RESPONSE OF HYPOTHALAMIC OXYTOCINERGIC NEURONS TO IMMOBILIZATION STRESS IS NOT DEPENDENT ON THE PRESENCE OF CORTICOTROPHIN RELEASING HORMONE (CRH): a CRH KNOCK-OUT MOUSE STUDY

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This study explores the quantitative patterns of immunolabeled Fos protein incidence in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON) oxytocinergic (OXY) neurons in response to immobilization (IMO) stress in corticotrophin releasing hormone deficient (CRH-KO) mice. Adult male mice, taken directly from cages or 120 min after a single IMO, were sacrificed by intracardial perfusion with fixative. Coronal brain sections of 30 µm thickness were processed for dual Fos/OXY immunohistochemistry. In control wild type (WT) and CRH-KO mice, scattered Fos immunoreactivity was observed in hypothalamus, including the PVN where scanty Fos signal occurred in both parvocellular and magnocellular PVN subdivisions. Dual Fos/OXY immunostainings revealed higher basal Fos expression in the PVN of control CRH-KO mice. IMO evoked a marked rise in Fos expression in OXY neurons of the PVN and SON in both WT and CRH-KO groups of mice. The present data demonstrate that 1/ CRH deficiency upregulates the basal activity of hypothalamic PVN OXY cells in CRH-KO mice and 2/ IMO stress in both WT and CRH-KO mice affects distinctly the activity of OXY cells in both SON and PVN. Our data indicate that CRH deficiency does not alter the responsiveness of PVN and SON OXY cells to IMO stress.

Key words: CRH knock-out mice, Fos-immunohistochemistry, hypothalamic paraventricular and supraoptic nuclei, immobilization stress, oxytocin

INTRODUCTION

Although corticotrophin releasing hormone (CRH) is a principal component of the hypothalamic-pituitary-adrenocortical axis (HPA) regulating the basal and stress-induced release of the pituitary adrenocorticotropin hormone (1-5), many other biologically active substances likewise prostaglandins (2) and nitric oxide (4), including oxytocin (OXY) (6-10), have been shown to be activated during stress (11-15). It has been also well documented that OXY neurons in the paraventricular nucleus (PVN) activated during stress are followed by both intra-PVN and plasma OXY releases (14, 16, 17). Moreover, immunostaining detections of different immediate early genes have indicated that OXY-containing neurons in the PVN can be activated by a broad range of stressors including restraint (11, 12) cold-swim (18), osmolality rise (15, 19, 20), and lipopolysaccharide administration (12). All these studies indicate that OXY belongs to a stress responsive brain neuropeptides.

Neuroanatomical interrelationships between the CRH-OXY neurons in the PVN have been indicated by demonstrating synaptic associations between magnocellular OXY-containing and parvocellular CRH-synthesizing neurons (21). CRH-containing fibers have been shown to be apposed to the OXY perikarya or making synapses with their dendritic processes (21). In addition, the presence of both type-1 and type-2 CRH receptors mRNAs has been demonstrated in the PVN and supraoptic nucleus (SON) OXY perikarya (22), indicating a capability of CRH and OXY cells to form direct and reciprocal CRH-OXY interactions. Indeed, acute restraint stress induced-up-regulation of CRH mRNA expression in the PVN has been shown to be significantly higher in OXY gene-deficient male mice than in their age-matched adult wild type (WT) controls (10), indicating that OXY may be involved in the regulation of stress-induced CRH gene expression in the PVN. Likewise, CRH intracerebroventricular (i.e.v.) administration has been shown to mimic OXY responses observed after ether or footshock stress (23), indicating that CRH may play a role in regulating stress-induced OXY activation and secretion in the rat.

The intent of the present study was to reveal whether CRH deficiency may influence the response of hypothalamic OXY-ergic cells to an acute restraint stress, i.e. immobilization stress, which is a potent stressor in several animal species accompanied by a complex of hormonal responses and functional changes in the central nervous system (24). Since the development of CRH knock-out mice model (25-27) has become an important tool for revealing the physiologic and pathologic roles of the endogeneous CRH, CRH knock-out and matched control WT mice were used in the present study. Response of OXY neurons was investigated in the PVN and SON by evaluating Fos expression incidence, as a general marker for cell activity (28), using dual Fos/OXY immunohistochemistry (19).
MATERIAL AND METHODS

Experimental animals

CRH-KO adult male mice (4-6 month old) weighting 24-30g were used. The animals, originally obtained from the Harvard Medical School Department of Endocrinology Boston USA, were bred in our facilities and maintained under controlled conditions of temperature (22 ± 1.0°C), lighting (12 h light/dark cycle with light on at 06:00 a.m.), and standard food chow and water ad libitum. To avoid the effect of diurnal variations on the Fos expression, the experiment was performed between 9:00 and 11:00 a.m. Principles of laboratory animal care and all experimental procedures were approved by the Animal Care Committee of the IEE SAS Bratislava, Slovak Republic. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 80 23, revised 1978).

Acute restraint stress

Both CRH-KO (n = 5) and matched control WT mice (WT, n = 7) were exposed to an acute restraint for 120 min, as described previously (29). Before starting the experiment, control CRH-KO (n = 3) and WT (n = 4) groups were subjected to handling for 3 days. The mice were anesthetized immediately after terminating the restraint while the controls were anesthetized immediately after removing them from the home cages. The mice were deeply anesthetized with a pentobarbital (50 mg/kg, i.p.) and rapidly perfused transcardially with 50 ml of 0.1 M phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde. Finally, the brains were removed, postfixed in the same fixative overnight at 4°C, and infiltrated with 15% sucrose in 0.025 M PB for 48 h at 4°C. After sectioning, the brains were rapidly (20 sec) frozen in cold isopentane (−40°C) followed by an additional cooling (1 min) in dry ice, and placed into a Reichert cryocut device for 1 h at −15°C. Four alternate series of coronal sections (30 µm thick) were cut from the hypothalami between the optic chiasm and the median eminence (coordinates from Bregma 0.02 to −2.3 mm) (30) and collected as free floating in cold (4°C) PB.

Fos-oxytocin immunohistochemistry

The sections were repeatedly washed in cold PB followed by a preincubation in 3% H2O2 (Fisher Scientific, Fair Lawn, NJ, USA) for 10 min at room temperature. Then they were incubated with a polyclonal Fos protein antiserum (1: 2000, No 94012), diluted in 0.1 M PB containing 4% normal goat serum (Gibco, Grand Island, NY, USA), 0.5% Triton X-100 (Koch-Light Lab. Ltd., Colnbrook Berks, England), and 0.1% sodium azide (Sigma Chemical Ltd. St. Louis MO, USA) for 48 h at 4°C. After several rinsings in PB, the sections were incubated with biotinylated goat-anti-rabbit IgG (1: 1000, VectorStain Elite ABC, Vector Lab, Burlingame, CA, USA) for 2h at room temperature. Next PB rinsings were followed by incubation with the avidin-biotin peroxidase complex (1: 500) for 2 h at room temperature. Thoroughgoing PB washing was followed by washing in 0.05 M sodium acetate buffer (SAB, pH 6.0). Fos antigenic sites were visualized with 0.0625% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) dissolved in SAB containing 0.375% hydrogen peroxide and 2.5% nickel ammonium sulfate (Sigma), for 15-20 min. The metal-intensification of DAB produced blackly labeled nuclei. After several washings in PB, the Fos-positive sections were incubated with OXY antibody using the same procedure as described above. The OXY (diluted 1:2000) immunoreaction was visualized in a single DAB chromogen solution in 0.1 M Tris buffer (pH 7.4) and 0.525% hydrogen peroxide. To reach the appropriate yellow color, the developing process of OXY was monitored under the light microscope. Finally, the sections were mounted into 1% of gelatine dissolved in 0.025 M SAB, air-dried, coverslipped with Permount (Sigma), and examined under Leica DMLS light microscope. Immunostaining of negative control, which did not show any antisera immunolabeling, included substitution of the primary antiserum with normal rabbit serum, and sequential elimination of the primary or secondary antibody from the staining series.

Antisera

Fos antisera was raised against N-terminal peptide similar to 2-17 of the rat Fos protein according to the protocol described elsewhere (31, 32). OXY antiserum was purchased from Chemicon International Inc. (Temecula, CA, USA).

Data evaluation

Counting of the single OXY immunoreactive and Fos-OXY double labeled neurons within the PVN and SON was performed bilaterally on the 30 µm thick serial coronal sections (minimally from 2 sections per animal) employing a computerized Leica DMLS light microscope equipped with a Canon Digital camera (PowerShot S40). The average number of OXY immunoreactive perikarya (including Fos labeled) used for countings was 206 ± 7.3 in PVN and 99.7 ± 6.3 in SON. The percentage of activated OXY neurons was calculated from the formula: 100 x (number of OXY perikarya displaying Fos signal/total amount of OXY immunolabeled perikarya).

Statistics

The results are reported as mean ± S.E.M. Two way ANOVA for factors immobilization and strain was applied. Post hoc comparisons were performed with Tukey’s test. For single comparisons between control WT and CRH-KO groups Student’s T-test was also used in PVN. Differences were considered as significant from P < 0.05. Statistical evaluation was performed by SIGMASTAT software for Windows (StatSoft) software.

RESULTS

Single Fos-expression

In the control WT and CRH-KO mice, scattered Fos immunoreactive signal was revealed over the whole extent of the hypothalamus, including the PVN. Only a scanty Fos immunoreactivity was detectable in both parvocellular and magnocellular subdivisions of the PVN (Fig. 1). IMO for 120 min evoked in WT as well as CRH-KO mice a marked Fos expression in many of the hypothalamic structures. However, after single IMO, distinct Fos expression was mainly observed in the PVN (Fig. 1). Actually, maximal number of Fos-positive profiles occurred explicitly in the middle portion of the PVN, which topographically corresponds to areas located caudal to Bregma (from −0.70 mm to −0.82 mm).

On the other hand, SON in the control WT mice did not exhibit any Fos labeled cell nuclei (Fig. 2). After acute IMO stress for 120 min, Fos labeled cell nuclei were also clearly detectable in the SON (Fig. 2).

IMO induced Fos-neuropeptide co-localizations

The middle portion of the PVN which displayed regularly most numerous Fos labeling also contained many OXY
immunopositive perikarya. In the control WT mice only 1.19 ± 0.6% (total number of calculated cells/group = 873) of OXY neurons displayed Fos presence in the PVN. On the other hand, control CRH-KO mice exhibited higher number of OXY cells with Fos signal (5.49 ± 0.9%) (total number of calculated cells/group = 596) in the PVN. The differences in the number of OXY activated cells between the WT and CRH-KO mice reached statistic significance (P <0.05). On the other hand, immobilization for 120 min significantly elevated the Fos expression in OXY perikarya in both groups of mice (Fig. 3), i.e. in WT mice up to 21.98 ± 4.4% (total number of calculated cells/group = 1327) and CRH KO ones up to 32.36 ± 9.8% (total number of calculated cells/group = 882). However, when analysing the difference between WT with CRH-KO immobilized mice, the rise of Fos in OXY perikarya did not show statistical significance.

As indicated by dual immunohistochemistry, the effect of IMO for 120 min was considerably lower in the Fos expression in the OXY immunopositive perikarya located in the SON. Computerised counting analysis revealed presence of Fos positive signals in less than 5% of OXY in both WT (4.38 ± 0.9%) (total number of calculated cells/group = 406) and CRH-KO (3.89 ± 0.9%) (total number of calculated cells/group = 339) mice. However, also in the case of the SON, IMO stress induced significant rise in the number of activated OXY neurons in both WT and CRH-KO mice (Fig. 3), i.e. up to 21.48 ± 2.7% (total number of calculated cells/group = 568) and 32.91 ± 6.9% (total number of calculated cells/group = 355), respectively. The rise

Fig. 1. Histological photomicrograph demonstrating Fos occurrence in the PVN of experimental animals. In WT (A) and CRH-KO (C) control mice, OXY immunoreactive perikarya and cell profiles predominate on the pictures while Fos presence is limited. IMO stress induced marked Fos expression in the PVN of both WT (B) and CRH-KO (D) mice. Bar scale 450 µm (A, B, C, D).

Fig. 2. Histological photomicrograph demonstrating Fos occurrence in the SON of experimental animals. In WT (A) and CRH-KO (C) control mice, OXY immunoreactive perikarya and cell profiles mainly occur. IMO stress induced marked Fos expression in the SON of both WT (B) and CRH KO (D) mice. Bar scales - 125 µm (A, C), 115 µm (B), and 135 µm (D).
of Fos in OXY perikarya in CRH-KO mice did not reach statistical significance.

**DISCUSSION**

The present data demonstrate that 1/ CRH deficiency in CRH-KO mice upregulates the basal activity of hypothalamic magnocellular OXY cells in the PVN and 2/ IMO stimulus in both WT and CRH-KO mice affects distinctly the activity of OXY in both SON and PVN. Overall, CRH deficiency had no impact on the responsiveness of OXY magnocellular population of cells to IMO stress indicating that CRH does not play important role in the regulation of OXY cells response to IMO challenge.

The anatomical interrelationships between the CRH and OXY neurons have been well established in the PVN by demonstrating the existence of synaptic contacts between OXY containing magnocellular neurons and parvocellular PVN neurons containing CRH (21). In addition, OXY neurons of SON and PVN have been shown to co-express mRNA for type-1 and type-2 CRH receptors (22), which clearly speak out for existence of a functional link between CRH-OXY cells. Indeed, OXY has been shown to elevate plasma ACTH levels, an effect that was abolished by immunoneutralization of endogenous CRH (33).

There are also many indications that OXY may play an important role in the regulation of neuroendocrine responses to stress. CRH i.c.v. administration has been shown to mimic OXY responses observed after ether or footshock stress (23), indicating that CRH may play a role in the modulation of stress-induced OXY activation and secretion in the rat. Actually, acute restraint stress induced up-regulation in CRH mRNA expression in the PVN has been shown to be significantly higher in OXY gene-deficient male mice than in their age-matched WT controls (10), which indicates that OXY may take a part in the regulation of stress-induced CRH gene expression in the PVN. Our data revealed significant increase (from 0.9 % to 5.9 %) in the Fos expression in PVN OXY cells already in control group of CRH-KO mice. Whether this marginal basal upregulation in the activity of OXY cells in CRH-KO mice may have significant peripheral effect is not clear. However it seems that this increased activity of OXY cells cannot be associated with CRH receptor involvement since the OXY messenger RNA expression in the PVN of CRH receptor 1 deficient mice did not differ from controls (34).

Different stress challenges, including swimming, restraint, ether, and footshock, have been shown to be able to stimulate OXY secretion (23, 35). In consistence with these observations, in our experiments both CRH-KO and WT mice showed marked response of OXY cells to acute IMO stress, whereas the response of OXY cells in CRH-KO mice appeared to be more noticeable (but not significantly different), in comparison with WT IMO mice. Nomura and co-workers (10) have found after 4 h of restraint stress significantly higher degree of CRH mRNA up-regulation in the PVN of OXY-KO in comparison with the wild-type mice. OXY has been also shown to be sensitive to both the osmotic and physical challenges (19). In addition, its release in response to various stressors has been demonstrated not only into blood but also within hypothalamic and extrahypothalamic areas indicating for its broad functional impact over different brain areas. PVN is a complex structure with multifarious cell compositions assigning projections to different central nervous system structures (36).
considered to be a primary source of OXY in the central nervous system. In fact, a direct PVN projection to the spinal cord has been demonstrated by retrograde and anterograde tracers, and more than the 50% of these projections appeared to be OXYergic (36). Since IMO stress, used in this study, is not an osmotic challenge and in contrast to hypertonic saline it does not contain even any osmotic component, it is quite reasonable to assume that at least part of the activated OXY neurons in the PVN might belong to that population of OXY neurons which project caudally and are involved in the modulation of autonomic nervous system response to stress (36-40). Moreover, it has been clearly shown that that PVN OXY is a part of the descending inhibitory control mechanisms displaying important antinociceptive actions (41, 42). On the other hand, the role of the SON OXY in stress response is less known although certain stress challenges including swimming stimulate the OXY release within the SON (43). This might suggest that OXY release in the SON during stress could be associated with certain intrinsic paracrine functions.

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