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CELLULAR AND MOLECULAR BIOLOGY

Effects of sub-chronic CRH administration into the hypothalamic paraventricular and central amygdala nuclei in male rats with a focus on food intake biomarkers

ATEFEH RAYATPOUR, MARYAM RADAHMADI, MINA S. IZADI & MAEDEH GHASEMI

Abstract: CRH neurons are found in the paraventricular nucleus(PVN) and central amygdala(CeA) nuclei. This study investigated the effects of sub-chronic CRH administration into the PVN and CeA nuclei on food intake biomarkers in rats divided into five groups: control, two shams, and two CRH-PVN and CRH-CeA groups(receiving CRH in nuclei for seven days). The CRH-PVN group had significantly higher cumulative food intake and food intake trends than the CRH-CeA group. The CRH-CeA and CRH-PVN groups exhibited significant increases in food intake during hours 1 and 2, respectively. Moreover, to be time-dependent, food intake is modulated by different brain nuclei. The CRH signaling pathway appeared to be activated later in the PVN than CeA. Both groups exhibited significantly higher leptin levels, the CRH-PVN group exhibited higher ghrelin levels and lower glucose levels. Repetitive administration of CRH into the PVN and CeA significantly reduced body weight differences. CRH administration into the PVN affected both leptin and ghrelin levels, but ghrelin had a greater impact on glucose variations and cumulative food intake than leptin. Finally, CRH administration into the PVN and CeA likely activated the HPA axis, and the CeA had a greater impact on the stress circuit than on food intake behavior.

Key words: Central amygdala, Corticotropin-releasing hormone, Food intake, Ghrelin, Leptin, Paraventricular nucleus.

INTRODUCTION

Food intake and energy expenditure interaction is regulated by complex mechanisms and different neuronal circuits (Richard et al. 2000). Although some studies have identified the various factors involved in food intake, the nutritional mechanisms involved remain far from clear (Smagin et al. 2001, Aguilera & Liu 2012). The corticotropin-releasing hormone (CRH) is a neurohormone that serves as one of the main brain mediators and/or neurotransmitters (Aguilera & Liu 2012, Gallagher et al. 2008, Owens &Nemeroff 1991) whose neural pathways might interact with other food intakes neural and energy expenditure circuits (Richard et al. 2000).

A high density of CRH-expressing neurons has been reported in the hypothalamic paraventricular nucleus (PVN) as the main CRH secretion region (Aguilera & Liu 2012, Richard et al. 2000). Nevertheless, CRH neurons are also found in such other regions as the limbic system, the bed nucleus of the stria terminalis, locus coeruleus, cerebral cortex, and, especially, in the central amygdala nucleus (CeA) (Aguilera & Liu 2012, Smagin et al. 2001). In addition, the CeA is an important accessory nucleus that might have contributed to CRH release (Kovacs

2013). This is while both PVN and CeA are rich in CRH receptors (Refojo & Holsboer 2009). Interestingly, activation of CRH receptors is also reportedly involved in food intake, body weight regulation, and metabolic control (Rabasa & Dickson 2016). Due to their CRH neurons and receptors, both PVN and CeA nuclei seem to be involved in food intake regulation (Cai et al. 2014). This underlies the assertion in some studies that central injection of CRH might affect both food intake and body weight (Richardson et al. 2002, Rabasa & Dickson 2016). For instance, acute intraventricular and intraparaventricular injections of CRH reportedly lead to anorexic effects represented by declining appetite and increasing energy expenditure in rats (Sobrino Crespo et al. 2014, Richardson et al. 2002, Krahn et al. 1988). Additionally, peripheral CRH administration has been found to increase both energy expenditure and fat oxidation (Smith et al. 2001). Scholars have suggested that central CRH administration might serve as both an autocrine and a paracrine to increase the expression of CRH mRNA and CRH receptors in the PVN (Makino et al. 2005). However, despite the critical effects of single CRH administration into different nuclei on food intake that of sub-chronic CRH administration remains far from clear. On the other hand, such peripheral factors as ghrelin (secreted by the gastrointestinal tract) and leptin (secreted by the adipose tissue) are involved in regulating food intake and body weight via synthesis and secretion of hypothalamic neuropeptides such as CRH (Klok et al. 2007). This is while some brain areas also have glucose-sensing neurons and are, thereby, sensitive to peripheral signals for regulating food intake, body weight, and energy balance (Stanley et al. 2005, Begg & Woods 2013, Routh et al. 2007). It may, therefore, be suggested that variations in food intake biomarkers such as ghrelin, leptin, and glucose must be related to CRH levels. Previous study has shown that CRH is involved not only in stress response initiation, but also in food intake, body weight, and energy expenditure regulation. To shed light on these processes and the related hypotheses, the present study was designed and implemented to investigate the effects of sub-chronic CRH administration into the PVN and CeA nuclei on food intake during three consecutive hours following CRH injection, food intake trend, body and adrenal weights, serum ghrelin and leptin levels, and blood glucose levels.

MATERIALS AND METHODS

Experimental animals

Thirty male Wistar rats with initial body weights of 200–250g were obtained from Pasteur Institute, Tehran, Iran. Following an initial laboratory adaptation period of at least one week before surgery, the rats were provided with *ad libitum* food and water under standard laboratory conditions of 12-hour light/dark cycles (lights on at 07:00–19:00) and controlled temperature (22±2° C) and humidity (50±5%). All the experimental conditions and procedures were approved by the Research and Ethics Committee of Isfahan University of Medical Sciences in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 2011 Revision). The animals were randomly divided into the following five groups (n=6):

1. Control group (Co): The rats were transferred to the laboratory with no special treatment provided throughout the study period.

2. Sham-operated PVN group (Sh-PVN): The rats underwent stereotaxic surgery and cannulation of PVN after seven days of vehicle (saline) administration.

3. Sham-operated PVN group (Sh-CeA): The rats underwent stereotaxic surgery and cannulation of CeA after seven days of vehicle (saline) administration.

4. CRH-treated intra-PVN (CRH-PVN): The rats received seven-day microinjections of CRH into their PVN.

5. CRH-treated intra-CeA (CRH-CeA): The rats received seven-day microinjections of CRH into their CeA.

Experimental procedures

The animals were anesthetized with chloral hydrate (400 mg/kg; i.p) (Hosseini et al. 2013) before being placed in a Stoelting stereotaxic apparatus (incisor bar ±3.3 mm with symmetrically positioned ear bars). The skull was exposed to drill small holes above the PVN (AP= –1.92, ML= ±0.4, and DV= 8) and the CeA (AP= –2.04, ML= ±3.6 and DV= –8.2) according to the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson 2005). Then, a stainless-steel guide cannula (23 gauge) was unilaterally implanted and fixed 1 mm above the PVN or CeA before a stainless steel stylet (30 gauge) was introduced to prevent any likely obstruction. Following the surgery, all the animals received gentamycin injections (5 mg/kg, i.p) to prevent infection (Singh & Kumar 2017, Bhardwaj et al. 2016) and were allowed for about five days to recover from surgery and the remnant effects of the anesthetic.

Drug microinjection into the PVN and CeA

Depending on the experimental group, each rat would receive intra-nuclear (PVN or CeA) injections of saline-dissolved CRH (2µg/kg/0.5µl saline for 7 days) (Sigma-Aldrich Co., USA). After stereotaxic surgery and PVN or CeA nuclei cannulation, equal volumes of only the vehicle (saline) would be administrated into either the PVN or the CeA nuclei of both sham groups (i.e., Sh-PVN and Sh-CeA). Given between 7:00-8:00 am for seven days, injection would be facilitated by lightly anesthetizing the rats only with ether. Moreover, the animals would be restrained in hand for the styles to be removed from the guide cannula and replaced with dental injection needles (27 gauge) 1 mm longer than the guide cannula to be subsequently connected to 10 µl Hamilton micro-syringes via polyethylene tubing (PE-20). CRH and saline (for the experimental and sham groups, respectively) would be injected into either PVN or CeA nuclei using an automated microinjection pump. The forward movement of a small air bubble inside the polyethylene tubing interposed between the upper end of the needle and the microinjection pump would be taken as drug flow. The injection solutions were administered in a total volume of 0.5 µl for 60 seconds. Following the injections, the needles would be left in place for an extended period of 60 seconds to facilitate drug diffusion.

Food intake paradigm

One possible paradigm for investigating food intake is to record the food mass eaten during a fixed period (Kristenssson et al. 2006), for which seven consecutive days were chosen in this study. Food intake trials were conducted normally between 9:00 AM and 12:00. To measure food intakes, the rats were starved for about 16-18 hours starting in the afternoon of the 7^{th} day of the experiment. On day 8, following the starvation period, the rats were transported to the laboratory at least one hour before the food intake trial began. The food pellets were weighed every hour over three hours (Izadi et al. 2018). Subsequently, each rat was individually placed in a Plexiglas cage covered at the bottom with a thick white paper lining; thus, the rats would have access to a pre-measured amount of their regular laboratory chow. During these three consecutive hours, the amount of leftovers, including crumbs, was measured in the first hour of the experiment to obtain the amount consumed so that the food intake trend

could be reported (Mirmohammadsadeghi et al. 2018, Salimi et al. 2015).

Measurement of body weight differences

Animal body weights were measured in the morning on days 1 and 7 before the starvation period. Body weight difference for each rat would then be calculated using the formula $BWD=BW_{Day7}-BW_{Day1}$.

Assessment of serum leptin and ghrelin as well as blood glucose levels

In the current study, the different food intake biomarkers (i.e., serum ghrelin and leptin levels as well as blood glucose) were measured on the final day of the experimental period (on day 8; blood glucose levels before food intake trials and leptin and ghrelin levels after the trials). Subsequently, after 16-18 hours of food deprivation, blood samples (500µl each) were collected on day 8 from their tail veins at 8:00-9:00 AM. Blood glucose levels were measured using a glucometer (On Call® Plus Co., USA). For hormonal analyses, blood samples were collected inside plastic vials and centrifuged at 6000 rpm for 20 minutes. The serum was separated from the blood samples and stored at −80° C until analysis. Also, serum ghrelin and leptin levels were determined using the commercial enzyme-linked immunosorbent assay (ELISA) and specific rat leptin and ghrelin kits (Zellbio GmbH Co., Germany) with a detection limit of 0.1–20 ng/ml for rat leptin and a sensitivity of 0.05 ng/ml (Intra-Assay precision CV<10% and Inter-Assay precision CV<12%). The detection limit for rat ghrelin was set at 0.4–12.8 ng/ml and sensitivity at 0.025 ng/ml (Intra-Assay precision CV<10% and Inter-Assay precision CV<12%).

Measurement of adrenal weight/100 gr body weight

The adrenal gland is a vital organ that produces corticosteroid hormones in response to stress. In the final stages of the experiment, the adrenal glands were removed and weighed for their weights to be used as the criterion for assessing CRH activity and stress index (Ranjbar et al. 2017).

Histology

Once the last experiment had been completed, the animals were sacrificed under deep anesthesia by decapitation between 13:00-14:00 p.m. before their brains were removed and stored in 10% formalin for at least 3 days. In addition, frozen brain serial transverse sections (60µm) were prepared and the injection sites were determined using a light microscope by a rat brain atlas (Fig. 1). Selecting a proper data percentage of 90%, all the data obtained on animals wrongly implanted were excluded from the analysis and new rats were replaced in each group to maintain the original number of rats per group.

Data analysis

All the data were reported as means ± SEM. The data for various groups were compared using Analysis of Variance (ANOVA) followed by the LSD post-hoc test for multiple comparisons. Differences between food intake trends were analyzed through repeated measures of ANOVA followed by the LSD post-hoc test. The intra-group differences such as pairwise comparisons made between different food intakes in each group at hours 1 *vs.* 2, 2 *vs.* 3, and 1 *vs.*3 were analyzed using a paired t-test. A p-value of less than 0.05 was declared as statistically significant. Ultimately, all the calculations were performed using IBM SPSS Statistics 23.

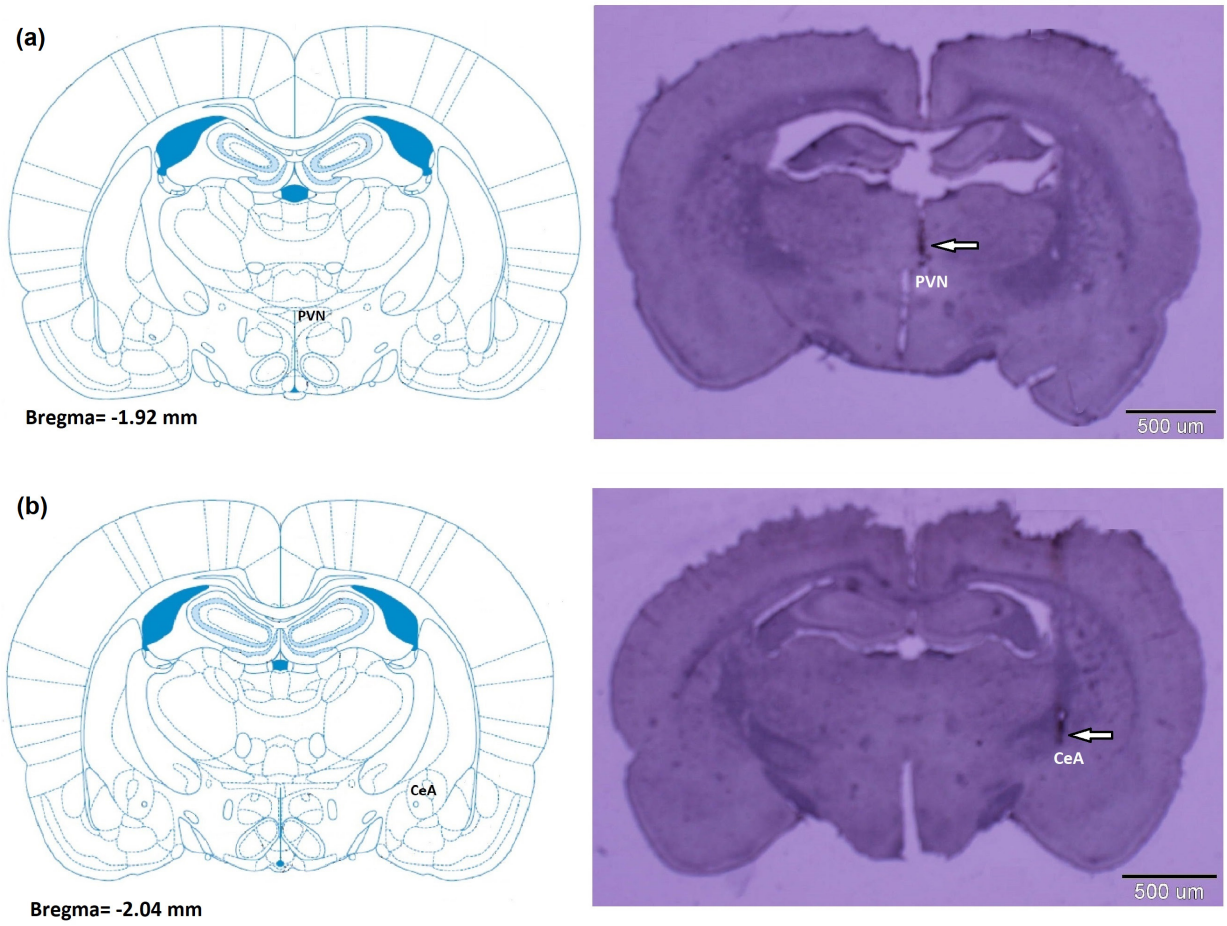

Figure 1. The position of the injection cannula tips in (a) the PVN and (b) the CeA regions for all rats included in the data analyses.

RESULTS

Not all the data revealed significant differences between the control (Co) and the sham (Sh- PVN and Sh-CeA) groups, indicating that neither the surgery nor the injections had any significant effects on the parameters investigated. Therefore, the control group was used as the reference in all comparisons (Figs. 2-9).

Effects of sub-chronic CRH administration into the PVN and CeA on cumulative food intake, food intake (after 1, 2, and 3 hours), and food intake trend

According to Figure 2, the CRH-PVN group exhibited a significantly ($p<0.01$) enhanced cumulative food intake when compared with the Co group. The CRH-CeA group displayed a significant ($p<0.05$) reduction in its cumulative food intake relative to that of the CRH-PVN group.

Figure 3 depicts the effects of sub-chronic CRH administration into PVN and CeA on food intake after 1, 2, and 3 hours. Clearly, compared to the Co group, the CRH-CeA group showed a significant ($p<0.01$) enhancement in food intake at hour 1 while the CRH-PVN group exhibited a significant ($p<0.01$) increase in its food intake at hour 2 (Fig. 3). Finally, compared to the CRH-PVN group, the CRH-CeA group showed significant differences ($p<0.05$, $p<0.01$ and $p<0.05$; respectively) in its food intake at hours 1, 2, and 3 (Fig. 3).

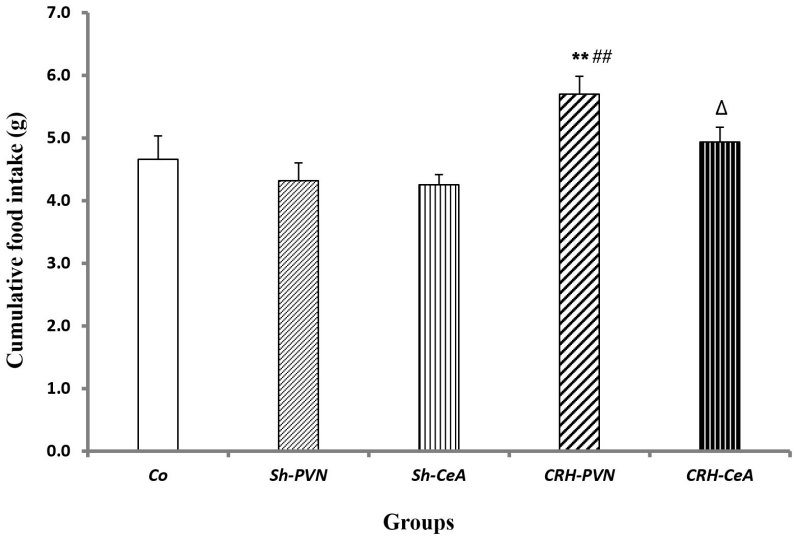

Figure 2. Comparison of cumulative food intake (g) in the experimental groups (n=6). Results are expressed as means ± SEM (One-way ANOVA followed by LSD post-hoc test). **p<0.01 compared to control group, ##p<0.01 compared to Sh-PVN group, Δp<0.05 compared to CRH-PVN. ***Co: control group, Sh-PVN: Sham-operated PVN group, Sh-CeA: Sham-operated CeA group, CRH- PVN: CRH-treated intra-PVN, CRH-CeA: CRH-treated intra-CeA.***

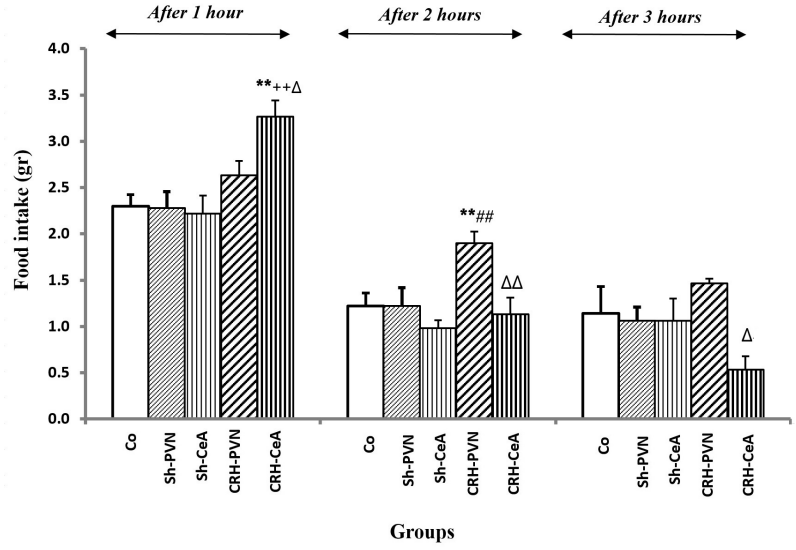

Figure 3. Comparison of food intakes (g) after 1st, 2nd, and 3rd hours in the experimental groups (n = 6). Results are expressed as means ± SEM (One-way ANOVA followed by LSD post-hoc test). **p<0.01 compared to control group, ##p<0.01 compared to Sh-PVN group, ++p<0.01, compared to Sh-CeA group, Δp< 0.05 and ΔΔp< 0.01 compared to CRH-PVN. ***Co: control group, Sh-PVN: Sham-operated PVN group, Sh-CeA: Sham-operated CeA group, CRH- PVN: CRH-treated intra-PVN, CRH-CeA: CRH-treated intra-CeA.***

Figure 4 shows the effects of sub-chronic CRH administration into the PVN and CeA on food intake trends while also comparing within-group food intakes at hours 1, 2, and 3. The repeated measures ANOVA followed by LSD post-hoc test revealed that food intake increasing trend in the CRH-PVN group was significantly higher ($p<0.01$) than that in the Co group (Fig. 4). This is while no significant difference was observed in food intake trend between the CRH-CeA and Co groups (Fig. 4). Compared to the CRH-PVN group, the CRH-CeA group recorded significant ($p<0.05$) decreases in its food intake trend (Fig. 4).

Paired t-tests were used to analyze food intakes in all three trials; hence, comparisons were made at hour 1 *vs.* 2, 2 *vs.* 3, and 1 *vs.* 3 (Fig. 4). As illustrated in Figure 3, food intakes at hour 1 *vs.* 2 and that at hour 1 *vs.*3 in the paired trials showed significant decreases (both $p<0.01$) relative to those in the Co group while no comparatively significant difference was detected between food intakes at hours 2 and 3.

The food intakes of the paired trials (i.e., hour 1 *vs.* 2 and hour 1 *vs.* 3) showed significant ($p<0.05$ and $p<0.01$, respectively) decreases in the Sh-PVN group and significant ($p<0.01$ and

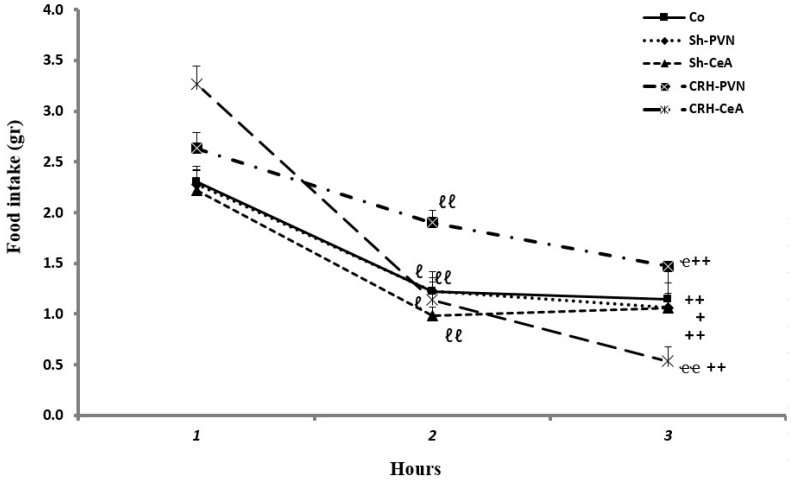

Figure 4. Comparison of food intake trends (g) in the experimental groups (between and within groups, n = 6). Results are expressed as means ± SEM (Repeated measure ANOVA followed by LSD post hoc test and paired t-test). $^{\ell}$p<0.05 and $^{\ell\ell}$p<0.01, compared 1 vs. 2 hours; $^{+}$p<0.05 and $^{++}$p<0.01, compared 1 vs. 3 hours; ^{e} p<0.05 and ^{ee} p<0.01, compared 2 vs 3 hours. ***Co: control group, Sh-PVN: Sham-operated PVN group, Sh-CeA: Sham-operated CeA group, CRH- PVN: CRH-treated intra-PVN, CRH-CeA: CRH-treated intra-CeA.***

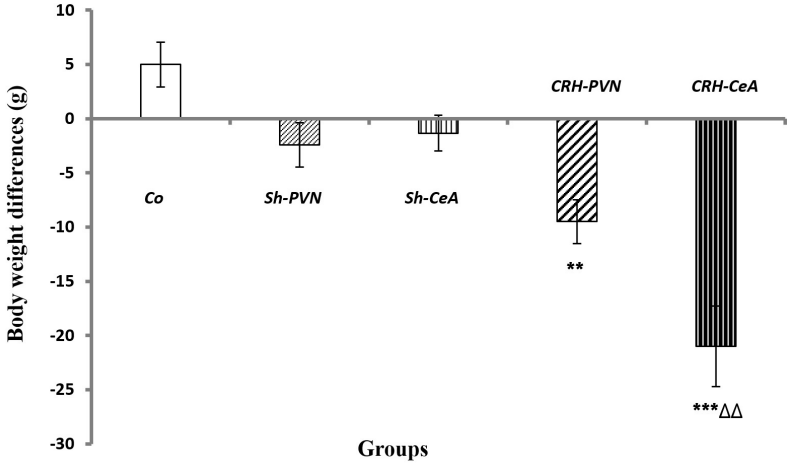

Figure 5. Comparison of body weight differences (g) in the experimental groups (n=6). Results are expressed as means ± SEM (One-way ANOVA followed by LSD post-hoc test). **p<0.01 and ***p<0.001, compared to Co group; $^{\Delta\Delta}$p<0.01 compared to CRH-PVN. ***Co: control group, Sh-PVN: Sham-operated PVN group, Sh-CeA: Sham-operated CeA group, CRH-PVN: CRH-treated intra-PVN, CRH-CeA: CRH-treated intra-CeA.***

p<0.05, respectively) differences in the Sh-CeA group. Nevertheless, no significant differences were observed between the Sh-PVN and Sh-CeA groups in intakes at hour 2 *vs.* 3, a result that was nearly similar to that obtained for the control group (Fig. 4).

Another point of interest in Figure 4 is the significant descending trends of food intake in the CRH-PVN group between hour 1 *vs.* 2, hour 1 *vs.* 3 (both p<0.01), and hour 2 *vs.* 3 (p<0.05). However, significant differences were observed in the CRH-CeA group between hour 1 *vs.*2 (p<0.05), hour 2 *vs.* 3 (p<0.01), and hour 1 *vs.* 3 (p<0.01).

Effects of sub-chronic CRH administration into the PVN and CeA on body weight differences

Compared to the Co group, the CRH-PVN and CRH-CeA groups exhibited significant (p<0.01 and p<0.001, respectively) declines in their body weight differences. However, compared to the CRH-PVN group, the CRH-CeA group showed a significant (p<0.01) decrease in body weight differences (Fig. 5).

Effects of sub-chronic CRH administration into the PVN and CeA on leptin, ghrelin, and glucose levels

As seen in Figure 6, the CRH-PVN and CRH-CeA groups showed significant (p<0.001 and p<0.01,

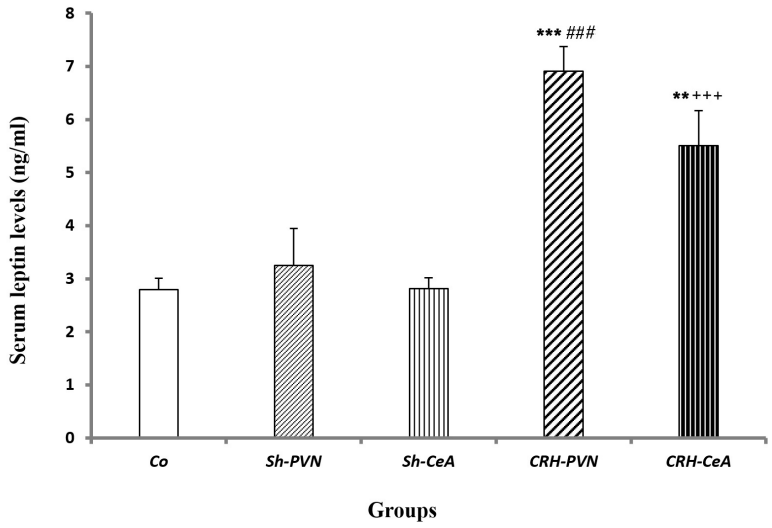

Figure 6. Comparison of on serum leptin levels (ng/ml) in the experimental groups (n = 6). Results are expressed as means ± SEM (ANOVA followed by LSD post hoc test). **p<0.01 and ***p<0.001, compared to Co group; ###p<0.001 compared to Sh-PVN, +++p<0.001, compared to Sh-CeA group. ***Co: control group, Sh-PVN: Sham-operated PVN group, Sh-CeA: Sham-operated CeA group, CRH- PVN: CRH-treated intra-PVN, CRH-CeA: CRH-treated intra-CeA.***

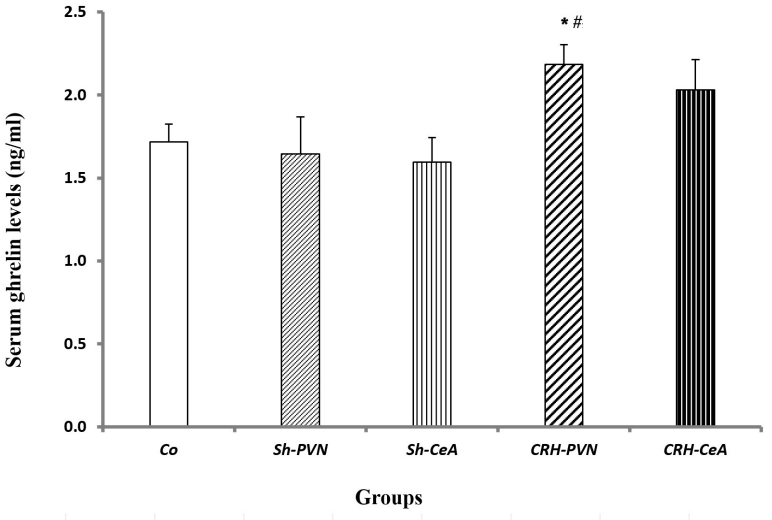

Figure 7. Comparison of on serum ghrelin levels (ng/ml) in the experimental groups (n = 6). Results are expressed as means ± SEM (ANOVA followed by LSD post hoc test). *p<0.05, compared to Co group; # p<0.05 compared to Sh-PVN group. ***Co: control group, Sh-PVN: Sham-operated PVN group, Sh-CeA: Sham-operated CeA group, CRH- PVN: CRH-treated intra-PVN, CRH-CeA: CRH-treated intra-CeA.***

respectively) increases in their leptin levels compared to the control group.

Serum ghrelin level increased significantly ($p<0.05$) only in the CRH-PVN group relative to that of the control (Fig. 7).

Finally, blood glucose levels declined significantly ($p<0.05$) in the CRH-CeA group when compared with the control (Fig. 8).

Effects of sub-chronic CRH administration into the PVN and CeA on adrenal gland weight per 100 g of body weight

Adrenal gland weight per 100 g of body weight was measured in the different groups. It was found that while neither the CRH-PVN group nor the CRH-CeA one showed any significant difference in its adrenal gland weight/100 g body weight relative to that of the other, both exhibited significant ($p<0.05$ and $p<0.01$, respectively) enhancements in this parameter relative to that measured in the Co group (Fig. 9).

DISCUSSION

The effects of sub-chronic CRH administration into the PVN and CeA nuclei on food intake, body weight differences, serum leptin and ghrelin levels, blood glucose levels, and adrenal

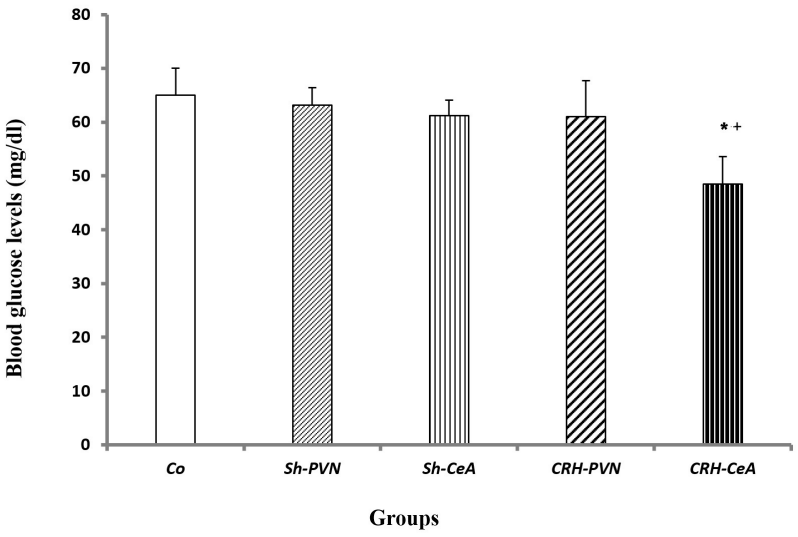

Figure 8: Comparison of on blood glucose levels (mg/dl) in the experimental groups (n = 6). Results are expressed as means ± SEM (ANOVA followed by LSD post hoc test). *p<0.05 compared to Co group, $^{+}$p<0.05 compared to Sh-CeA group. ***Co: control group, Sh-PVN: Sham-operated PVN group, Sh-CeA: Sham-operated CeA group, CRH-PVN: CRH-treated intra-PVN, CRH-CeA: CRH-treated intra-CeA.***

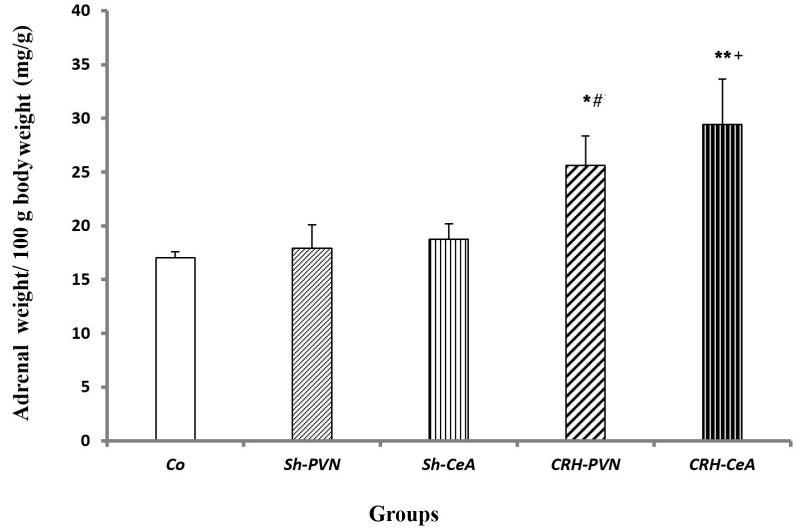

Figure 9. Comparison of adrenal glands weight/ 100 g body weight (mg/g) in the experimental groups (n = 6). Results are expressed as means ± SEM (One-way ANOVA followed by LSD post-hoc test). *p<0.05 and **p<0.01 compared to Co group, $^{\#}$p<0.05 compared to Sh-PVN group, $^{+}$p<0.05, compared to Sh-CeA group. ***Co: control group, Sh-PVN: Sham-operated PVN group, Sh-CeA: Sham-operated CeA group, CRH-PVN: CRH-treated intra-PVN, CRH-CeA: CRH-treated intra-CeA.***

weight/100 g of body weight were investigated in rats.

Effects of sub-chronic CRH administration into the PVN and CeA on cumulative food intake

The data obtained showed that cumulative food intake mainly increased under sub-chronic and/or repetitive CRH administration into the PVN rather than in the CeA nucleus, confirming the important role of the PVN in cumulative food intake (Fig. 2). As an integrating sensor for multiple central and peripheral signals, PVN was found to serve an important function in regulating energy homeostasis (Sutton et al. 2016). In contrast to these findings, Moris and Pavia maintained that acute CRH administration into the PVN exhibited anorexic effects through the neuropeptide Y (NPY) (Morris and Pavia 1998). Hence, despite previous reports of decreased food intake following an acute CRH injection, the present study suggests that food intake reasonably increases as a result of sub-chronic CRH administration into the PVN due to the changes produced in both appetite and NPY (as an orexigenic neuropeptide) (Morris & Pavia 1998). Moreover, sub-chronic CRH administration

into the CeA was also observed to decrease cumulative food intake. Jochman et al. found that the single dose of CRH administration into the basolateral amygdala, rather than into the central amygdala, diminished food intake (Jochman et al. 2005). It should be mentioned in passing that different mechanisms might be involved in food intake. For example, CRH injection frequency or adaptation to CRH anorexic effects might affect food intake behavior due to changes produced in the sensitivity and number of CRH receptors (Owens and Nemeroff 1991). In addition, complex neural circuits (those with neuroendocrine axes) might be involved in regulating appetite and nutrition.

Effects of sub-chronic CRH administration into the PVN and CeA on food intake (after 1, 2, and 3 hours)

It was found that food intake increased in the CRH-CeA group after 1 hour but that it increased in the CRH-PVN group after 2 hours (Figs. 2-3), suggesting that the CRH signaling pathway involved in food intake would be activated later in the PVN than in CeA nucleus (Fig. 2). To explain this, Iemolo et al. claimed that the elevated CRH expression in the CeA might be due to food deprivation (Iemolo et al. 2013). Alternatively, it may be claimed that this elevated CRH expression and CRH injection into CeA had a reinforcing effect on food intake after 1 hour. In addition, some studies suggested that the central amygdala (as an extra-hypothalamic CRH systems) would be able to activate the serotonergic and dopaminergic (being involved in the food intake reward neurotransmitters) systems, leading to elevated food consumption levels (Douglass et al. 2017, Cameron et al. 2017, Iemolo et al. 2013, George et al. 2012). Hence, the enhanced activity of serotonergic neurons in the CeA might have possibly led to enhanced food intake within the first hour of food intake.

Effects of sub-chronic CRH administration into the PVN and CeA on food intake trend

The data obtained in the current study showed that declining food intake trends in the CRH-PVN group and particularly in the CRH-CeA group were observed to occur during later hours (Fig. 4). The declining physiological food intake response at later hours seems to be in compensation for the elevated food intake, probably because these groups had their stomachs already filled during the preceding hours. Furthermore, Zhang et al. demonstrated that CRH release in the CeA followed food intake (Zhang et al. 2011). This suggests that increased food intake in the first hour possibly inhibited food intake in the CRH-CeA group during the following hours. Further support for this claim is provided by Fekete et al. who maintained that increased expression of CRH receptors in the central amygdala would lead to anorexia effects (Stengel and Tache 2014). The declining food intake after the initial hour might be, therefore, related to regulatory changes in the CRH receptors of the central amygdala. Nevertheless, the food intake trend marked higher records in the CRH-PVN group than it did in the control (Fig. 4), suggesting that the CRH signaling pathway of food intake would be probably activated not only later but also more permanently in the PVN than in the CeA nucleus. It seems that the effect of sub-chronic CRH administration into the brain nuclei on food intake is time-dependent and depends on the brain nuclei as the injection sites. This is in agreement with the findings of Moris and Pavia who showed different food intake responses by various nuclei in acute CRH injection (Sutton et al. 2016).

Effects of repetitive CRH administration into the PVN and CeA on body weight differences

Another important finding of the present study is the decreased body weight differences in the PVN-CRH group and the graver differences

in the CeA-CRH group (Fig. 5); this is in agreement with growing reports demonstrating that the CeA is another brain region involved in food intake/body weight regulation. LeFeuvre et al. demonstrated that CRH had a strong effect on the thermogenesis of fatty tissues (LeFeuvre et al. 1987). Moreover, either central or intra-paraventricular CRH injection reportedly leads to brown fat thermogenesis stimulation by initially activating the sympathetic output (Owens & Nemeroff 1991, Cullen et al. 2001, Benoit et al. 2000, Rothwell et al. 1991), thereby causing a negative energy balance and weight loss (Cullen et al. 2001). Thus, although cumulative food intake increased mainly under CRH administration into the PVN (rather than under injection into the CeA nucleus), body weight changes in the CRH-PVN group recorded a lower rate than those observed in the CRH-CeA group and the following cumulative food intake (Figs. 2 and 5). In other words, elevated cumulative food intake was associated with greater inhibition of weight loss in the CRH-PVN group.

Effects of sub-chronic CRH administration into the PVN and CeA on serum leptin and ghrelin levels as well as blood glucose level

It is worth noting that despite the significantly elevated serum leptin levels in both CRH-PVN and CRH-CeA groups, serum ghrelin levels increased only in the CRH-PVN group (Figs. 6 and 7). In contrast to our findings, Rezai-Zadeh et al. (2014) proposed that leptin level did not affect whatsoever on food intake. Some studies reported both leptin and ghrelin levels to affect energy homeostasis; leptin suppressed food intake and, thereby, induced weight loss though ghrelin enhanced food intake and led to weight gain (Klok et al. 2007, Castaneda et al. 2010). Furthermore, it is possible that ghrelin and other ghrelin synergic biochemical factors played more effective roles in cumulative food intake and body weight changes under sub-chronic CRH administration into the PVN, as evidenced by the increased cumulative food intake and the more gravely inhibited weight loss in the CRH-PVN group due to ghrelin observed in the present study (Figs 5-7). These observations justify the identification of ghrelin as a key factor in body weight regulation (Korbonits et al. 2004, Jensen et al. 2016). Central and systemic administrations of ghrelin have also been found to enhance food intake and weight gain to result in energy homeostasis regulation (Castaneda et al. 2010).

On the other hand, both the decreased cumulative food intake and the weight loss observed underwent major changes in the sub-chronic CRH-CeA group in the present study whereas changes in ghrelin level were insignificant in this group (Figs. 2, 5, and 7). This might be explained with recourse to other hitherto unidentified mechanisms associated with food intake that may be involved in the administration of CRH into the CeA nucleus; this is confirmed, for instance, by Mihalache et al. who indicated that weight loss was accompanied by an increase in ghrelin level (Mihalache et al. 2016). Generally speaking, it is possible that different hormones, such as leptin and ghrelin, serve as dominant regulators in various brain nuclei.

In this study, CRH administered into the CeA, or its slight amounts administered into the PVN, was found likely to lead to hypoglycemia (Fig. 8). This is confirmed by the findings reported elsewhere showing leptin's direct modulatory effect on glucose metabolism through improving insulin sensitivity and lower blood glucose levels (Bluher and Mantzoros 2009, Brennan and Mantzoros 2007). Castañeda et al. showed that high ghrelin levels decreased plasma insulin but increased plasma glucose levels (Castaneda et al. 2010). It may, thus, be claimed

that the changes in glucose levels in the CRH-PVN group occurred under the effects of both leptin and ghrelin levels and/or their ratio, with the ghrelin effect being stronger. However, it is uncertain yet whether any specific physiological or pharmacological dose of ghrelin would change insulin and glucose levels (Castaneda et al. 2010). It must be noted, however, that no major hypoglycemia was observed in the CRH-PVN group in the present study.

Effects of sub-chronic CRH administration into the PVN and CeA on adrenal gland weight

The adrenal gland weight per 100 g body weight (representing CRH activation and stress index) showed significant increases not only in the CRH-PVN group but also in the CRH-CeA group in particular (Fig. 9). In contrast to acute CRH injection, sub-chronic CRH administration into the PVN and CeA would probably activate the HPA axis acting as stressful conditions. This is confirmed by a previous study that indicated the HPA axis could be activated by such extra-hypothalamic CRH systems as the central amygdala (Anthenelli 2010).

It may be concluded that a major enhancement occurred in cumulative food intake as a result of sub-chronic CRH administration into the PVN and that food intake was time-dependent, varying with the brain nuclei affected. Moreover, the CRH signaling pathway of food intake was probably activated later and more permanently in the PVN than in the CeA. Sub-chronic CRH administration into the PVN was also found to influence both leptin and ghrelin levels, with the ghrelin effect being stronger than that of leptin on glucose variations. Ghrelin had a greater role in cumulative food intake under CRH administration into the PVN. Finally, it may be concluded that sub-chronic CRH administration into the PVN and CeA probably activated the HPA axis and that the CeA had a more important impact on the stress circuit than on the food intake behavior. The role played by the expression of receptors and other hormones involved in food intake as well as the long-term impacts of CRH injection may be suggested for future study.

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