

# Hypothalamic Corticotropin-Releasing Factor is Centrally Involved in Learning Under Moderate Stress

Morgan Lucas<sup>1</sup>, Alon Chen<sup>2</sup> and Gal Richter-Levin<sup>\*,1,3,4</sup>

<sup>1</sup>The Institute for the Study of Affective Neuroscience (ISAN), Haifa, Israel; <sup>2</sup>Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel; <sup>3</sup>Department of Psychology, University of Haifa, Haifa, Israel; <sup>4</sup>Sagol Department of Neurobiology and Ethology, Haifa, Israel

The corticotropin-releasing factor (CRF) neuropeptide is found to have a pivotal role in the regulation of the behavioral and neuroendocrine responses to stressful challenges. Here, we studied the involvement of the hypothalamic CRF in learning under stressful conditions. We have used a site-specific viral approach to knockdown (KD) CRF expression in the paraventricular nucleus of the hypothalamus (PVN). The two-way shuttle avoidance (TWSA) task was chosen to assess learning and memory under stressful conditions. Control animals learned to shuttle from one side to the other to avoid electrical foot shock by responding to a tone. Novel object and social recognition tasks were used to assess memory under less stressful conditions. KD of PVN-CRF expression decreased the number of avoidance responses in a TWSA session under moderate (0.8 mA), but not strong (1.5 mA), stimulus intensity compared to control rats. On the other hand, KD of PVN-CRF had no effect on memory performance in the less stressful novel object or social recognition tasks. Interestingly, basal or stress-induced corticosterone levels in CRF KD rats were not significantly different from controls. Taken together, the data suggest that the observed impairment was not a result of alteration in HPA axis activity, but rather due to reduced PVN-CRF activity on other brain areas. We propose that hypothalamic CRF is centrally involved in learning under moderate stressful challenge. Under 'basal' (less stressful) conditions or when the intensity of the stress is more demanding, central CRF ceases to be the determinant factor, as was indicated by performances in the TWSA with higher stimulus intensity or in the less stressful tasks of object and social recognition.

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## INTRODUCTION

Inappropriate regulation of the central stress response is known to be associated with higher prevalence of different psychopathologies (eg, anxiety, depression, PTSD) and to affect learning and memory performances. Corticotropin-releasing factor (CRF) has a crucial role in mediating behavioral, autonomic, and endocrine responses to stressful challenges, and deregulation of the CRF system has been involved in stress-related psychopathologies (Kehne and Cain, 2010; Lloyd and Nemeroff, 2011). A significant group of CRF-containing neurons is located in the paraventricular nucleus of the hypothalamus (PVN). These neurons target the median eminence (Swanson *et al.*, 1983), triggering the release of ACTH from the anterior pituitary, which ultimately leads to glucocorticoid secretion from the adrenal cortex (the HPA axis). In addition, CRF-containing neurons are found in the hippocampus, the central nucleus of the amygdala and the bed nucleus of the stria terminalis (BNST).

This central CRF system has been demonstrated to be involved in emotion regulation and stress-related learning and memory (Bangasser and Shors, 2010). CRF release facilitates hippocampus-dependent memory (Lee *et al.*, 1993; Row and Dohanich, 2008), while post-training CRF antagonists administered in the BLA impair fear conditioning and inhibitory avoidance memory consolidation (Hubbard *et al.*, 2007; Roozendaal *et al.*, 2002). Using conditional mutant mice, limbic CRHR1 has been shown to mediate anxiety-related behaviors independent of the effects on the HPA axis (Muller *et al.*, 2003), suggesting dissociation between central CRF system involvement in stress- and anxiety-related behaviors and the hypothalamic CRF involvement in neuroendocrine regulation. CRF-containing neurons in the PVN are also known to target brain structures like the locus coeruleus (LC) and the ventral tegmental area (VTA) (Reyes *et al.*, 2005; Rodaros *et al.*, 2007), both are known to be involved in emotional modulation of memory and stress-related behaviors (eg, McGaugh, 2004; Pezze and Feldon, 2004). We thus hypothesized that hypothalamic CRF could take part in learning when stress is involved. To explore this assumption, we have used a lentiviral vector system to specifically KD CRF expression in the PVN and subsequently assess rats' learning abilities in three different tasks, exhibiting different levels of stress intensity.

\*Correspondence: Professor G Richter-Levin, Sagol Department of Neurobiology and Ethology, University of Haifa, Mt Carmel, Haifa 31905 Israel, Tel: +972 48240962, Fax: +972 48288578, E-mail: galrichterlevin@gmail.com

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The two-way shuttle avoidance (TWSA) task was chosen to assess learning abilities under stressful conditions. In this task, rats learn to shuttle from one compartment to the other when a conditioned stimulus is presented, to avoid a footshock. Intensities of 0.8 and 1.5 mA were used as moderate and strong footshock, respectively. We also evaluated rats' learning ability in novel object and social recognition tasks, known as memory tasks involving lower levels of stress (Terranova *et al*, 1999; Straube *et al*, 2003). In parallel, the functionality of the HPA axis was assessed by measuring 'basal' and stress-induced corticosterone levels.

## MATERIALS AND METHODS

### Animals

Male Sprague–Dawley rats (Harlan Laboratories, Jerusalem, Israel) weighing 200–224 g on arrival were group-housed at room temperature ( $21 \pm 2^\circ\text{C}$ ) on a 12:12 light–dark cycle (lights on: 0700 hours), with water and food pellets *ad libitum*. Animals were randomly separated in two groups: one group was injected with a control virus, whereas the other was injected with CRF KD lentivirus. All behavioral procedures adhered to the NIH Guide for the care and use of laboratory animals and were approved by the University of Haifa ethical committee.

### Lentivirus Construction and Validation

Lentiviral vector was constructed to express shRNA against CRF transcript. *In vivo* efficacy of infection and CRF expression KD was verified by *in situ* hybridization and immunohistochemistry as described previously (Elliot *et al*, 2010; Regev *et al*, 2012). Non-related siRNA was used as control lentivirus. Both viral vectors constructs also express GFP reporter. GFP signal was used to ensure proper infection. Only rats with infection restricted to the PVN and encompassing at least 50% of it were included in the analysis.

### Surgical Procedure

Rats were anesthetized with chloral hydrate (15 mg/kg, intraperitoneally) and placed in a stereotaxic frame, on a heating blanket to maintain body temperature. Antibiotic (Vetrimoxin, 15%, 0.2 ml/kg, subcutaneously; Vetmarket, Petah Tikva, Israel) and analgesic (calmagine, 0.3 ml/kg, subcutaneously; Vetmarket) treatments were given at the beginning of the surgery. Stereotaxic injections were performed bilaterally in the PVN (Paxinos and Watson, 1997) (relative to bregma: AP:  $-1.8$ ; ML:  $\pm 1.8$ ; DV:  $-8.1$  from the skull,  $\alpha = 10^\circ$ ). After placing the needle in the target area, 5 min were given for stabilization. Then, 0.5  $\mu\text{l}$  of viral vector suspension were injected (0.1  $\mu\text{l}/\text{min}$ ) through a 2  $\mu\text{l}$  Hamilton syringe (30 g) connected to a motorized nanoinjector (Stereotaxic Injector; Stoelting, Wood Dale, IL). The needle remained in place for 5 additional minutes before being slowly withdrawn. Animals were allowed 3 weeks of recovery before behavioral procedures.

## Conditioning Paradigms

All experiments were conducted between 0800 and 1400 hours in dim light and sound-attenuated conditions.

**Experiment 1: TWSA.** The TWSA box (Panlab, Harvard Apparatus, Barcelona, Spain) is placed in a dimly-lit, ventilated, sound-attenuated cupboard. The rectangular chamber ( $60 \times 26 \times 28 \text{ cm}^3$ ) is divided by an opaque partition with a passage ( $10 \times 8 \text{ cm}^2$ ) connecting two equal size compartments. Both compartments' metal grid floors are weight-sensitive; microswitches transmit information on the rat's location to an automated data collection program managing both the conditioned stimulus (75 db, 3000 Hz tone) and the unconditioned stimulus (electrical footshock, 0.8 or 1.5 mA) presentations, as well as recording the rats' responses. Training session consisted of 75 trials. Exploration behavior was initially assessed for 10 min by the quantification of shuttles from one compartment to the other. Each trial started with the delivery of the conditioned stimulus for 10 s, immediately followed by the unconditioned stimulus (10 s maximum) with an intertrial interval of  $30 \pm 10$  s. Rats could shuttle during the tone (avoidance, conditioned response), during the shock (escape), or perform no response (no escape).

**Experiment 2: Novel object recognition task.** Rats were first habituated to the open-field arena ( $89 \times 89 \text{ cm}^2$ ) for 3 days, 5 min per day. Then, on familiarization day (day 1), rats were presented with two objects. After 24 h (day 2), rats were presented with one of the previously encountered objects on day 1 and a novel object. Exploration time of each object (animal nose  $< 1$  cm from objects) was recorded during the 5-min sessions on days 1 and 2, and then analyzed offline by an experimenter blind to rats' treatment.

**Experiment 3: Social recognition task.** Rats were first habituated to the open-field arena for 3 days, 5 min per day. After 24 h, rats were simultaneously presented with two rats within the open-field arena (one familiar rat from the same cage and one non-familiar rat from a different cage kept in a different room). These two rats were placed in two individual small cages (20 cm diameter, 18 cm height, 2.5 cm spaced bars), 18 cm apart. Exploration time of both rats (animal nose  $< 1$  cm from the explored rat) was recorded during a 5-min session and analyzed offline by an experimenter blind to rats' treatment.

### Blood Collection for Corticosterone Quantification

Blood samples were taken from the tail through 1 mm incision at the tip of the tail, in freely moving animals (Fluttert *et al*, 2000). The experimenter is meticulously trained to this procedure to not restrain the rat and to be fast and accurate. Gentle pushes on the tail allow collecting 200  $\mu\text{l}$  per sample. There is no bleeding from the tail when the rat is back in the home cage. For circadian rhythm measurements, samples were collected every morning (0700 hours) and evening (1900 hours) over 2 days. For stress-induced corticosterone secretion, blood samples were collected one time 2 days before the TWSA session (at the time of the day the conditioning session will take place) and a second time at

the end of the 0.8 mA TWSA conditioning session. Samples were immediately centrifuged (4 °C, 3600 r.p.m., 20 min) and 70  $\mu$ l of serum were collected and stored at -80 °C until further analysis. Corticosterone concentration was quantified by ELISA (IBL International Kit).

### Immunohistochemistry

After the completion of behavioral tests, rats were anesthetized (chloral hydrate overdose) and transcardially perfused with 100 ml of 0.9% sodium chloride, followed by 250 ml of 4%, 4 °C paraformaldehyde in 0.01 M phosphate-buffered saline (PBS). Brains were removed, postfixed overnight at 4 °C in the same fixative, and immersed in graded series of PBS sucrose solutions (10%, 20%). Free floating, 30- $\mu$ m-thick coronal sections were collected in a cryostat (Leica) in PBS azide 0.05% and stored at 4 °C until further analysis. GFP signal analysis was performed for each rat to check for appropriate PVN infection. All steps were performed at room temperature with mild shaking. Brain sections were rinsed in PBS Triton 0.3% (PBSt) (3  $\times$  10 min) and incubated with a blocking reagent (background sniper; Biocare Medical, Israel) to avoid nonspecific background staining. After PBSt washes (3  $\times$  10 min), sections were incubated overnight with the primary antibody at room temperature (rabbit anti-GFP (ClonTech), 1/400 in PBSt). Sections were then rinsed (3  $\times$  10 min), incubated with the secondary antibody (biotinylated-goat anti-rabbit, 1/1000 (Jackson ImmunoResearch), 1 h), rinsed (3  $\times$  10 min), and incubated with the peroxidase-conjugated streptavidin (1/1000 (Jackson ImmunoResearch), 1 h). After

PBSt washes (3  $\times$  10 min), sections were incubated with DAB (Biocare Medical), rinsed (3  $\times$  10 min), and mounted on slides.

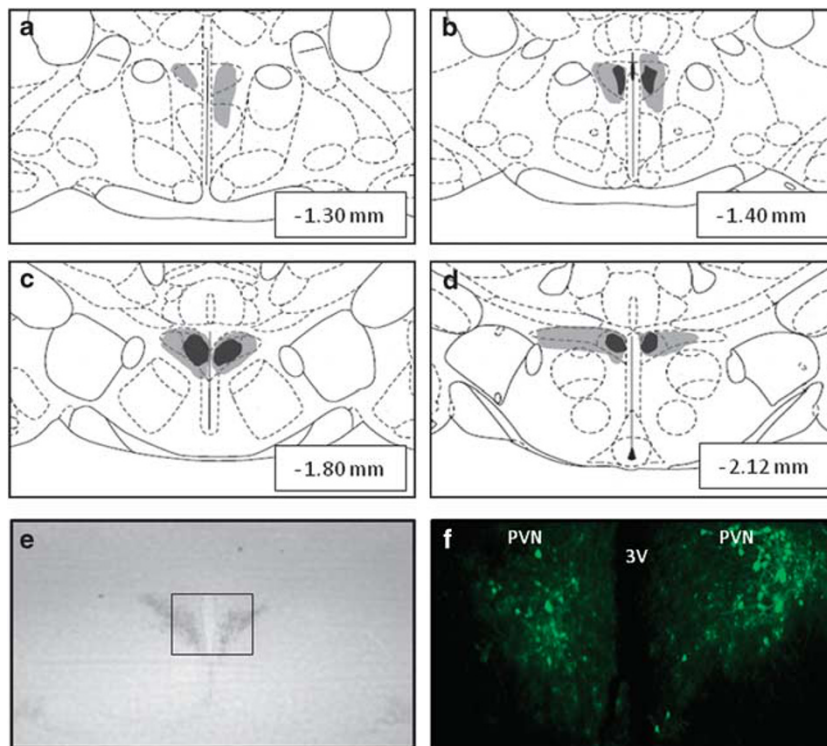
### Data Analysis

The results are presented as mean  $\pm$  SEM and all statistical analyses are performed with SPSS 18. For the TWSA task, given that some variables are not following normal distribution, behavioral data were analyzed by non-parametric ANOVA (Kruskal-Wallis), with treatment and footshock intensity as factors. Corticosterone levels were analyzed by two-way ANOVA with time points as a within factor and treatment as a between factor. For novel object recognition and social recognition tasks, a two-way ANOVA was used, with treatment as a between factor, and object or rat familiarity (familiar/novel) as a within factor.

## RESULTS

### Histological Validation

Rats were bilaterally injected into the PVN. *In vivo* GFP signal showed the spread of the infection, mainly localized in mid and posterior part of the PVN (Figure 1). The brains of the shCRF- and shControl-injected rats were used for GFP immunostaining to validate the site and degree of infection. This localization step was essential for excluding off-target injections and evaluate the degree of PVN infection. Only rats displaying infection site restricted to the PVN were included in the analysis for both control virus- and CRF KD virus-injected rats. Animals with



**Figure 1** Injection sites in the hypothalamus. (a–d) Brain section maps showing the extent of green fluorescent protein (GFP) infection in the paraventricular nucleus of the hypothalamus (PVN) (-1.3 to -2.12 mm from bregma, adapted from the Paxinos and Watson rat brain atlas). Black fill represents the minimum extent of infection in all the rats, and gray fill represents the maximum extent. (e and f) Example of GFP immunostaining at the site of injection, illustrated at low (e) and high (f) magnification.

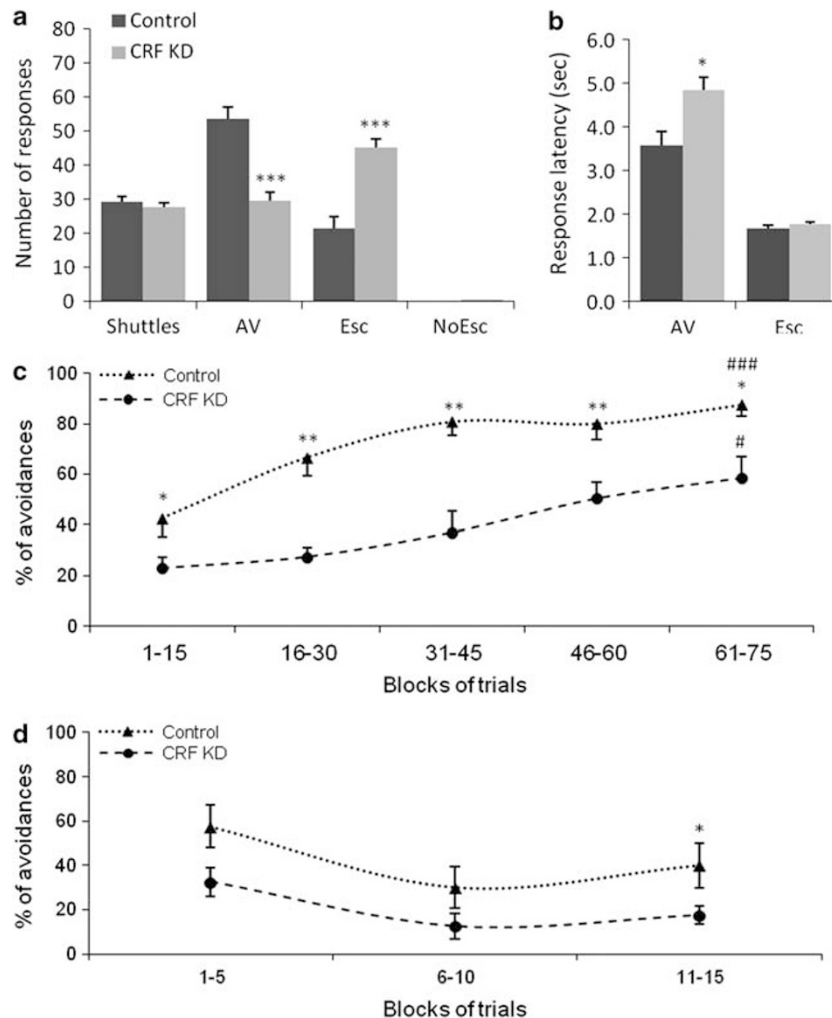
infection outside of the PVN were used to control for the anatomical specificity of behavioral effects (eg, Figure 3a).

As the lentiviral construct was designed to contain both the shCRF and the GFP cDNA sequences (Elliott *et al*, 2010), they are coexpressed in each of the infected cells. Therefore, one can use the GFP distribution to estimate the degree of CRF KD by calculating the percentage of PVN infection. Screening of the PVN sections obtained from the experimental rats, we calculated an approximately 65–70% coverage of the PVN, which suggest similar levels of CRF KD in our experimental group.

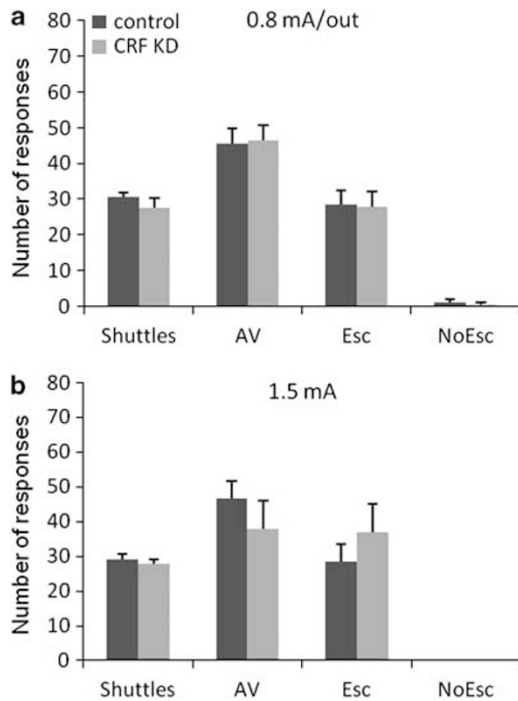
### CRF KD alters learning abilities in the TWSA task under moderate shock intensity conditions

Rats were trained in the TWSA task using either 0.8 (control  $n=8$ ; CRF KD  $n=9$ ) or 1.5 mA (control  $n=8$ ; CRF KD  $n=6$ ). There was no significant difference in the number of

shuttles during the exploration period (Figures 2a and 3a, b; treatment effect: nonsignificant (NS)). When animals were trained with 0.8 mA footshock, CRF KD rats exhibited a decreased number of avoidance responses compared to controls (Figure 2a,  $p<0.01$ ), and increased the number of escapes (Figure 2a,  $p<0.01$ ). These animals also exhibited longer avoidance latencies (Figure 2b). Analyzing more specifically the course of the learning by dividing the session by blocks of 15 trials showed that CRF KD rats do perform more avoidance responses in the last block as compared with the first (Figure 2c). However, their percentage of avoidance remained lower than controls within each block. To rule out any difference in reactivity during the first trials, we further analyzed the very beginning of the session by blocks of five trials (Figure 2d). There was no significant difference between KD and controls during the first 10 trials (block 1–5:  $p>0.05$ , NS; block 6–10,  $p>0.05$ , NS), whereas during the



**Figure 2** Corticotropin-releasing factor (CRF) knockdown (KD) in the paraventricular nucleus of the hypothalamus (PVN) impairs learning in the 0.8 mA two-way shuttle avoidance (TWSA) task. (a) CRF KD decreases the number of avoidance (AV) responses and increases the number of escape (Esc) compared with control (AV:  $p<0.01$  control vs KD; Esc:  $p<0.01$  control vs KD). (b) CRF KD increases AV response latency compared with control ( $p<0.05$ ), but has no effect on Esc latency (control vs KD: nonsignificant). (c) This curve represents the percentage of avoidance in each block of 15 trials during the conditioning session. Although CRF KD rats do exhibit, as controls, higher percentage of avoidance responses at the end of the session than at the beginning ( $^{\#}p<0.05$ ,  $^{\#\#\#}p<0.001$ , blocks 61–75 vs blocks 1–15), this group performs significantly less avoidance responses at each point of the learning curve ( $^*p<0.05$ ,  $^{**}p<0.01$ , control vs KD, within each block). (d) Percentage of avoidance by block of five trials for the first 15 trials of the session. CRF KD rats start to differ from controls from trials 11 to 15 ( $^*p<0.05$ ).



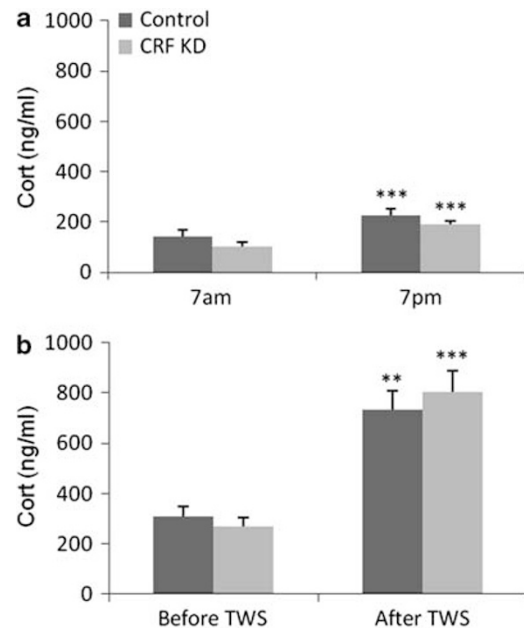
**Figure 3** Corticotropin-releasing factor (CRF) knockdown (KD) in the paraventricular nucleus of the hypothalamus (PVN) has no effect at 1.5 mA or when injected outside of the PVN. (a) Injection out of the PVN shows no unspecific effect of CRF KD on learning abilities. (b) CRF knockdown has no effect on avoidance (AV) and escape (Esc) responses number in a single session using 1.5 mA footshock unconditioned stimulus (control vs CRF KD: nonsignificant).

five next trials, CRF KD rats started to significantly differ from controls (block 11–15,  $p < 0.05$ ). On the other hand, there was no significant difference between control and CRF KD rats responses when using a higher footshock intensity (1.5 mA (Figure 3b); avoidance:  $p > 0.05$ , NS; escape:  $p > 0.05$ , NS). When CRF KD viral vector was injected outside of the PVN (control  $n = 9$ ; CRF KD  $n = 9$ ), CRF KD rats performed the task as well as controls in the 0.8 mA condition (Figure 3a; control vs KD rats: NS for all responses).

With respect to the effect on HPA axis activation, circadian rhythm of basal levels of corticosterone was present in both groups (Figure 4a; nadir in the morning, peak in the evening, time-of-the-day effect:  $F_{(1, 14)} = 19.662$ ,  $p < 0.01$ ), with a NS trend for lower level in CRF KD rats (treatment effect:  $F_{(1, 14)} = 3.575$ ,  $p = 0.08$ ). In addition, both control and CRF KD rats exhibited the same increase of corticosterone blood level after a single TWS conditioning session (Figure 4b; two-way ANOVA, time effect:  $F_{(1, 15)} = 52.838$ ,  $p < 0.001$ ; treatment effect:  $F_{(1, 15)} = 0.91$ , NS).

### Novel Object Recognition task

To further evaluate memory abilities following hypothalamic CRF KD, rats (control  $n = 14$ ; CRF KD  $n = 10$ ) were tested for learning under low stress condition, using the novel object recognition task. First, there was no significant difference in the exploration time of each object on day 1 (Figure 5a; two-way ANOVA, within factor (objects exploration)  $F_{(1, 22)} = 2.317$ , NS; between factor (treatment)



**Figure 4** Corticotropin-releasing factor (CRF) knockdown (KD) in the paraventricular nucleus of the hypothalamus (PVN) does not significantly affect corticosterone levels. (a) CRF KD and control rats display similar basal levels of circulating corticosterone both in the morning and in the evening (treatment effect: nonsignificant (NS); time effect:  $***p < 0.001$ ), 0700 vs 1900 hours, for control and KD animals. (b) Also, a similar increase of circulating corticosterone is observed in control and KD animals after the conditioning session (treatment effect: NS; time effect:  $**p < 0.01$  before vs after in controls,  $p < 0.001$  in CRF KD). TWS, two-way shuttle.

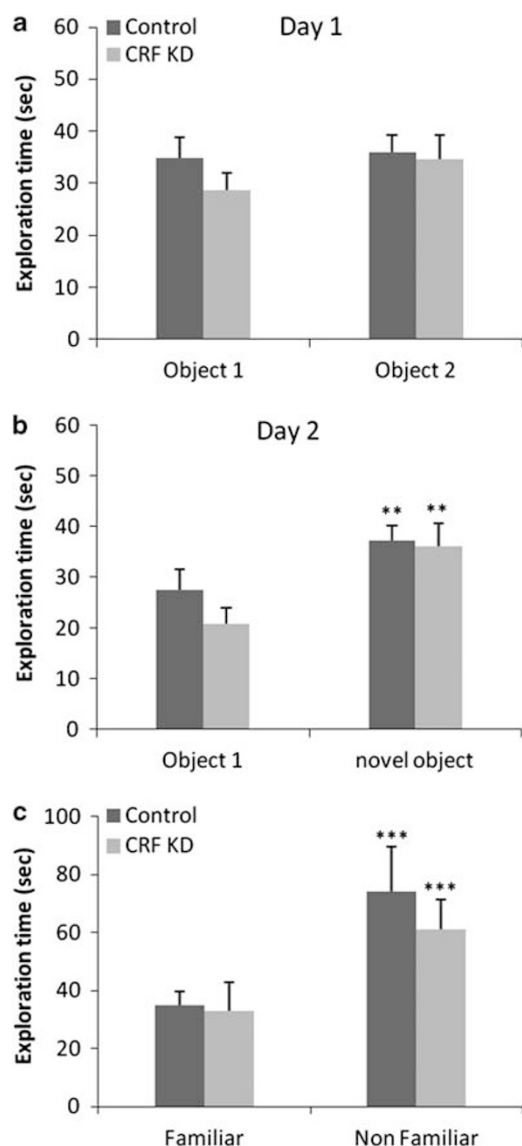
$F_{(1, 22)} = 1.287$ , NS). On day 2, both groups explored significantly more the new object compared with the familiar one (Figure 5b; two-way ANOVA, within factor (objects exploration)  $F_{(1, 22)} = 11.474$ ,  $p < 0.01$ ; between factor (treatment)  $F_{(1, 22)} = 1.628$ , NS).

### Social Recognition Task

Another low stress memory test used was the social recognition test. When rats (control  $n = 8$ ; CRF KD  $n = 9$ ) were presented simultaneously with a familiar and a non-familiar rat, both groups explored significantly more the non-familiar one (Figure 5c; two-way ANOVA, within factor  $F_{(1, 15)} = 22.072$ ,  $p < 0.001$ ; between factor (treatment)  $F_{(1, 22)} = 0.821$ , NS).

### DISCUSSION

To examine the contribution of hypothalamic CRF to stress induce learning deficits, we have KD CRF in the rat PVN. Subsequently, we tested learning and memory abilities in three different behavioral tasks and assayed circulating levels of corticosterone. Our data clearly showed that CRF KD in the PVN impaired learning of the TWSA task when moderate footshock intensity was used. However, CRF KD rats performed similarly to controls when trained with higher footshock intensity. In addition, CRF KD did not affect memory performances in the novel object recognition task nor did it alter social recognition. The results suggest



**Figure 5** Corticotropin-releasing factor (CRF) knockdown (KD) in the paraventricular nucleus of the hypothalamus (PVN) has no effect on memory performance in non-stressful memory tasks. (a) Control and CRF KD rats explore similarly the two objects presented on day 1. (b) Exploration on day 2 of a familiar object vs a novel object, both groups explored significantly more the novel object than the familiar one. (difference between time to explore familiar vs novel object: \*\* $p < 0.01$ ) (c) During the social recognition task, both groups explore significantly more a non-familiar rat than a familiar one. (difference between time to explore familiar vs non-familiar rat: \*\*\* $p < 0.001$ ).

that hypothalamic CRF is involved in learning under moderate stressful conditions but not in learning under low or high stress conditions. Hypothalamic CRF is associated with the modulation of the HPA axis. However, the effects found here were not a result of alterations of the HPA axis, as corticosterone levels in CRF KD rats, whether 'basal' or stress-induced, were not different from controls. Taken together, these results suggest that hypothalamic CRF may be centrally required for appropriate learning when rats are challenged by moderate levels of stress.

CRF has been associated with the response to stress exposure, mainly through its role in the activation of the HPA axis. However, KD of CRF, as conducted here, had no

significant effect on circulating corticosterone levels, suggesting that relatively low levels of PVN-CRF are sufficient for the activation of pituitary CRF receptors and the subsequent secretion of corticosterone from the adrenal cortex. Although it was previously shown that CRF KD strongly decreases *in vivo* CRF expression levels in infected neurons (Elliott *et al*, 2010; Regev *et al*, 2012), low CRF expression could still be evident in infected neurons. In addition, as the infection does not encompass the entire PVN, sparing in particular the anterior part of the parvocellular PVN, a compensatory potentiation of the effect of remaining CRF by vasopressin could sustain the observed HPA axis activity (Antoni, 1993). Moreover, even partial CRF receptor occupancy can lead to full ACTH release (for a review see Aguilera *et al*, 2004). Corticosterone level following 1.5 mA training was not measured as we only assessed corticosterone as readout of CRF KD effect on HPA axis activity and not as a direct measure of stress intensity. Corticosterone is often presented as a marker of stress response, but the relevance of corticosterone level by itself to account for stress intensity is debatable (Natelson *et al*, 1981, 1987). For instance, Merino *et al* (2000) found similar corticosterone increase following 0.4 and 1 mA footshock presentation. More interestingly, corticosterone increase is similar following 0.5 and 1.5 mA foot shocks, whereas weight gain, entries in closed arms, and total entries in the elevated plus maze were clearly different between 0.5 and 1.5 mA (Rabasa *et al*, 2011), indicating that parameters other than corticosterone are required to classify stress intensity. In addition, behavioral scoring during footshock delivery (flinching, jumping, and vocalization) showed significant difference between 0.8 and 1.6 mA, while corticosterone levels were similar (Kant *et al*, 1983).

On the basis of our current findings, it is suggested that training at moderate or high footshock intensity engages different neuronal processing, as hypothalamic CRF was found to be involved only at 0.8 mA. It is well known that many neurotransmitters, peptides, and steroid hormones mediate the stress response in the brain in a complex but well-synchronized manner (Joels and Baram, 2009). CRF neurons in the PVN are mainly known as initiating the HPA axis, projecting to the median eminence and leading to glucocorticoids' release. However, these neurons also innervate the LC and the VTA (Reyes *et al*, 2005; Rodaros *et al*, 2007). It has been shown that PVN neurons projecting to the LC are not projecting to the median eminence (Reyes *et al*, 2005). Affecting mainly LC projecting neurons in the PVN could explain the dissociation we observed between behavioral and endocrine effects of CRF KD. In addition, no anatomical study, to our knowledge, specifically looked for potential projections variations between the anterior part (between  $-1.4$  and  $-1.8$  mm from bregma) and the posterior part of the parvocellular PVN (between  $-1.8$  and  $-2.1$  from bregma). However, as dissociation between CRF neurons innervating the median eminence and the LC was evidenced previously (Reyes *et al*, 2005), we may similarly propose that anterior and posterior part of parvocellular PVN could be functionally dissociated from each other, maybe through different efferent projections, as our injection sites mainly reached the posterior part of parvocellular PVN compared with the relative sparing of its anterior part.

The LC, the main source of noradrenaline in the brain, is involved in emotional modulation of memory (eg, McGaugh, 2004). Damaging the noradrenergic system impaired learning of the two-way active avoidance task (Radwanska *et al*, 2010). Here, we show that KD of CRF in the PVN impairs performances in this task when moderate footshock intensity is used. Thus, hypothalamic CRF could modulate LC activity, leading to noradrenaline release in the amygdala and/or hippocampus; both structures receiving noradrenergic inputs from the LC and being required for two-way active avoidance conditioning (Choi *et al*, 2010). In agreement with this, noradrenaline release in the amygdala was found to increase with increased footshock intensity (Quirarte *et al*, 1998). Training at 1.5 mA could trigger higher noradrenaline release compared with 0.8 mA, and thus compensating for hypothalamic CRF KD and allowing animals trained at 1.5 mA to perform similarly to controls. However, a residual effect of remaining CRF could still participate in the learning. CRF KD does impair learning at 0.8 mA, but not at 1.5 mA. The noradrenaline compensation we propose here could also be mediated through the activation of remaining non-infected CRF neurons, given that noradrenaline is also a potent regulator of CRF neurons' activity (Pacak *et al*, 1995). As higher footshock intensity increases noradrenaline release, it could also further activate remaining CRF in a more effective way than following 0.8 mA training.

Altogether, these data suggest that CRF is a critical player in TWSA acquisition at 0.8 mA, whereas at 1.5 mA, although we cannot rule out involvement of CRF, additional mechanisms are at work, thereby leading to performances similar to controls. Bilateral temporary inactivation of the hippocampus immediately after the inhibitory avoidance conditioning was shown to impair memory retention following training at 0.8 mA, but not at 1 mA (Quiroz *et al*, 2003). This suggests that even very close footshock intensities can be behaviorally dissociated provided that relevant manipulations were carried out. CRF projections from the PVN are also known to innervate the VTA (Rodaros *et al*, 2007). CRF is released in the VTA in response to footshock (Wang *et al*, 2005), and it was proposed that CRF, through potentiation of NMDAR in the VTA, could switch dopaminergic neurons from regular firing to burst firing, which in turn could result in dopamine release in the amygdala, nucleus accumbens, or prefrontal cortex (Ungless *et al*, 2003). VTA dopamine was also shown to be critical for learning the TWSA task (Oades *et al*, 1987). The reduction of hypothalamic CRF could thus affect performance in the TWSA task by reducing the activation of VTA dopaminergic neurons. It remains to be tested which of these pathways is more directly involved in the observed effects of CRF KD.

Performances in the novel object recognition task and the social recognition, involving low level of stress, were also examined. Although several studies addressed the effect of stress on novel object recognition and showed that CRF was a mediator of such effects, no study, to our knowledge, evaluated the involvement of CRF itself, without concomitant stress effects, in this task. We found no effect of PVN CRF KD on object recognition, suggesting that hypothalamic CRF may be specifically involved in learning under moderate, but not low, stress conditions.

Regarding social recognition, previous studies have hinted to the involvement of CRF in this a task. For instance, mice overexpressing CRF showed higher social investigation and improved long-term social recognition compared with wild-type (Kasahara *et al*, 2011), whereas the intracerebroventricular manipulation of the CRF system demonstrated its requirement for short-term social recognition (Heinrichs, 2003). In this study, the specific KD of hypothalamic CRF had no significant effect on social recognition in our conditions. The discrepancy with previous studies could be due to a lack of specificity of transgenic mice or intracerebroventricular injections, which affect CRF-containing neurons in other brain areas, including the hippocampus, BNST and central amygdala. This could also be due to prolonged housing of familiar rats together, allowing particularly robust social memory of the group members to take place.

Stress effects on learning and memory are complex and highly dependent on parameters like intensity, controllability, or duration (Bergado *et al*, 2011; Koolhaas *et al*, 2011). In line with this idea, we suggest here that hypothalamic CRF is involved in learning under moderate stress conditions. It is not required for learning under low level of stress, and when a higher amount of stress is encountered, hypothalamic CRF KD could be compensated by other neuronal systems for appropriate learning. Such a fine-tuning in the stress response has been previously suggested (Joels and Baram, 2009), pointing out the advantage of complementary functions of the different stress mediators to adjust ongoing behavior to environmental demands.

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## DISCLOSURE

The authors declare no conflict of interest.

## REFERENCES

- Aguilera G, Nikodemova M, Wynn PC, Catt KJ (2004). Corticotropin releasing hormone receptors: two decades later. *Peptides* 25: 319–329.
- Antoni FA (1993). Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. *Front Neuroendocrinol* 14: 76–122.
- Bangasser DA, Shors TJ (2010). Critical brain circuits at the intersection between stress and learning. *Neurosci Biobehav Rev* 34: 1223–1233.
- Bergado JA, Lucas M, Richter-Levin G (2011). Emotional tagging—a simple hypothesis in a complex reality. *Prog Neurobiol* 94: 64–76.
- Choi JS, Cain CK, LeDoux JE (2010). The role of amygdala nuclei in the expression of auditory signaled two-way active avoidance in rats. *Learn Mem* 17: 139–147.

- Elliott E, Ezra-Nevo G, Regev L, Neufeld-Cohen A, Chen A (2010). Resilience to social stress coincides with functional DNA methylation of the CRF gene in adult mice. *Nat Neurosci* 13: 1351–1353.
- Flutterm M, Dalm S, Oitzl MS (2000). A refined method for sequential blood sampling by tail incision in rats. *Lab Anim* 34: 372–378.
- Heinrichs SC (2003). Modulation of social learning in rats by brain corticotropin-releasing factor. *Brain Res* 994: 107–114.
- Hubbard DT, Nakashima BR, Lee I, Takahashi LK (2007). Activation of basolateral amygdala corticotropin-releasing factor 1 receptors modulates the consolidation of contextual fear. *Neuroscience* 150: 818–828.
- Joels M, Baram TZ (2009). The neuro-symphony of stress. *Nat Rev Neurosci* 10: 459–466.
- Kant GJ, Mougey EH, Pennington LL, Meyerhoff JL (1983). Graded footshock stress elevates pituitary cyclic AMP and plasma beta-endorphin, beta-LPH corticosterone and prolactin. *Life Sci* 33: 2657–2663.
- Kasahara M, Groenink L, Kas MJ, Bijlsma EY, Olivier B, Sarnyai Z (2011). Influence of transgenic corticotropin-releasing factor (CRF) over-expression on social recognition memory in mice. *Behav Brain Res* 218: 357–362.
- Kehne JH, Cain CK (2010). Therapeutic utility of non-peptidic CRF1 receptor antagonists in anxiety, depression, and stress-related disorders: evidence from animal models. *Pharmacol Ther* 128: 460–487.
- Koolhaas JM, Bartolomucci A, Buwalda B, de Boer SF, Flugge G, Korte SM et al (2011). Stress revisited: a critical evaluation of the stress concept. *Neurosci Biobehav Rev* 35: 1291–1301.
- Lee EH, Lee CP, Wang HI, Lin WR (1993). Hippocampal CRF, NE, and NMDA system interactions in memory processing in the rat. *Synapse* 14: 144–153.
- Lloyd RB, Nemeroff CB (2011). The role of corticotropin-releasing hormone in the pathophysiology of depression: therapeutic implications. *Curr Top Med Chem* 11: 609–617.
- McGaugh JL (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annu Rev Neurosci* 27: 1–28.
- Merino JJ, Cordero MI, Sandi C (2000). Regulation of hippocampal cell adhesion molecules NCAM and L1 by contextual fear conditioning is dependent upon time and stressor intensity. *Eur J Neurosci* 12: 3283–3290.
- Muller MB, Zimmermann S, Sillaber I, Hagemeyer TP, Deussing JM, Timpl P et al (2003). Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. *Nat Neurosci* 6: 1100–1107.
- Natelson BH, Creighton D, McCarty R, Tapp WN, Pitman D, Ottenweller JE (1987). Adrenal hormonal indices of stress in laboratory rats. *Physiol Behav* 39: 117–125.
- Natelson BH, Tapp WN, Adamus JE, Mittler JC, Levin BE (1981). Humoral indices of stress in rats. *Physiol Behav* 26: 1049–1054.
- Oades RD, Rivet JM, Taghzouti K, Kharoubi M, Simon H, Le Moal M (1987). Catecholamines and conditioned blocking: effects of ventral tegmental, septal and frontal 6-hydroxydopamine lesions in rats. *Brain Res* 406: 136–146.
- Pacak K, Palkovits M, Kopin IJ, Goldstein DS (1995). Stress-induced norepinephrine release in the hypothalamic paraventricular nucleus and pituitary-adrenocortical and sympathoadrenal activity: *in vivo* microdialysis studies. *Front Neuroendocrinol* 16: 89–150.
- Paxinos G, Watson C (1997). *The Rat Brain in Stereotaxic Coordinates*. 3rd edn. Academic Press: New York.
- Pezze MA, Feldon J (2004). Mesolimbic dopaminergic pathways in fear conditioning. *Prog Neurobiol* 74: 301–320.
- Quirarte GL, Galvez R, Roozendaal B, McGaugh JL (1998). Norepinephrine release in the amygdala in response to footshock and opioid peptidergic drugs. *Brain Res* 808: 134–140.
- Quiroz C, Martinez I, Quirarte GL, Morales T, Diaz-Cintra S, Prado-Alcala RA (2003). Enhanced inhibitory avoidance learning prevents the memory-impairing effects of post-training hippocampal inactivation. *Exp Brain Res* 153: 400–402.
- Rabasa C, Munoz-Abellan C, Daviu N, Nadal R, Armario A (2011). Repeated exposure to immobilization or two different footshock intensities reveals differential adaptation of the hypothalamic-pituitary-adrenal axis. *Physiol Behav* 103: 125–133.
- Radwanska K, Nikolaev E, Kaczmarek L (2010). Central noradrenergic lesion induced by DSP-4 impairs the acquisition of avoidance reactions and prevents molecular changes in the amygdala. *Neurobiol Learn Mem* 94: 303–311.
- Regev L, Tsoory M, Gil S, Chen A (2012). Site-specific genetic manipulation of amygdala corticotropin-releasing factor reveals its imperative role in mediating behavioral response to challenge. *Biol Psychiatry* 71: 317–326.
- Reyes BA, Valentino RJ, Xu G, Van Bockstaele EJ (2005). Hypothalamic projections to locus coeruleus neurons in rat brain. *Eur J Neurosci* 22: 93–106.
- Rodaros D, Caruana DA, Amir S, Stewart J (2007). Corticotropin-releasing factor projections from limbic forebrain and paraventricular nucleus of the hypothalamus to the region of the ventral tegmental area. *Neuroscience* 150: 8–13.
- Roozendaal B, Brunson KL, Holloway BL, McGaugh JL, Baram TZ (2002). Involvement of stress-released corticotropin-releasing hormone in the basolateral amygdala in regulating memory consolidation. *Proc Natl Acad Sci USA* 99: 13908–13913.
- Row BW, Dohanich GP (2008). Post-training administration of corticotropin-releasing hormone (CRH) enhances retention of a spatial memory through a noradrenergic mechanism in male rats. *Neurobiol Learn Mem* 89: 370–378.
- Straube T, Korz V, Frey JU (2003). Bidirectional modulation of long-term potentiation by novelty-exploration in rat dentate gyrus. *Neurosci Lett* 344: 5–8.
- Swanson LW, Sawchenko PE, Rivier J, Vale WW (1983). Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. *Neuroendocrinology* 36: 165–186.
- Terranova ML, Cirulli F, Laviola G (1999). Behavioral and hormonal effects of partner familiarity in periadolescent rat pairs upon novelty exposure. *Psychoneuroendocrinology* 24: 639–656.
- Ungless MA, Singh V, Crowder TL, Yaka R, Ron D, Bonci A (2003). Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons. *Neuron* 39: 401–407.
- Valentino RJ, Van Bockstaele E (2008). Convergent regulation of locus coeruleus activity as an adaptive response to stress. *Eur J Pharmacol* 583: 194–203.
- Wang B, Shaham Y, Zitzman D, Azari S, Wise RA, You ZB (2005). Cocaine experience establishes control of midbrain glutamate and dopamine by corticotropin-releasing factor: a role in stress-induced relapse to drug seeking. *J Neurosci* 25: 5389–5396.