1, and CRHR2. The CRHR1's CRH effect is the main responsible for the synthesis and secretion of ACTH regulation, which stimulates the release of glucocorticoids from the adrenal cortex (Majzoub, 2006). However, studies have observed that CRH overproduction in mice is directly related to the development of anxiety-like behavior (Stenzel-Poore, Heinrichs, Rivest, Koob & Vale, 1994; van Gaalen, Stenzel-Poore, Holsboer & Steckler, 2003). Also, CRHR1 and CRHR2 appear to modulate the expression of stressors differently. CRH1 is related to the initial activation of the HPA axis to stress stimulus and anxiogenic response (Bale & Vale, 2004; Heinrichs, Lapsansky, Lovenberg, De Souza & Chalmers, 1997). In contrast, CRHR2 activation mediates a stress adjustment response, promoting anxiolytic and antidepressant effects (Bale & Vale, 2004; Majzoub, 2006).

It has been suggested that endogenous Ouabain (OUA) levels are modulated by stress conditions (Goto, Yamada, Nagoshi, Terano & Omata, 1995), however little is known about the effect of this cardiosteroid in stress situations. The plasma membrane protein Na^+,K^+-ATPase has the function of maintaining cellular ion homeostasis (Blanco, 1998; Gloor, 1997) and is constituted by the α (catalytic), β, and γ subunits (Lingrel & Kuntzweiler, 1994; Sweadner, 1979). The $α_1$ isoform is expressed in all cells of nervous tissue, while $α_2$ is found in astrocytes, and $α_3$ is exclusively expressed in neurons (Dobretsov & Stimers, 2005). Several Central Nervous System (CNS) disorders are related to alterations in the Na^+,K^+-ATPase activity, such as depression and bipolar disorder (Goldstein et al., 2012; Goldstein et al., 2009; Goldstein et al., 2006).

In this work, we aimed to evaluate the role of intermittent treatment with OUA in chronic unpredictable stress (CUS)-induced HPA axis hyperactivity and extinction of fear memory in animals. Our results demonstrated that intermittent treatment with OUA is effective in reversing CUS-induced HPA axis hyperactivity by reducing glucocorticoid circulating levels and CRH-CRHR1 expression, whilst there was an increase in CRHR2 expression, without interfering with low-grade neuroinflammation induced by the CUS. Also, OUA intermittent treatment improved the extinction of aversive memory in CUS animals, possibly by altering the expression of CRH and its receptors in the hypothalamus and hippocampus.

Results

Ouabain interferes with HPA axis hyperactivity induced by CUS

Chronic stress induces an HPA axis hyperactivity that is modulated by increased sustained corticosterone levels (Ulrich-Lai & Herman, 2009). Thus, the effect of chronic intermittent treatment (every other day) with OUA on serum corticosterone levels in animals submitted to the CUS protocol was evaluated. It was observed that only animals that received chronic intermittent treatment with OUA at the dose of 1.8 μg/kg by intraperitoneal injection did not have altered basal levels of corticosterone (Figure 2A). However, animals submitted to CUS showed an increase in corticosterone levels 24 h after the last stressor stimulus when compared to the control group (Figure 2A). In this study, the animals submitted to CUS+OUA exhibited a reduction in corticosterone levels when compared to the CUS only group (Figure 2A).

Nonetheless, alterations in serum CRH and ACTH levels 24 h after the CUS protocol when compared to the control group (CTR) were not observed. Also, the treatment with OUA did not interfere with the level of these hormones (Figure 2B, C).

Pro-inflammatory state induced by chronic unpredictable stress (CUS) in the hippocampus and hypothalamus was modified by ouabain

Based on the evidence that stress can induce inflammatory responses in neurons (Karagkouni, Alevizos & Theoharides, 2013), TNF-α and IL-1β levels were measured in

145 the hypothalamus and hippocampus. It was observed that the animals submitted to CUS,

146 as well as animals treated with OUA and subjected to CUS-protocol, did not show

147 differences in the levels of IL-1β and TNF-α in the hypothalamus and hippocampus when

148 compared to the control (Figure 3A, B, C, D). However, the chronic intermittent treatment

149 with OUA did not interfere with the basal levels of IL-1β and TNF-α in both brain areas

150 (Figure 3).

151 Furthermore, no variations in the activity of total NOS and neuronal isoform (nNOS)

152 were observed in the hypothalamus (Figure 4A, B) and hippocampus (Figure 4D, E) of

153 all the groups. However, when the activity of induced NOS (iNOS) was measured, even

154 though there were no changes observed in the hypothalamus (Figure 4C), there was an

155 increase of iNOS enzyme activity in the hippocampus of animals submitted to CUS,

156 which was reduced by ouabain treatment (Figure 4F).

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158 ***OUA did not alter the effects of CUS-exposure on antioxidant enzymes expression***

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160 Chronic stress promotes reactive oxygen species (ROS) generation, causing oxidative

161 stress and consequent neurodegeneration. Furthermore, a reduction in antioxidant

162 enzymes activity has been observed in an unpredictable chronic stress model (Bilici, Efe,

163 Köroğlu, Uydu, Bekaroğlu & Değer, 2001; Lucca et al., 2009). Accordingly, qPCR was

164 performed to measure the gene transcription levels related to antioxidant enzymes. The

165 results demonstrated that there were no changes in the hypothalamic mRNA expression

166 of superoxide dismutase 1 and 2 (SOD1, SOD2) and glutathione reductase (GSR) (Figure

167 5A, B, C). Also, no variation in mRNA levels was detected for *Sod1* in the hippocampus

168 (Figure 5D). However, a reduction in *Sod2* and *Gsr* mRNA expression was observed in

169 the hippocampus of animals exposed to CUS when compared to the control, which was

170 not reversed by OUA treatment (Figure 5E, F).

171

172 ***The Crh, Crhr1, and Crhr2 gene expression was modified by both CUS and Chronic***

173 ***Intermittent Ouabain treatment in the hippocampus and hypothalamus***

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175 The CRH and its receptors are crucial to the regulation process of the HPA axis. Thus

176 *Chr, Crhr1,* and *Crhr2* mRNA expression were assayed in the hippocampus and

177 hypothalamus through qPCR to evaluate OUA treatment's effect on animals submitted to

178 CUS. In the hypothalamus, the results demonstrate an increase in the *Crh* expression in

the animals submitted to the CUS in comparison to the control group (CTR), and OUA treatment had this effect reduced (Figure 6A). However, there were no changes in the expression of Crhr1 in the hypothalamus of the groups evaluated (Figure 6B). Interestingly, we observed that chronic intermittent OUA treatment promoted an increase in the expression of *Crhr2* in the animals submitted to the CUS when compared to the CUS group (Figure 6C). When we evaluated the hippocampus, a reduction in the expression of *Crh1* of the animals submitted to CUS was observed, as well as the ones treated with OUA (Figure 6D). It was also noticed that the animals subjected to CUS had a reduction in the expression of *Crhr1* in the hippocampus but not in the hypothalamus when compared to CTR (Figure 6E and 6B). Besides, chronic intermittent treatment with OUA reduced the expression of *Crhr2* in the hippocampus of the animals submitted to the CUS when compared to the CUS group (Figure 6F).

CUS -induced long-term memory impairment reduced by OUA

The novel object recognition test revealed that the CUS group exhibited a long-term memory impairment since they had a reduced capacity to discern the presence of a new object when compared to the control group. Besides, chronic intermittent treatment with OUA alone did not interfere with long-term memory formation (Figure 7). Interestingly, the pretreated animals with OUA that were exposed to CUS had the deficit induced by CUS in the long-term memory prevented (Figure 7).

OUA promotes a rapid extinction of fear memory without interfering with the acquisition of aversive memory

Finally, OUA and CUS promoted a rapid extinction of fear memory without interfering with the acquisition of aversive memory. The contextual fear conditioning and extinction tests were performed to evaluate the effects of OUA onto the process of acquisition and extinction of conditioned contextual fear memory. Controls and stressed animals, either treated with chronic intermittent OUA or saline, received a 1 mA foot shock. To evaluate whether conditioned fear memory was associated with the context of the traumatic event, another group of animals was transferred to a previously unknown container 24 h after receiving the footshock (unpaired control), and the freezing behavior was analyzed.

These unpaired control animals did not present as much freezing behavior as the ones that were placed in the footshock context (Figure 8A). These results support our data, indicating that animals were freezing in response to the aversive context (Figure 8A). In addition, the animals submitted to CUS, as well as to chronic intermittent OUA, did not contrast from CTR in the acquisition of fear memory in relation to the percentage of freezing compared between both groups at 24 hours after the acquisition training (day 1) (Figure 8B). Interestingly, regarding the process of memory extinction, the OUA, OUA + CUS, and CUS groups presented a reduction of freezing after successive re-exposures in the contextual fear conditioning arena from the third day (Figure 8C), in comparison to the control group. Given this, it is possible to infer that intermittent treatment with OUA has played an important role in the enhancement of aversive memory extinction, as well as CUS does in the absence or presence of OUA.

Discussion

Ouabain (OUA), a ligand of Na^+,K^+-ATPase, has been identified as an endogenous substance present in human plasma and appears to be involved in response to acute stress in animals and humans (Goto, Yamada, Nagoshi, Terano & Omata, 1995). Moreover, chronic stress is a known important aggravating factor of psychiatric disorders.
The CUS protocol was used to evaluate the role of intermittent OUA treatment in the modulation of the HPA axis and the extinction of fear memory, both parameters are well known to be altered in depression, anxiety disorders, and PTSD. Results suggested that chronic intermittent OUA treatment diminished CUS-induced HPA axis hyperactivity by reducing circulating levels of glucocorticoid (Figure 2A). Furthermore, it was demonstrated that, despite the 14-day CUS increasing CRH expression in the hypothalamus, the CRHR1 receptor expression in the hypothalamus and hippocampus was lessened, even though OUA reduced CRH expression (Figure 5). In addition, the present work shows for the first time that the rats treated with chronic intermittent OUA had an improvement in the long-term memory that was impaired by CUS (Figure 7). Interestingly, both of them, CUS paradigm and OUA treatment have, independently, led to the rapid extinction of fear memory (Figure 8)
Chronic stress promotes prolonged activation of the HPA axis, providing long-term adaptive changes in tone and responsiveness, leading to increased corticosterone secretion and CRH expression (Herman, Adams & Prewitt, 1995). The 14-day CUS

247   protocol leads to a moderate increase in glucocorticoid levels (Munhoz, Sorrells, Caso,

248   Scavone & Sapolsky, 2010), which was reduced by chronic intermittent OUA treatment.

249   Previous data have shown that acute OUA does not interfere with the levels of

250   corticosterone released in acute stress situations (Kinoshita et al., 2014), thus suggesting

251   that the OUA is administered in the chronic intermittent schedule can regulate the activity

252   of the HPA axis only in response to chronic stressors.

253   Different studies have reported that OUA is an essential regulator of the inflammatory

254   response at the peripheral and central nervous systems (Kinoshita et al., 2014; Leite et al.,

255   2015). Additionally, it is well known that chronic stress promotes, in humans and animals,

256   an inflammatory state in the peripheral and central nervous systems (Goshen et al., 2008;

257   Grippo, Francis, Beltz, Felder & Johnson, 2005; Miller et al., 2008). Studies have

258   associated the elevated levels of glucocorticoids with the presence of increased

259   inflammatory response in cells such as macrophages and microglia in the CNS (Dinkel,

260   MacPherson & Sapolsky, 2003). Also, IL-1β participates in the induction of memory

261   impairment as well as in the release of CRF (Gonzalez et al., 2013; Karalis, Sano,

262   Redwine, Listwak, Wilder & Chrousos, 1991). However, in the chronic stress model that

263   was performed in this study, it was not possible to observe changes in pro-inflammatory

264   cytokines levels in the hypothalamus and hippocampus. Furthermore, intermittent

265   treatment with OUA did not develop changes in the levels of these cytokines.

266   Besides the participation of pro-inflammatory cytokines in the development of

267   neuroinflammation induced by chronic stress, the involvement of nitric oxide in the

268   development of anxiety has been demonstrated, as treatment with L-NAME, a NOS-

269   inhibitor capable of reversing the chronic stress-induced increase in anxiety-like behavior

270   (Sevgi, Ozek & Eroglu, 2006). On the other hand, nNOS activity in the hippocampus

271   induces a decrease in the expression of glucocorticoid receptors (GR) in the

272   hypothalamus, thereby reducing the negative feedback induced by corticosterone (Zhou

273   et al., 2011). Given this, the effects of CUS and OUA treatment on NOS activity in the

274   hypothalamus and hippocampal were investigated, although no changes were observed.

275   The participation of iNOS in chronic stress has been previously described (Wang,

276   Kamphuis, Huitinga, Zhou & Swaab, 2008). This study results showed that CUS

277   increased iNOS activity in the hippocampus (Figure 4), which was reverted by

278   intermittent treatment with OUA. Therefore CUS may interfere with immunity through

279   system overactivation, leading to low-grade inflammation. These data are in agreement

280   with findings previously shown by Munhoz and colleagues (2006), where the 14-day

CUS model exacerbates activation induced by LPS of factor NF-κB nuclear factor in the frontal cortex and hippocampus via glucocorticoid secretion. Furthermore, OUA has been related to its anti-inflammatory potential in several models (Kinoshita et al., 2014; Leite et al., 2015), and this study demonstrates that OUA treatment reduces iNOS activity in animals submitted to CUS protocol.

Chronic stress may increase ROS levels as well as reduce the activity of antioxidant enzymes such as glutathione (GSH) and superoxide dismutase (SOD) (Bilici, Efe, Köroğlu, Uydu, Bekaroğlu & Değer, 2001; Schiavone, Jaquet, Trabace & Krause, 2013). The imbalance between free radical production and the body's antioxidant capacity has been presented as an important factor in the development of neuropsychiatric diseases, including depression in humans and in rodents (Kumar, Kuhad & Chopra, 2011; Maes, Galecki, Chang & Berk, 2011). This study demonstrates that the CUS group showed a reduction in the expression of the antioxidant enzymes SOD2 and GSH in the hippocampus. However, the OUA treatment did not interfere in the expression of these antioxidant enzymes, although other studies have shown that OUA treatment reduced oxidative stress in rat hippocampus in a model of LPS-induced neuroinflammation (Garcia et al., 2019).

Patients with PTSD exhibit difficulty in suppressing responses to stimuli associated with trauma (Wessa & Flor, 2007; Blechert, Michael, Vriends, Margraf & Wilhelm, 2007). Classic experimental models of fear conditioning based on memory acquisition and extinction are used to study pathophysiology and search for PTSD treatment. The HPA axis activity in response to stressors stimuli and contextual fear memory depends on a neuronal circuitry involving the hippocampus, medial prefrontal cortex, and amygdala, as well as the participation of glucocorticoid and the CRF neuropeptide (Maren, Phan & Liberzon, 2013; Smith & Vale, 2006).

Recent studies point to the participation of CRHR1 in conditioned fear memory, and it has been shown that antalarmin, a CRHR1 antagonist, when administered systemically attenuated the fear response, as well as rescued HPA axis activity in rats (Skórzewska et al., 2019). Mice with a specific deletion of the GABA (A) α 1 receptor in CRH neurons exhibited an increase in CRH levels in the amygdala and developed anxiety and impairment in the extinction of fear memory (Gafford, Guo, Flandreau, Hazra, Rainnie & Ressler, 2012).

This study's results showed that OUA treated animals submitted to CUS could consolidate the contextual memory in response to a traumatic event and still managed to

315 perform the extinction of this conditioned fear memory. These findings may correlate
316 with the reduction in the expression of CRHR1 and the increase of CRHR2 in the
317 hippocampus of CUS and OUA-CUS treated animals.

318 In summary, OUA promotes the facilitation of fear memory extinction with
319 corresponding decreases in *Crh* and *Crhr1*, as well as increases of *Crhr2* gene expression
320 in the hippocampus and hypothalamus and reduced glucocorticoid serum levels of rats.
321 Further, OUA decreased iNOS activity in the hippocampus by altering CUS-induced low-
322 grade neuroinflammation. Furthermore, these findings suggest, for the first time, the
323 participation of the OUA as a regulator of contextual fear memory in animals submitted
324 to chronic stress via modulation of the HPA axis and neuroinflammation.

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327 **Methods**

328 ***Animal and chronic unpredictable stress***

329 Male Wistar Rats (250–350 g) (Biomedical Sciences Institute, University of São Paulo)
330 were kept under a 12 h light/dark cycle (lights on at 7:00 a.m.) and fed *ad libitum*. Rats
331 were randomly assigned into four groups; all of them had intraperitoneal (i.p.)
332 administration of either vehicle (PBS) or ouabain (1.8 μg/kg) one hour before the stress
333 protocol every other day. Chronic unpredictable stress (CUS) was performed as described
334 (Munhoz *et al.*, 2006) (Figure 1A). All animals were euthanized 24 hours after the last
335 stressor protocol. Trunk blood was collected and centrifuged at 3000 rpm for 10 min to
336 obtain serum, and the hippocampus and hypothalamus were dissected for biochemical
337 studies. All procedures were also approved by the Ethical Committee for Animal
338 Research (CEUA) of the Biomedical Sciences Institute of the University of São Paulo.

339

340 ***Measurement of Corticosterone, CRF, ACTH, and Cytokine levels***

341 ELISA kits measured serum levels of Corticosterone, CRF, ACTH following
342 manufacturer's instructions of Corticosterone EIA kit (Enzo Life Sciences International,
343 Inc., USA), ACTH CRF kit (PHOENIX), and ELISA kit (PHOENIX). Hippocampal and
344 Hypothalamus levels of TNF-α and IL-1β were measured by ELISA (eBioscience, USA).
345 The absorbance was measured using a spectrophotometer at 450 nm (Epock, Biotech),
346 and the concentrations of the cytokines were measured by correlating with the standard
347 curve.

Measurement of NOS activity

For the NOS activity assay, the hypothalamus and hippocampus were homogenized in a buffer containing: 20 mM HEPES pH 7.4; 0.32 M sucrose; 0.1 mM EDTA; 1.0 mM DTT; 1.0 mM PMSF; 10 µg/mL leupeptin; 2 µg/mL aprotinin. Then, the tissue suspension was centrifuged at 1000 x g for 10 min, and the supernatants were centrifuged again at 12,000 x g for 20 min, 4 °C. The supernatants were passed through a Dowex AG 50 Wx-8 (Na$^+$ form) column to remove the endogenous arginine. The arginine-free eluent was used to assay the NOS activity. After determining the protein concentration of the sample by the Bio-Rad kit, the samples were diluted to a concentration of 1 µg/µL, and samples were incubated for 30 minutes at 37 °C in 200 µL reaction medium containing: 20 µM arginine (0.5 µCi), 4 µM FAD, 4 µM FMN, 10 µM BH4, 10 µg/mL Calmodulin, and 1mM NADPH and 100 µl sample. To verify NOSi activity, a new reaction medium containing 5 mM EGTA was made in place of calmodulin. To stop the reaction, the tubes were placed on ice, and 1 mL of 20 mM HEPES pH 5.5 was added. The total volume contained in the reaction portion was transferred to a column with 0.3 ml Dowex AG 50 Wx-8 (Na$^+$ form), collecting the eluate in a scintillation spectrophotometer container. To wash the resin and ensure maximum product recovery, another 1 mL of pH 5.5 HEPES and 1 mL of distilled water was applied to the column, and the eluate was collected in the same container. 8 mL of scintillation liquid (Ultima Gold ™) was added to a container, and the activity present in the samples was determined with the aid of a radiation counter (McKee, Scavone & Nathanson, 1994).

Real-Time PCR

Total tissue RNA (Hypothalamus and Hippocampus) was found and purified using the total KIT RNA I (OMEGA, Georgia, USA). RNA was quantified, and 1µg were treated with DNAse I and subjected to reverse transcription using oligo (12-18), random primer, and an IMPROM II reverse transcriptase according to manufacturer's instructions (Promega Corporation, USA). The *Crh*, *Crhr1*, *Crhr2*, *Sod1*, *Sod2,* and *Gsr* gene expressions were measured by quantitative PCR (qPCR) using the TaqMan gene expression assay (Thermo Fisher Scientific).

The qPCR reaction was performed in the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CAUSE). Each duplicate reaction contained 4uL of cDNA, 6.25uL of TaqMan FAST Advanced Master Mix (Applied Biosystems), 0.625uL of the

381   TaqMan probe, and 1.62uL of water nuclease-free (Applied Biosystems), totaling a
382   volume of 12.5uL per well. The first step of the reaction was to amplify at 95° C for 20
383   seconds, followed by 40 cycles at 95° for 3 seconds (denaturation) and 60° C at 30
384   seconds (annealing and extension). The comparative method of delta-delta-Ct was used
385   to quantify the difference between the samples normalized by the calibrator of
386   (endogenous control). As endogenous control of the qPCR reaction, the TaqMan probe
387   for the Hprt-1 gene (hypoxanthine phosphoribosyltransferase 1) was used. Information
388   on all probes used is listed in Table 1.

389

390   **Table1. List of primers used for qPCR.**

Gene	Primer ID
Crh	NM_031019.1
Crhr1	XM_006247542.2
Crhr2	NM_022714.2
Sod1	NM_017050.1
Sod2	NM_017051.2
Gsr	NM_053906.2
Hprt-1	NM_012583.2

391

392   ***Behavioral Analysis***

393

394   ***Novel Object Recognition (NOR)***

395   Learning and memory tests were performed in open field apparatus and were divided into
396   three phases: habituation (10 min), training (5 min), and test (5 min). The habituation
397   phase was performed 24 h after the stressor stimulus. For three consecutive days, the rats
398   were placed in the center of the apparatus, where they could explore an open field arena
399   in the absence of any objects. A familiarization session was done after 24 hours of
400   habituation to the test cage; the rats explored 2 identical objects for 5 minutes. 24 hours
401   later, the animals returned to the apparatus, but one of the previous objects was replaced

402 by a new one. The test was filmed, and later the time of exploration of each object was

403 measured with the aid of a stopwatch. Animals that had the exploration time under 10

404 seconds were excluded from the experiment. The scaled index was calculated as the

405 difference between the time of exploring the new and the old object, exposed as a ratio to

406 the total time spent (Roozendaal, Okuda, Van der Zee & McGaugh, 2006).

407

408 ### *Contextual Fear Conditioning and Memory Extinction*

409 In order to assess context-dependent fear memory and extinction, the different groups of

410 rats were exposed to a fear conditioning protocol and subsequently subjected to a context-

411 dependent extinction protocol. Briefly, 24 hours after the last stressor stimulus, the

412 animals were placed individually in the conditioning box, which was comprised of three

413 white walls, a cover and a transparent acrylic wall (28 x 26 x 23 cm), as well as a base

414 composed of bars (diameter of 0.4 cm and spacing between them of 1.05 cm) that were

415 connected to an electric shock generator (Insight Equipamentos, Pesquisa e Ensino,

416 Ribeirão Preto-SP).

417 The rats were allowed to freely explore for 2 min the test box before receiving a 1-second

418 foot shock (FS, 1 mA). The rats were removed from the chamber 15 seconds after foot

419 shock. The rats were tested 24 hours after conditioning for recall and extinction of

420 context-dependent fear for seven consecutive days. Each extinction session consisted of

421 re-exposing the animals to the conditioning context for 10 minutes without negative

422 reinforcement. The measure of fear behavior analysis was the freezing time, defined as

423 complete immobility of the animal, with no movement of vibrissae and sniffing. Besides,

424 a group of animals was conducted to the unpaired arena, 24 hours after the foot shock,

425 being the control of the experiment. After each test, the matched and unpaired

426 conditioning box was cleaned with 5% alcohol. A video camera recorded all training and

427 testing procedures, and behavior analysis was blinded.

428

429 ### *Statistics*

430 The qPCR results were analyzed via the delta-delta-Ct method. Normality was assessed

431 through the D'Agostino & Pearson omnibus normality test, and, for parametric analyses.

432 Parametric analyses were conducted through two-way ANOVA followed by Newman-

433 Keuls post-test. Non-parametric analyses were conducted through the Kruskal-Wallis

434 test, followed by Dunn's post hoc test. Differences were significant at $p<0.05$, and all

435   results are expressed as the mean ± standard error of the mean (SEM) of the indicated

436   number of experiments. All analyses were performed using the Prism 6 software package

437   (GraphPad Software, San Diego, CA, USA).

438

446

447   **Author contributions**

448   Jacqueline Alves Leite, Elisa Mitiko Kawamoto, Carolina Demarchi Munhoz, and

449   Cristoforo Scavone conceived and designed the experiments. Jacqueline Alves Leite, Ana

450   Maria Orellana, Diana Zukas Andreotti, Amanda Matumoto, Vinicius Watanabe Nakao,

451   and Larissa de Sá Lima performed the experiments. Jacqueline Alves Leite analyzed data.

452   Jacqueline Alves Leite and Cristoforo Scavone composed the manuscript.

453

454   **Conflicts of interest**

455   Authors declare no conflict of interest.

456

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649 **Figure captions**

650 **Figure 1** Schematic representation of the unpredictable chronic stress protocol and

651 behavioral tests performed to assess the effects of CUS and intermittent OUA treatment

652 on memory formation. (A) The animals were divided into four groups: PBS, OUA, CUS,

653 and CUS + OUA. Intermittent treatment was performed, as early as day 1 the animals of

654 the CUS and CUS + OUA group were exposed only to the stressor stimulus, and on day

655 2 the animals of the CUS and OUA + CUS were treated with PBS or Ouabain 1 hour

656 before the stimulus stressor, so treatment was maintained alternately for 14 days. Control

657 animals (PBS and OUA) were also treated on alternate days, but after treatment, they

658 were returned to the cages. Twenty-four hours after the last stressor, the animals were

659 euthanized, and the hippocampus and hypothalamus were stored for biochemical studies.

660 (B, C) Different groups were exposed to behavioral testing 24 hours after the last CUS

661 protocol. (B) Scheme representative of the new object recognition test performed on the

662 fourteenth day with the adaptation and 24 h after the test. (C) Illustrative scheme of

Contextual Fear Conditioning and Memory Extinction, first, the animals received a foot shock (1mA) 24 hours after the last stress stimulation. After 1 day, the animals were re-exposed to the arena, and the memory consolidation measure was performed. Subsequently, the extinction of fear memory was evaluated.

Figure 2 Ouabain reduces serum corticosterone levels in animals submitted to CUS. (A) Animals submitted to the CUS display an increase in serum corticosterone levels (ng/dL), 24 hours after the last stressor stimulus, in relation to the CTR group (n = 7), and chronic treatment with OUA reduces corticosterone concentration in relation to the CUS group. CRF (ng/mL) (n = 13-15) and ACTH (ng//mL) (n = 9-10) had no alterations in the different groups studied, 24 hours after last stressor stimulus. Data are presented as mean ± SEM. *** $p < 0.001$. (Two-way ANOVA followed by Newman-Keuls post hoc test revealed a significant for ACTH and CRF or Kruskal-Wallis test followed by Dunn's post hoc test for corticosterone).

Figure 3 Chronic unpredictable stress increases proinflammatory cytokines levels in the hypothalamus. (A, B) show levels of the hypothalamus (blue bars) (IL-1β (n= 4-5) and TNF-α (n= 9-10), animals submitted to CUS had increased levels of IL-1β and TNF-α in the hypothalamus compared to the control groups. (C, D) levels of hippocampal (red bars) IL-1β (n= 4-5) and TNF-α n= 8-10), animals submitted to CUS display increased levels of IL-1β in the hypothalamus compared to the control groups. Data are presented as mean ± SEM. (Two-way ANOVA followed by Newman-Keuls post hoc test revealed a significant for TNF-α from HP or Kruskal-Wallis test followed by Dunn's post hoc test for IL-1β from HT and HP).

Figure 4 Chronic intermittent treatment with ouabain reduces iNOS activity in the hippocampus of animals submitted to CUS. (A, B) The activity of tNOS and nNOS were not altered in the hypothalamus of the different groups studied (n=5) (blue bars). (B) Unpredictable chronic stress increases iNOS activity in the hypothalamus when compared to control groups (P = 0.01) (n=5) (blue bars). (D, E) The graphs show that there was no change in NOS total and nNOS activity in the hippocampus of the different groups studied (red bars) (n=4-5). (F) CUS increased iNOS activity relative to control groups that were reduced by OUA treatment (red bars) (n=4-5). Results are presented as mean ± SEM.** $p < 0.01$, *$p < 0.05$.(Two-way ANOVA followed by Newman-Keuls post hoc test revealed a significant).

Figure 5 Modulation of antioxidant enzyme expression in rat hippocampus and hypothalamus by CUS protocol. (A, B, C) the results show no alteration in the expression of SOD1, SOD2, and GSR in the hypothalamus of the different groups studied (blue bars) (n=4-5). (D) The results suggest an absence of modulation in SOD1 expression in the hippocampus of the different groups studied (n=4-5). (E, F) SOD2 and GSR expression were reduced in the groups submitted to CUS in relation to the control groups in the hippocampus (n= 4-5). Data are presented as mean ± SEM.*** $p < 0.001$ (Two-way ANOVA followed by Newman-Keuls post hoc test revealed a significant).

Figure 6 Decreased expression of *Crh* mRNA and their receptors in the hippocampus and hypothalamus of stressed rats and treated with ouabain. (A, B, C) expression of *Crh* mRNA (n= 4-5), C*rhr1* mRNA (n= 4-5) and C*rhr2* mRNA (n= 4-5) in the hypothalamus (blue bars). (E, F, G) expression of C*rh* mRNA (n= 4-5), *Crfr1* mRNA (n= 4-5) and *Crhr2* mRNA (n= 4-5) in the hippocampus (red bars). Data are presented as mean ± SEM.** $p < 0.01$, * $p < 0.05$. (Two-way ANOVA followed by Newman-Keuls post hoc test revealed a significant).

Figure 7 Ouabain (OUA) prevents Chronic unpredictable stress-induced memory impairment. After 24 h of the last stressor stimulus, the animals were submitted to the test where they were exposed to two equal objects. After three days, an object was replaced, and the time of exploration of the two objects was quantified to evaluate the long-term memory. Data are represented by the percentage of the discrimination index (n= 6-9). Data are presented as mean ± SEM.** $p < 0.01$, * $p < 0.05$ (Kruskal-Wallis test followed by Dunn's post h

Figure 8 The effects of chronic ouabain administration and chronic unpredictable stress in the formation and extinction of fear memory. (A) show the unpaired test, where the CTR animals presented lower freezing when packed in the unpaired arena (n=4-10). (B) shows that there was no difference in the freezing percentage between the groups studied 24 hours after foot shock in the animals of the different groups (n=10). (C) Animals from control group (CTR) (n=10) presented higher percentage of freezing on days 3 and 4 compared to OUA (n=10), CUS (n=10) and CUS + OUA (n=10) groups. Data are

730   presented as mean $\pm$ SEM.** p <0.01, *p < 0.05. (Two-way ANOVA followed by

731   Newman-Keuls post hoc test revealed a significant).

732

733   **Figure 9** Schematic drawing of the proposed action upon OUA treatment in rats subjected

734   to chronic unpredictable stress. Intermittent treatment with OUA reduced the activity of

735   the HPA axis, since it reduced the expression of *Crf* and its *Crfr1* receptor, leading to a

736   reduction in the serum release of glucocorticoids, in addition, ouabain showed anti-

737   inflammatory activity, observed in the reduction of the activity of iNOS enzyme. The

738   interference of treatment with OUA on the HPA axis promoted a rapid extinction of

739   memory due to the fear of animals subjected to chronic stress.

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