eISSN 1303-1775 • pISSN 1303-1783

Original Article

DOC and GCA blocks the reduction of hypothalamic CRF containing neurons due to repetitive stress

Published online 28 February, 2007 © http://www.neuroanatomy.org

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Received 2 May 2006; accepted 15 February 2007

ABSTRACT

Stress causes multiple adaptive hormonal responses including the secretion of CRF, ACTH and corticosteroids. These responses to repetitive stress could be blocked by naloxone, corticosteroids and glycyrrhizic acid (GCA). The effect of corticosteroids and GCA on the hypothalamic CRF containing neurons with repetitive stress has not been studied.

Sprague-Dawley rats were given either dexamethasone (DEX), deoxycorticosterone (DOC) or GCA. On the eleventh day of treatment, the rats were exposed to two hours of immobilization stress and the procedure was repeated daily for another twelve days. The rats were sacrificed on days 2, 4, 8 and 12 after immobilization. On the days of sacrifice, the rats were transfused transcardially and the hypothalami were dissected for immunohistochemical analysis.

With repetitive stress, the number of CRF containing neurons decreased significantly from 1631 ± 58 neurons/mm^2 to 595 ± 71 neurons/mm^2 ($p<0.0005$) on second exposure to stress and gradually increased to basal values 2058 ± 109 neurons/mm^2 by the 8–12 exposures to stress. DOC and GCA completely inhibited this decrease in CRF containing neurons. DEX initially caused a significant decrease in CRF containing neurons compared to controls with repetitive stress.

The release of CRF, which initiates the hypothalamus pituitary adrenal responses to stress, could be blocked by mineralocorticoids as well as glucocorticoids. ***Neuroanatomy; 2007; 6: 17–20.***

Key words [deoxycorticosterone] [dexamethasone] [glycyrrhizic acid] [CRF containing neurons] [paraventricular nucleus] [repetitive stress]

Introduction

Stress stimulates multiple adaptive hormonal responses including the secretion of *corticotrophin releasing factor* (CRF), *adrenocorticotrophic hormone* (ACTH), β-endorphin and corticosteroids. There are two major systems involved in stress responses, the CRF system or the *hypothalamo pituitary adrenal axis* (HPA) system and the locus caeruleus or *sympathetic adrenal medullary axis* (SAM) system [1,2]. Hence in stress, both HPA and SAM axes are stimulated simultaneously at each level of the stress system, including the frontal cortex, amygdala and *paraventricular nucleus* (PVN) of the hypothalamus.

CRF is a predominant regulator of the neuroendocrine, autonomic and behavioral responses to stress. CRF-containing neurons are in the parvocellular subdivisions of the PVN in the hypothalamus [3,4] and they play a pivotal role in the regulation of the HPA. These cells project to the *median eminence* [4,5] where they are released into the primary plexus of the hypothalamo-hypophyseal portal circulation and hence to the ACTH-producing cells of the anterior pituitary. Stress impulses are sent to the PVN of the hypothalamus, leading to the release and synthesis of CRF [6,7]. A large increase in hypothalamic CRF occurs within minutes of applying a wide variety of stressful stimuli [8]. It seems probable that there are a wide number of mechanisms by which CRF synthesis and release can be achieved by various stressors. In response to the various stressors, CRF release would result in the release of glucocorticoids from the adrenal cortex.

Glucocorticoids are the hormonal mediators of stress. Glucocorticoids inhibit the activity of HPA directly and indirectly at the various levels of stress response structures such as the anterior pituitary, PVN and amygdala. Glucocorticoid receptors are present at these structures. Glucocorticoid effects at the tissues however may be modulated by binding proteins and by enzymes such as *11β-hydroxysteroid dehydrogenase* (11β-HSD), which reversibly converts the active glucocorticoids (cortisol, corticosterone) to the inactive moieties (cortisone, 11-dehydrocorticosterone). 11β-HSD has so far two identified isoenzymes that interconvert hormonally active cortisol and inactive cortisone [9,10]. *Glycyrrhizic acid* (GCA) is a well-known inhibitor of the enzyme 11β-HSD [11,12]. Previous studies by the group had demonstrated that stress resulted in marked responses such as decrease in blood pressure (BP) and decrease in locomotor activity [13,14]. Repetitive stress however, resulted in adaptation by the 4th to 7th exposure. These stress responses could be ameliorated by corticosteroids, naloxone or GCA [13,14]. In another study by our group [15] we demonstrated that DEX treated rats resulted in a reduction in the number of CRF containing neurons. Similar pattern was seen in the DOC and GCA treated rats but the reduction was less. However the effects of these steroids and GCA on the PVN-CRF containing neurons in response to repetitive stress are not known. The objective of this study was to

determine whether the corticosteroid modulation of the CRF containing neurons could be affected by stress and GCA.

Material and Methods

Male Sprague-Dawley rats (180-230 g) from a pathogen-free colony bred in the Animal House, Institute of Medical Research of Malaysia were used in this study. The rats were housed two per cage, and maintained in a regular day/night cycle; with natural light period from 07:00–19:00 hours with free access to rodent chow and tap water.

The rats were randomly divided into 4 treatment groups of 6 rats each. Group 1 control rats (C) were given vehicle injections (0.1 ml olive oil). Group 2 rats were treated with intramuscular injection of dexamethasone (DEX 120 μg/kg) and group 3 with intramuscular injection deoxycorticosterone (DOC 2.4 mg/kg). These doses were previously shown to have maximal effects on the secretion and synthesis of ACTH at the pituitary and hypothalamus [16,17]. Group 4 rats, received drinking water containing 1.0 mg/ml GCA (Sigma, St. Louis, MO, USA). The average amount consumed was 40-50 ml/day per rat and at these doses have been previously shown to inhibit the responses to repetitive stress [14]. The steroid hormone injections were given daily ranging from a minimum period of 10 days and a maximum period of 22 days depending on the day of sacrifice.

Immobilization stress

The rats were subjected to repeated immobilization stress following previous method [18]. This was accomplished by placing the rats into plastic restrainers with a diameter of 5 cm and length of 12 cm. Daily immobilization stress consisted of exposing rats to 2 hours/day of immobilization (from 09:00 to 11:00) until the day of sacrifice. The rats were unable to move forward nor backward but were able to twist their bodies. Following each immobilization stress session, rats were returned to their respective cages and were able to eat and drink ad libitum for the remainder of the day. The rats were sacrificed the day after the last administration of the immobilization stress. The days of sacrifice were carefully chosen whereby the days 2–4 corresponded to the maximal responses to acute stress and days 8–12 to the periods of adaptation to the repetitive stress.

Tissue collection

Prior to sacrifice, the rats were injected intraperitoneally with 500 units of heparin. The rats were then deeply anaesthetized with 25% urethane, 0.6 ml/100g body weight intraperitoneally. Perfusion was done transcardially initially with 200 ml of phosphate buffer solution (PBS) (0.15M NaCl, 10^{-4}M KH_2PO_4, 10^{-3}M $Na_2HPO_4 12H_2O$, 10^{-3}M $NaNO_3$, pH=7.6, heparin 5000 units/l) or until the effluent remained clear, and immediately followed by Bouin's fixative (750 ml picric acid, 250 ml 40% formaldehyde, 50 ml glacial acetic acid) for 10–15 minutes. The hypothalami were then postfixed in the same fixative solution for an hour at 4°C before they were stored in 10% sucrose in PBS overnight at 4°C. On the following morning, the tissues were embedded in albumin/gelatin (30%:0.5%) embedding medium for 4 hours, before they were immersed in 30% sucrose solution overnight at 4°C. Frozen transverse serial sections of the hypothalami were cut at a thickness of 5 μm using a cryostat (Reichert Jung) at 20°C. The sections then were mounted on poly-L-lysine (Sigma Chemical Co. USA) coated glass slides. The slides were kept at 20°C until the staining procedure.

Immunohistochemical studies

Immunohistochemical staining was based on the labeled streptavidin-biotin (LSAB) method using Dako LSAB 2 Kit (USA). The sections were soaked in Tris buffer solution (TBS) pH=7.6 for 30 minutes to remove the remaining Bouin's fixative. The endogenous peroxidase activity was then blocked by incubation with 3% H_2O_2 followed by incubation with 0.5% normal goat serum in TBS for 20 minutes to block non-spesific immunoglobin (Ig) binding sites on the sections. Tissue sections were then exposed to the primary antibody, anti-CRF (rat) whereby the antibody was diluted to 1:1000 in TBS, with 1% bovine serum albumin for 60 minutes. Positive immunostaining was identified by the addition of a biotinylated anti-mouse and anti-rabbit IgG secondary antibody followed by streptavidin peroxidase (DAKO LSAB 2 Kit, Dako, USA) for 10 minutes. The sections were then exposed to 10 mg 3,3' diaminobenzidine tetrahydrochloride in 15 ml TBS buffer, with 12 μl, 30% hydrogen peroxide for up to 5 minutes. Sections were then washed under running tap water, counterstained with Mayer's haematoxylin, dehydrated and mounted in synthetic mounting media (DPX 8711, Difco Lab. UK). The absence of non-specific staining for the primary antibody, anti-CRF antibody was demonstrated by negative control sections (i.e. incubations carried out as above but without the anti-CRF antibody).

Counting of CRF containing neurons and quantification

The PVN of the hypothalamus was defined anatomically and the number of the CRF containing neurons was counted in the area of 2 mm^2. The positive CRF containing neurons were identified as those neurons with brown stained cytoplasm (Figure 1). All the positive CRF containing neurons within the 2 mm^2 area of the PVN were counted using the computerized image analyzer with the KS-400 (Carl Zeiss, Germany) program.

Results

Dense populations of CRF containing neurons were easily identified in the PVN of the hypothalamus as shown by other researchers [19]. Pretreatment with DEX significantly ($p<0.05$) decreased the number of CRF containing neurons, but not in the rats pretreated with DOC or GCA. Following the first two acute exposures to immobilization stress, there was a significant ($p<0.0005$) decrease in the number of CRF containing neurons in the PVN of the control rats. No significant decrease was seen in the number of CRF containing neurons in the DOC or GCA pretreated rats following stress, whilst the number of CRF containing neurons in the DEX treated rats showed a trend towards an increase.

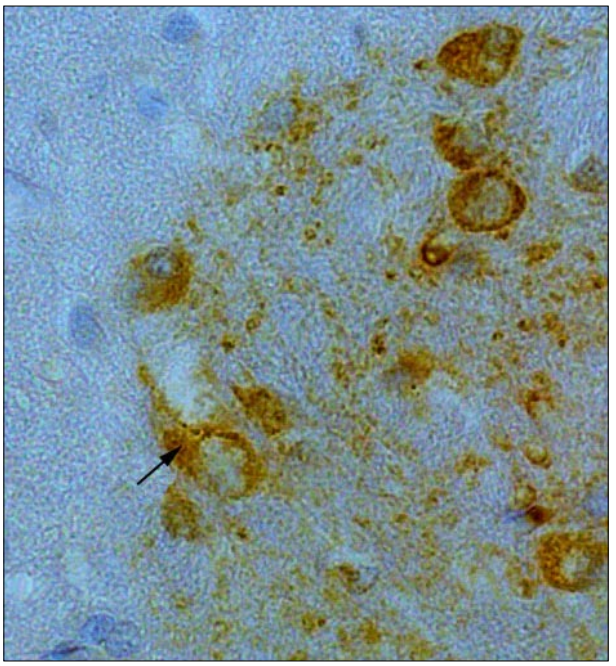

Figure 1. Immunohistochemical staining of CRF containing neurons in a section of PVN of the hypothalamus. The positive CRF containing neurons were identified with the presence of brown stained cytoplasms x 400. Color version of figure is available online. *(**arrow:** cytoplasm of one stained neuron)*

With repetitive stress, the number of CRF containing neurons in the control group then increased towards pre-stressed values and became comparable to the pre-stressed values by the 8th day of repetitive stress, when adaptation to the stress had occurred. In the GCA and DOC treated rats, there was no significant change in the number of CRF containing neurons in the PVN even with repetitive stress, whilst the DEX treated rats had significantly increasing number of CRF containing neurons with the repetitive stress (Table 1 and Figure 2).

Discussion

Responses to repetitive stress in the Sprague-Dawley rats have been studied using various parameters, namely decrease in BP, decrease in locomotor activity and increased levels of corticosterone and endorphins have been studied [13,14]. These responses to immobilization stress typically consisted of a marked decrease in BP or locomotor activity and an increase in plasma corticosterone and β-endorphin levels. With repetitive stress, the rats have a process of adaptation whereby by the 4th to 5th exposure, the BP or locomotor activity began to increase towards basal values whilst the plasma corticosterone and β-endorphin decreased. By the 8th to 12th exposure, the animals were fully adapted and further exposure to stress did not decrease the BP or locomotor activity nor increase the β-endorphin or corticosterone. These typical responses were totally blocked by administration of naloxone, an opioid antagonist, or by pretreatment with DEX, DOC or GCA. For locomotor activity, the responses to stress were partially blocked by DEX.

In this study, it was found that the control rats had significant decrease in the number of CRF containing neurons in the PVN with the first 2 exposures to repetitive immobilization stress, and that the number of CRF containing neurons then increased back to basal levels with further exposure to repetitive stress. Thus, the process of acute response to stress in normal rats are associated with a decrease in the number of CRF containing neurons implying a marked release in CRF, causing the increase in plasma ACTH, endorphins and corticosterone seen in previous studies [13–18]. With increasing repetitive stress, the control rats eventually became adapted and did not exhibit the stress responses. This coincides with the increasing number of CRF containing neurons in the hypothalamus, and the decrease in the endorphins and corticosterone seen previously.

On the other hand, DOC blocked the release of CRF by the insignificant change in the number of CRF containing neurons in the PVN even with repetitive stress and thus blocked the acute drop in BP or a decrease in locomotor activity with the stress seen previously. Ten days pretreatment with daily injections of DEX decreased the number of CRF containing neurons; but with stress, the

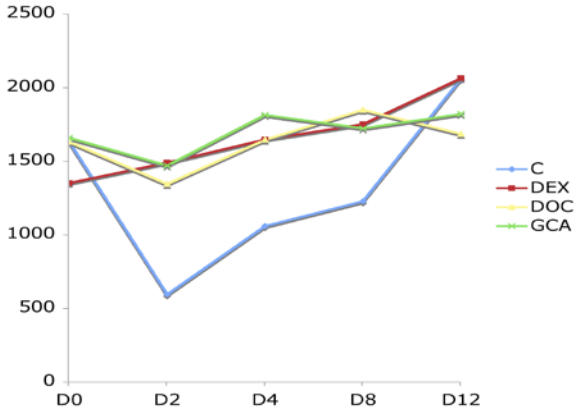

Figure 2. A graph showing the number of CRF containg neurons (unit/mm^2) according to the post-immobilization (days). Color version of figure is available online.

Table 1. Effects of DEX, DOC and GCA on the number of CRF containing neurons in the PVN after the immobilization stress.

PI (days)	Number of CRF containing neurons (unit/mm^2)			
	C	DEX	DOC	GCA
D0	1631 ± 58	1351 ± 15	1632 ± 15	1658 ± 126
D2	595 ± 71	1490 ± 95	1344 ± 82	1469 ± 66
D4	1058 ± 105	1646 ± 34	1645 ± 149	1813 ± 51
D8	1227 ± 16	1751 ± 62	1848 ± 152	1722 ± 87
D12	2058 ± 109	2062 ± 268	1684 ± 80	1820 ± 69

Values are mean ± SEM.
(PI: post-immobilization; C: control; DEX: dexamethasone; DOC: deoxycorticosterone; GCA: glycyrrhizic acid)

number increased and it was not significantly different to that of DOC.

GCA is the potent inhibitor of the enzyme 11β-HSD which reversibly converts corticosterone to 11-dehydrocorticosterone in tissues. Thus GCA effectively increased the amount of endogenous corticosterone in the tissues including hypothalamus. The effects on CRF neurons were similar to that seen in the DOC treated, not to the DEX treated rats. Thus GCA increased corticosterone to bind more to the mineralocorticoid receptors rather than glucocorticoid receptors in the hypothalamus. Mineralocorticoid and glucocorticoid receptors have previously been reported in the hypothalamus [20,21].

These data implies that stress results in release of CRF from the neurons in the PVN of the hypothalamus, and that if these CRF release could be blocked by DEX, DOC or GCA, the stress responses could be blocked. Furthermore, adaptation coincides with increasing CRF neurons in the PVN, such that further stress could not cause the release of the CRF from the neurons. How this is achieved is not known from this set of experiments.

In conclusion, this study has shown that with repetitive stress, DOC and GCA completely inhibited the decrease in CRF containing neurons, whereas DEX initially caused a significant decrease in CRF containing neurons. Therefore, by blocking the CRF release in repetitive stress, it potentially blocked the stress response.

Acknowledgement

This study was supported by Universiti Kebangsaan Malaysia research grant F27/96. The authors gratefully acknowledge the technical assistance of Puan Hairi.

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