

Research report

Stress-induced sensitization of CRH-ir but not P-CREB-ir responsivity in the rat central nervous system

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Accepted 8 May 2001

Abstract

There is some evidence that a traumatic life event can induce long-term alterations in corticotropin-releasing hormone (CRH) producing neurons in humans, which may play a role in the pathophysiology of anxiety disorders, including post-traumatic stress disorder (PTSD). To study the long-term effects of a traumatic event on brain CRH-immunoreactivity (CRH-ir) and phospho-cAMP response element binding protein-immunoreactivity (P-CREB-ir), rats were exposed to a single session of foot shocks (preshocked) or no shocks (control). Two weeks later half of the control rats and half of the preshocked rats received an electrified prod in the home cage for 15 min and behavior was recorded. Fifteen minutes after the removal of the prod rats were perfused and brain sections were stained for CRH-ir and P-CREB-ir. There was no basal difference between preshocked and control rats in brain CRH-ir and P-CREB-ir. Exposure to the electrified prod induced a significant increase in CRH-ir in the paraventricular nucleus of the hypothalamus, the median eminence and the central amygdala in preshocked rats, but not in control rats. The electrified prod increased the number of P-CREB-ir neurons in the paraventricular nucleus of the hypothalamus and the locus coeruleus, but the preshock experience did not affect this response. In an additional experiment with a similar design plasma hormone levels were measured 14 days after the foot shocks. The preshock experience sensitized the shock prod-induced ACTH and corticosterone response. No behavioral differences between preshocked and control rats were found during the shock prod tests. We suggest that long-term stress-induced changes in neuropeptide dynamics of CRH-ir neurons may play a role in long-term stress-induced neuroendocrine sensitization. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Neural basis of behaviour

Topic: Stress

Keywords: cAMP response element binding protein; Corticotropin-releasing hormone; Stress-induced sensitization; Paraventricular nucleus of the hypothalamus

1. Introduction

Exposure to an unusually distressing event induces alterations in neuronal substrates and may ultimately lead to the development of some anxiety disorders, including post-traumatic stress disorder (PTSD) [51]. Exposure of rats to a single session of uncontrollable foot shocks is a well-validated animal model to study the long-term effects of a stressful life event in humans [59].

Preshocked rats can display long-term sensitized be-

havioral, neuroendocrine, autonomic and central nervous system responses to novel stimuli. Sensitization is defined as an elementary form of nonassociative learning in which an animal learns to strengthen its defensive reflexes and to respond vigorously to a variety of previously neutral or indifferent stimuli after it has been exposed to a potentially threatening or noxious stimulus [21]. Preshocked rats display an increased ACTH and immobility response in the noise test, in which the rats are placed in a novel environment with a loud background noise (80–85 dB), followed by a period in which the noise is turned off [52]. Moreover, preshocked rats show increased avoidance behavior when placed in a dark box in a brightly lit environment up to 10 weeks after the foot shock session

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[6]. The expression of the sensitized behavioral responses is, however, dependent on the test conditions. Behavioral differences are not expressed in the shock prod test conducted in the home cage [3], but are clearly expressed when the test is conducted in a novel cage [Stam et al., unpublished observations], indicating that behavioral sensitization is more clearly expressed in challenges outside the familiar home cage environment. In the low arousal home cage situation, however, sensitized cardiovascular and colonic responses to a novel electrified prod can be seen which are not linked to behavioral sensitization [5,48]. These observations match some findings in PTSD patients. PTSD patients display increased avoidance behavior to circumvent any situation or activity that might revive memories of the trauma [15]. Exaggerated autonomic responses to non-trauma related stimuli like a burst of white noise have been found in PTSD patients [35]. Moreover, alterations in the hypothalamus–pituitary–adrenal (HPA) axis have been found in PTSD patients [47]. Relatively little is known about the neuroanatomical substrates involved in these long-term stress-induced alterations. However, recent studies suggest that sensitization of corticotropin-releasing hormone (CRH) producing neurons might play a role in stress-induced sensitization of behavioral and physiological responses.

Since the characterization by Vale et al. [50], the function of CRH in the brain has been extensively studied. CRH produced by parvocellular neurons in the paraventricular nucleus of the hypothalamus is a major regulator of the HPA axis. However, CRH is also expressed in extrahypothalamic regions and seems to play an important role in the appraisal of a stressor and the regulation of the corresponding autonomic responses [49]. Recent studies suggest that a traumatic experience can induce long-term alterations in CRH producing neurons. A blunted adrenocorticotrophic hormone (ACTH) response to CRH has been found in PTSD patients, which might be a result of desensitization of CRH₁ receptors at the anterior pituitary due to a hypersecretion of CRH by parvocellular hypothalamic neurons [47]. Moreover, increased CRH levels have been found in the cerebrospinal fluid of Vietnam veterans who met the full criteria for PTSD [2]. Despite the fact that CRH might play a role in the development and expression of PTSD, long term effects of a single stressful experience on basal and challenge-induced CRH levels in brain areas involved in behavioral, neuroendocrine and autonomic control have not been extensively studied yet.

Studying the activation of molecular signaling pathways by external and internal stimuli might also shed light on the neuronal networks underlying stress-induced sensitization. The transcription factor cAMP response element binding protein (CREB) can bind to the promoter sequence of cAMP responsive genes at the cAMP response element binding site, and activation of the cAMP–protein kinase A pathway results in the phosphorylation of CREB (P-CREB) [16]. P-CREB may ultimately modulate the tran-

scription of stress sensitive genes including the immediate early gene *c-fos* and the CRH gene [45,56]. Recent studies demonstrate that ether stress increases the number of P-CREB-immunoreactive (P-CREB-ir) cells in the paraventricular nucleus of the hypothalamus (PVH) [26]. However, whether an electrified prod inserted in the home cage, which is considered a relatively mild stimulus, can also affect the amount of P-CREB positive cells in the PVH and extrahypothalamic areas involved in behavioral and autonomic control is not known yet.

In a previous study [3] we demonstrated that a preshock experience sensitizes the electrified prod-induced expression of Fos-ir in brain areas involved in neuroendocrine and autonomic control. It is, however, not yet known if a preshock experience can affect neuropeptide responses to a novel electrified prod inserted in the home cage. The aim of the present study was to investigate whether a single foot shock experience can induce long-term alterations in basal and electrified prod-induced CRH-ir levels in brain areas involved in neuroendocrine and autonomic control. In additional experiments we measured plasma ACTH and corticosterone levels under resting conditions and at different time points after the introduction of the electrified prod in the home cage. In order to gain more insight in the mechanisms underlying the expression of stress-sensitization we also studied the effect of a previous stressful experience on the basal and stimulus-induced expression of the transcription factor P-CREB, which is involved in the transcription of *c-fos* [34].

2. Material and methods

2.1. Animals and housing

Male Wistar rats, weighing 250–300 g were used. The rats were individually housed in perspex cages measuring 40×26×20 cm (l×w×h) with sawdust bedding at 21°C under regulated lighting conditions (light on at 07:00 h, off at 19:00 h). Food (complete laboratory chow: Hope Farms, Woerden, The Netherlands) and water were freely available. Experimental procedures were approved by the Ethical Committee on Animal Experiments of the Faculty of Medicine, Utrecht University.

2.2. Experimental procedures

2.2.1. Foot shocks and shock prod

Rats in a ‘preshocked’ group were placed in a metal grid cage (32×32×38 cm, l×w×h) between 17:00 and 18:30 h, and underwent a single 15-min session of scrambled electric foot shocks (10×6 s, 0.5 mA, with randomized interval of 20–210 s). Rats in a ‘control’ group spent 15 min in the grid cage without receiving shocks. To avoid exposure of the control and preshocked rats to the sound or odor of rats being exposed to foot shocks, the grid cages

were placed in a separate room and the grid cages were cleaned after each stress session. Afterwards, rats were returned to their home cages, and left undisturbed apart from weekly changing of bedding material. Thirteen days after the shock box session, rats were transported to a separate experimental room and on the afternoon of the following day at 15:00 h an electrified prod ($\varnothing=1$ cm, $l=6.5$ cm) that delivered a 2-mA shock immediately upon touch [10], was inserted through a hole in the home cage (modified prod challenge).

Behavior was recorded on video and afterwards analyzed blindly by an experienced observer using The Observer (version 3.0, Noldus Information Technology, Wageningen, The Netherlands). The duration (s) of the following behavioral elements was measured: locomotion (movement of the whole body not directed towards prod), rearing (upright posture), immobility, attention (sitting while head and whiskers are moving), prod (locomotion or stretching in direction of prod), burying (shoveling of bedding material in direction of prod) and consumatory behavior (eating or drinking).

2.2.2. Immunocytochemistry (experiment 1)

Fifteen minutes after the removal of the prod from the home cage, the rats were deeply anaesthetized with 0.1 ml/100 g (i.p.) of sodium pentobarbital (Nembutal[®], Sanofi Santé B.V., Maassluis, The Netherlands) and perfused via the ascending aorta with 200 ml saline followed by 400 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were postfixed for an additional 20 h. Sections were cut on a vibratome (coronal sections of 40 μ m), and thereafter the free-floating sections were rinsed with 0.01 M phosphate-buffered saline (PBS). The sections were stored in 0.01 M PBS with 0.1% sodium azide at 4°C.

Sections were thoroughly washed with PBS, rinsed in PBS containing 0.3% hydrogen peroxide, and preincubated with 5% normal goat serum. Subsequently, the sections were incubated overnight with the primary antibody, CRH rabbit polyclonal (kindly donated by Professor Dr F. Tilders, Department of Pharmacology, Free University, Amsterdam, The Netherlands; 1:10 000) or P-CREB rabbit polyclonal (Upstate Biotechnology, Waltham, MA, USA; 1:1000) in 1% normal goat serum (NGS) and 0.3% Triton. After incubation, the sections were exposed to biotinylated secondary goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:800) in 1% NGS and 0.3% Triton for 90 min, and incubated with avidin DH: biotinylated horseradish peroxidase H complex (Vectastain ABC kit from Vector Laboratories, CA, USA; 1:800) for 90 min. Between the incubations the sections were rinsed with 0.01 M PBS and all the incubations were carried out at room temperature on a slow shaker. A peroxidase reaction was performed to visualize the immunolabeling of CRH and P-CREB by incubating the sections in 0.03% 3,3'-diaminobenzidine tetrahydrochloride+0.3% nickel ammonium sulphate+0.008% hydrogen peroxide in 0.05

M Tris-HCl for 10 min. Finally, the sections were mounted, air-dried, dehydrated in ethanol, cleared in xylene and coverslipped.

CRH-ir (cell bodies and fibers; area expressed in arbitrary units, a.u.) and the number of P-CREB-ir positive neurons were quantified automatically at a magnification of 100 in a frame of 4 mm² using image analysis software (MCID-M5, Imaging Research Inc, St. Catherine's, Ontario, Canada). Two cell groups, the locus coeruleus and the lateral parabrachial nucleus, were analyzed using a hand made frame covering the complete area of interest. Measurements were done bilaterally on two coronal brain sections from each animal and the average of the four sides served as representative for CRH-ir or the number of P-CREB-ir neurons in a selected area. For reproducible comparison between the experimental groups, measurements were always performed at the same coordinates with respect to bregma according to the atlas of Paxinos and Watson [39]: Bed nucleus of the stria terminalis (BNST), -0.80; basolateral amygdaloid complex, -2.56; central amygdala, -2.56; median eminence, -2.80; paraventricular nucleus of the hypothalamus, -1.80; locus coeruleus, -9.68; lateral parabrachial nucleus central, -9.68; lateral parabrachial nucleus external, -9.68;

2.2.3. ACTH and corticosterone radioimmunoassays (experiment 2)

Blood samples were collected in two separate experiments. In the first experiment rats were decapitated under resting conditions or 5 min after the introduction of the prod (Exp. 2A). In the second experiment rats were decapitated under resting conditions or 15 min after the introduction of the prod (Exp. 2B). ACTH concentrations in plasma were measured as previously described [54], using a specific rabbit antiserum directed to the mid-portion of ACTH (code Ft 8514), kindly donated by Dr G.B. Makara (Budapest, Hungary). Synthetic human ACTH (1-39) (Peninsula Laboratories, Belmont, CA, USA) was used as standard and ¹²⁵I-labeled as tracer. Sample dilution curves paralleled the standard curve. The sensitivity of the assay was 10 pg/ml plasma (0.5 pg/tube). Intra- and interassay variations were 5 and 8%, respectively. Plasma corticosterone was measured using an ImmChem[™] double antibody kit (ICN Biomedicals, Costa Mesa, CA, USA).

2.2.4. Statistical analysis

The Mann-Whitney *U*-test was used to analyze the number of times the rats touched the shock prod and the unpaired Student's *t*-test to analyze the duration of each of the eight behavioral components studied during the shock prod challenge. Two-factor multivariate analysis of variance (MANOVA) was used to test the effect of foot shocks and the electrified prod-challenge on ACTH, corticosterone, CRH-ir and the number of P-CREB-ir neurons.

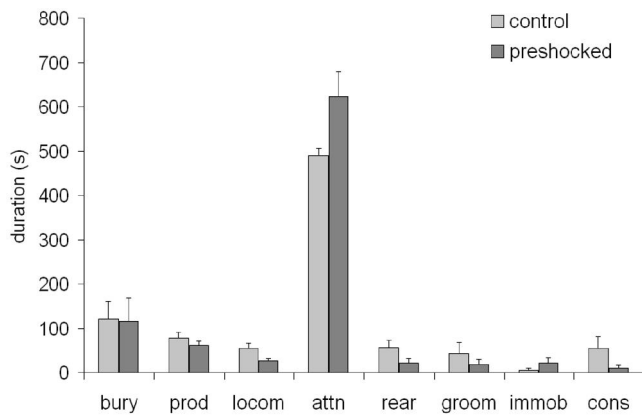


Fig. 1. Behavioral response to the shock prod challenge 2 weeks after receiving a single session of foot shocks (preshocked) or no shocks (control). Mean duration (\pm S.E.M.) of the eight behavioral components during the 15 min presence of the shock prod are given (locom, locomotion; rear, rearing; immob, immobility; attn, attention; prod, prod directed behavior; bury, burying; cons, consumatory behavior). n =eight rats per group.

3. Results

3.1. Immunohistochemistry (experiment 1)

The animals were usually asleep at the beginning of the test, but awoke at the introduction of the shock prod. The shock prod was touched by all rats within the first minute and there was no significant difference in the number of prod touches between the groups (mean control 1.8; mean preshocked 2.6). The behavioral response to the shock prod is shown in Fig. 1. No significant difference in the duration of any behavioral component between preshocked and control rats was found.

Generally, before exposure to the electrified prod a very

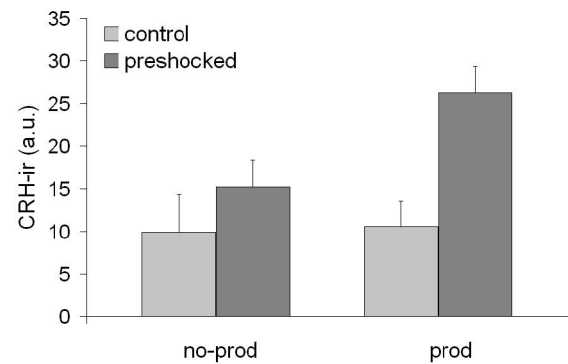


Fig. 2. CRH-ir (arbitrary units, a.u., \pm S.E.M.) in the paraventricular nucleus of the hypothalamus (PVH) 15 min after exposure to an electrified prod (prod) or no prod (no-prod), in rats exposed to a single session of foot shocks (preshocked) or no shocks (control) 2 weeks earlier. Preshock $P=0.005$; n =six to eight rats per group.

intense staining of CRH-ir fibers was detected in the external zone of the median eminence, and a moderate density of CRH-ir fibers was detected in the central amygdala and the bed nucleus of the stria terminalis. Only a very low density of fibers was found in the paraventricular hypothalamic nucleus and almost no fibers were detected in the locus coeruleus. The most intense staining of CRH-ir cell bodies was found in the paraventricular hypothalamic nucleus. On the other hand, only a few scattered CRH-ir cells were detected in the central amygdala and the bed nucleus of the stria terminalis. The amount of CRH-ir in the brain areas studied is shown in Table 1. A significant preshock effect ($F(1,28)=9.38$, $P=0.005$) on CRH-ir in the paraventricular hypothalamic nucleus (Figs. 2 and 4A,B) appeared mainly due to a large prod-induced increase in CRH-ir in the preshocked rats. Statistical analysis also revealed a preshock effect for

Table 1

CRH-ir (arbitrary units) 15 min after exposure to an electrified prod (prod) or no prod (no-prod), in rats exposed to a single session of foot shocks (preshocked) or no shocks (control) 2 weeks earlier

Brain area	Control		Preshocked		Statistics
	No-prod	Prod	No-prod	Prod	
Forebrain					
BNST	106 \pm 22	149 \pm 29	129 \pm 22	102 \pm 13	N.S.
Amygdala					
Central nucleus	111 \pm 23	102 \pm 14	131 \pm 17	163 \pm 19	prs: $P=0.040$
Hypothalamus					
Paraventricular nucleus	10 \pm 4	11 \pm 3	15 \pm 3	26 \pm 3	prs: $P=0.005$
Median eminence	136 \pm 8	126 \pm 11	147 \pm 10	172 \pm 12	prs: $P=0.012$
Brainstem					
Locus coeruleus	0.8 \pm 0.1	0.9 \pm 0.2	0.7 \pm 0.2	0.9 \pm 0.2	N.S.
Lateral parabrachial nucleus central	69 \pm 23	75 \pm 15	100 \pm 19	69 \pm 19	N.S.
Lateral parabrachial nucleus external	23 \pm 9	22 \pm 8	44 \pm 13	30 \pm 6	N.S.

Multivariate analysis of variance was used to test the effect of previous foot shocks and the prod-challenge on CRH-ir. Significant effects are given in the right-hand column. F values with degrees of freedom can be found in Section 3. Abbreviations: prs, preshock treatment.

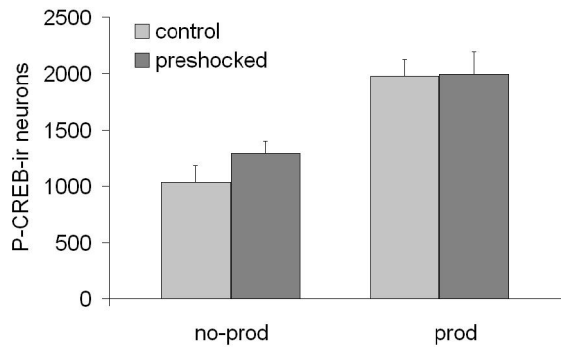


Fig. 3. Mean number (\pm S.E.M.) of P-CREB-ir neurons in the paraventricular nucleus of the hypothalamus (PVH) 15 min after exposure to an electrified prod (prod) or no prod (no-prod), in rats exposed to a single session of foot shocks (preshocked) or no shocks (control) 2 weeks earlier. Prod $P < 0.001$, $n =$ six to eight rats per group.

CRH-ir in both the median eminence ($F(1,25) = 7.47$, $P = 0.012$) and the central amygdala ($F(1,28) = 4.66$, $P = 0.040$), also appearing due to a prod-induced increase in CRH-ir in the preshocked rats while there was no clear effect of the prod-challenge on CRH-ir in the control rats. No significant differences in CRH-ir were found in the other brain areas studied.

In control rats, not exposed to the prod-challenge, large numbers of P-CREB-ir nuclei were detected in a wide range of brain areas. Especially large numbers of P-CREB-ir nuclei were seen in the dentate gyrus and the CA1, CA2 and CA3 areas. Large numbers of P-CREB-ir nuclei were also found in the cortex, the hypothalamus and the amygdala complex. The number of P-CREB positive nuclei in the brain areas studied is shown in Table 2. Despite the high basal expression of P-CREB-ir a significant prod-effect was found for the number of P-CREB-ir nuclei in the paraventricular nucleus of the hypothalamus ($F(1,27) = 28.31$, $P < 0.001$; Figs. 3 and 5A,B) and the

locus coeruleus ($F(1,27) = 8.47$, $P = 0.007$). No significant differences were found in other areas described in Table 2.

3.2. Plasma hormone levels (experiment 2)

In the first experiment (Exp. 2A) rats were decapitated under resting conditions or 5 min after the introduction of the prod. The prod was touched by all rats within the first minute and there was no significant difference in the number of prod touches between the groups (mean control 2.3; mean preshocked 2.3). There were no significant differences in the duration of any behavioral component between preshocked and control rats (data not shown). No significant differences were found in baseline levels of ACTH (Fig. 6A) and corticosterone (Fig. 6B) between control and preshocked rats. Statistical analysis revealed a significant preshock \times prod interaction for ACTH ($F(1,38) = 9.83$, $P = 0.003$; Fig. 6A), preshocked rats displaying higher ACTH levels 5 min after the introduction of the prod. A significant preshock \times prod interaction was also found for corticosterone ($F(1,38) = 6.07$, $P = 0.018$; Fig. 6B). The corticosterone levels were increased in the preshocked rats 5 min after the introduction of the prod, but the somewhat lower basal corticosterone levels in the preshocked rats may have contributed to the preshock \times prod interaction.

In the second experiment (Exp. 2B) rats were decapitated under resting conditions or 15 min after the introduction of the prod. The prod was touched by all rats within the first minute and there was no significant difference in the number of prod touches between the groups (mean control 2.5; mean preshocked 2.5). There were no behavioral differences between preshocked and control rats (data not shown) and no differences in basal levels of ACTH (Fig. 7A) and corticosterone (Fig. 7B). However, statistical analysis revealed a significant preshock \times prod interaction ($F(1,28) = 5.10$, $P = 0.032$; Fig.

Table 2

Mean number (\pm S.E.M.) of P-CREB-ir positive neurons 15 min after exposure to an electrified prod (prod) or no prod (no-prod), in rats exposed to a single session of foot shocks (preshocked) or no shocks (control) 2 weeks earlier

Brain area	Control		Preshocked		Statistics
	No-prod	Prod	No-prod	Prod	
Forebrain					
BNST	1689 \pm 80	1587 \pm 65	1634 \pm 88	1658 \pm 79	N.S.
Amygdala					
Basolateral complex	148 \pm 10	158 \pm 15	156 \pm 10	158 \pm 15	N.S.
Central nucleus	861 \pm 46	1013 \pm 51	946 \pm 67	943 \pm 32	N.S.
Hypothalamus					
Paraventricular nucleus	1035 \pm 147	1981 \pm 146	1291 \pm 113	1995 \pm 198	prod: $P < 0.001$
Brainstem					
Locus coeruleus	244 \pm 22	334 \pm 24	267 \pm 32	346 \pm 34	prod: $P = 0.007$

Multivariate analysis of variance was used to test the effect of previous foot shocks and the prod-challenge on the number of P-CREB-ir neurons. Significant effects are given in the right-hand column. F values with degrees of freedom can be found in Section 3.

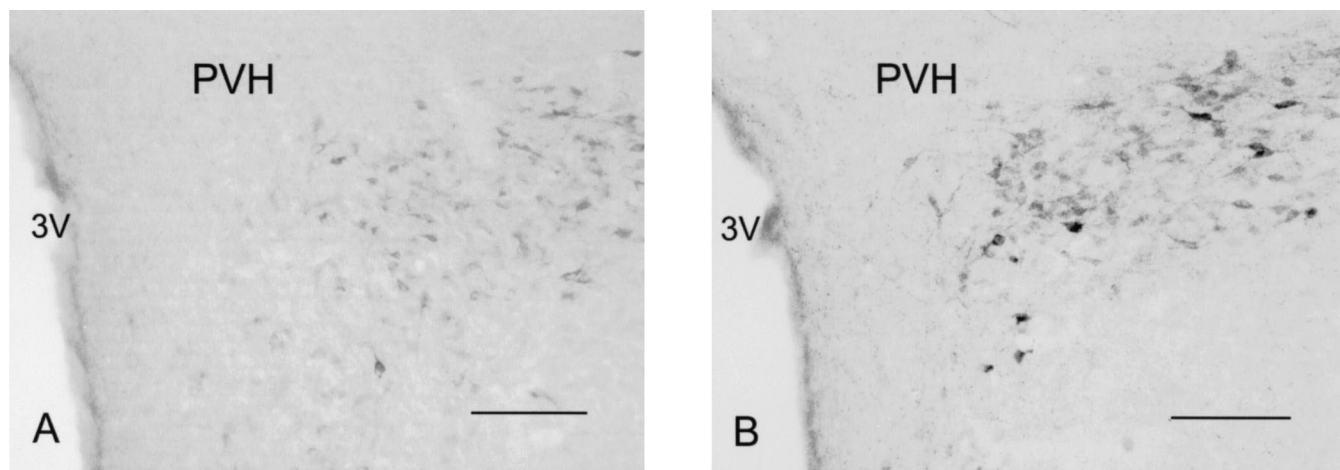


Fig. 4. CRH-ir in the paraventricular nucleus of the hypothalamus (PVH) in control (A) and preshocked (B) rats after exposure to the electrified prod. Scale bars: 130 μ m.

7B) for corticosterone. No preshock or prod effects were found for ACTH 15 min after the introduction of the prod (Fig. 7A).

4. Discussion

In the present study we investigated the long-term effects of single foot shock experience on HPA axis responsivity to a novel electrified prod inserted in the home cage 2 weeks later. The preshock experience sensitized the electrified prod-induced ACTH and corticosterone response. Moreover, in the paraventricular nucleus of the hypothalamus (PVH), the median eminence (ME) and the central amygdala (CeA) a significant preshock effect was found for CRH-ir, primarily as a result of a shock prod-induced increase in CRH-ir in preshocked rats. A significant shock prod-induced increase in the number of

P-CREB-ir nuclei was found in the PVH and the locus coeruleus (LC). However, the preshock experience did not affect the basal or electrified prod-induced expression of P-CREB-ir. No significant difference in behavior between preshocked and control rats during the shock prod challenges was found.

The results of this study showed that exposure of control rats to an electrified prod in the home cage does not affect the amount of CRH-ir in the PVH. However, the electrified prod induced an increase in CRH-ir in the PVH of preshocked rats. Most of the parvocellular CRH-ir neurons in the PVH give rise to axons which terminate in the external zone of the ME, where CRH is released in the hypophyseal portal circulation and can stimulate the release of ACTH from the anterior pituitary [8,38]. Many stressful stimuli like restraint [20] and foot shock [43] induce activation of the HPA axis, as measured by ACTH and corticosterone levels in plasma [1]. Moreover, stress-

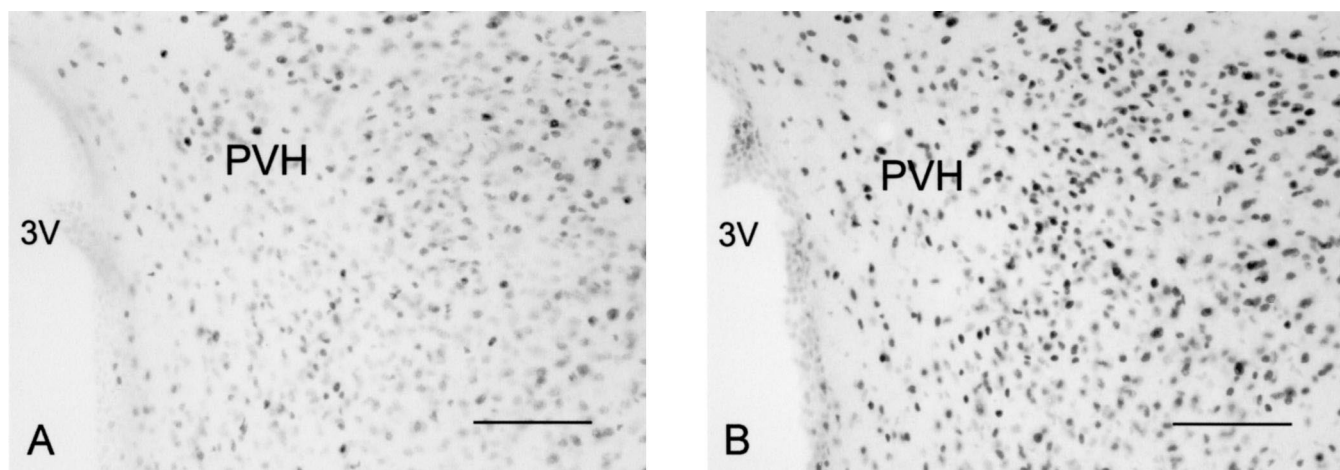


Fig. 5. P-CREB-ir in the paraventricular nucleus of the hypothalamus (PVH) in preshocked rats exposed to the electrified prod (B) or no prod (A). Scale bars: 130 μ m.

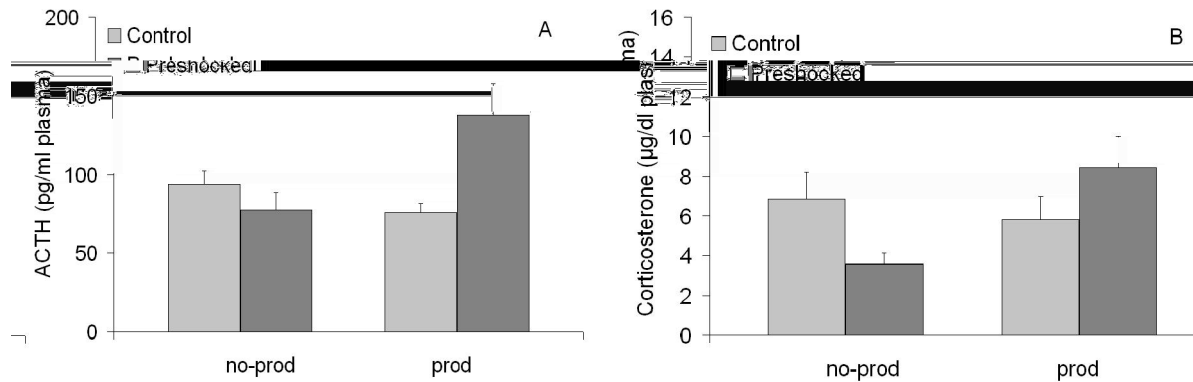


Fig. 6. ACTH (A) and corticosterone (B) levels 5 min after exposure to an electrified prod (prod) or no prod (no-prod), in rats exposed to a single session of foot shocks (preshocked) or no shocks (control) 2 weeks earlier. ACTH preshock \times prod $P=0.003$; corticosterone preshock \times prod $P=0.018$, n =nine to thirteen rats per group.

induced alterations in CRH-ir, CRH mRNA and CRH receptor type 1 (CRH-R1) mRNA in the PVH have been found [19,25,33]. Exposure to a stressful stimulus like foot shock is also reflected in a strong increase in the expression of Fos-ir in CRH-ir neurons in the PVH [43]. However, in the above mentioned studies stressors are used which induce a strong activation of the HPA axis, including CRH-ir neurons in the PVH. The shock prod challenge, as used in our experiment, appeared not to affect the amount of CRH-ir in the PVH of control rats, but increased CRH-ir in the PVH of preshocked rats, indicating that a previous stressful stimulus sensitized the responsivity of CRH-ir neurons in the PVH to a relatively mild stimulus. Interestingly, there is also indirect evidence for a hyperresponsivity of CRH producing neurons in the PVH of PTSD patients. A blunted ACTH response to CRH has been found in traumatized patients, which might be a result of desensitization of CRH₁ receptors at the anterior pituitary due to a hypersecretion of CRH by parvocellular hypothalamic neurons [47]. Our findings point in the same direction, suggesting that a traumatic experience might enhance the responsivity of CRH neurons in the PVH to external stimuli.

In our study the preshock experience sensitized the

shock prod-induced ACTH and corticosterone response. No difference in ACTH levels between preshocked and control rats was found 15 min after the onset of the shock prod test. This is in agreement with a previous study [52] demonstrating that the enhanced release of ACTH in preshocked rats is only found at the peak of the response approximately 5 min after the onset of the stressor. Interestingly, the present study demonstrated that a single foot shock experience does not affect the behavioral response during the shock prod test but sensitizes the response of the HPA axis. In this regard, it is also of interest that a restraint session induces a similar increase in anxiety-like behavior in CRH knockout mice and wild-type mice [13], while the same stressor induces an increase in corticosterone levels in wild-type mice but not in CRH knock out mice [36]. This is in line with our finding that sensitized neuroendocrine and behavioral responses are not necessarily linked. Unfortunately, little research has been done into the responsivity of the HPA axis of PTSD patients to non-trauma related stimuli. In a recent study it was demonstrated that PTSD patients do not display a sensitized ACTH response to trauma related (combat sounds) and non-trauma related stimuli (white noise) [30]. However, in that study baseline measures were collected

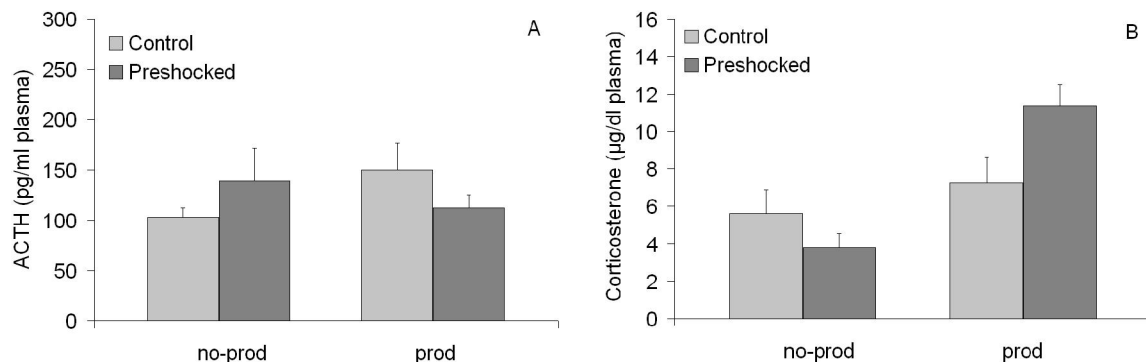


Fig. 7. ACTH (A) and corticosterone (B) levels 15 min after exposure to an electrified prod (prod) or no prod (no-prod), in rats exposed to a single session of foot shocks (preshocked) or no shocks (control) 2 weeks earlier. Corticosterone preshock \times prod $P=0.032$, n =eight rats per group.

minutes before the stress tests and therefore it cannot be excluded that the baseline ACTH levels were already increased due to anticipatory anxiety [37].

We found a significant preshock effect for CRH-ir in the CeA as a result of a shock prod-induced increase in CRH-ir in preshocked rats. The amygdala is considered an important brain area for the regulation of stress-induced behavioral and autonomic responses [24]. CRH neurons in the CeA project to important brainstem areas involved in autonomic control, including the nucleus of the solitary tract and the dorsal motor nucleus of the vagus [55]. The hyperresponsivity of CRH-ir neurons in the CeA could therefore play a role in the sensitized blood pressure response as found in preshocked rats in the shock-prod test [4]. The infusion of CRH in the CeA induces an increase in heart rate which might be a result of a shift in the sympathetic/parasympathetic balance [58]. CRH produced by CeA neurons might also stimulate non-CRH neurons in the CeA involved in autonomic control or indirectly affect CeA neurons via diffusion into the BLA, which in turn innervates the CeA [40,44]. The BLA is rich in CRH₁ receptors compared to the CeA [12,41] and is considered an important interface between sensory brain areas and the CeA [9,32].

The hyperresponsivity of CRH-ir neurons in the CeA to external stimuli may also play a role in the increased immobility response of preshocked rats when placed in a novel environment [53]. CRH released in the CeA is recognized as a mediator of fear and arousal responses. Firstly, CRH injected in the CeA induces behavioral inhibition in a novel environment or behavioral activation in the home cage, indicating enhanced fear or behavioral arousal [29,57]. Secondly, infusion of α -helical CRH in the CeA reverses the social defeat or ethanol withdrawal-induced decrease in open arm exploration in the elevated plus maze [17,42]. Thirdly, a stressful experience induces an increase in CRH and CRH mRNA in the CeA and chronic treatment with a benzodiazepine anxiolytic induces a decrease in basal CRH mRNA levels in the CeA and a decrease in CRH-R1 mRNA and CRH receptor binding in the BLA [31,46].

The results of our study showed that exposure of rats to a shock prod challenge increased the number of P-CREB-ir neurons in the PVH and the LC. However, the preshock experience did not affect the basal and shock prod-induced expression of P-CREB-ir. The electrified prod-induced increase in the number of P-CREB-ir neurons in the PVH is in agreement with a previous study demonstrating an ether-stress-induced increase in P-CREB-ir in the PVH [23]. However, despite the fact that a preshock experience sensitizes the shock prod-induced expression of Fos-ir [3], no preshock effect was found for the shock prod-induced expression of P-CREB-ir in either PVH or LC. The differences are not likely to be caused by a suboptimal time point to measure P-CREB-ir, since both Fos-ir and P-CREB-ir are measured at time points when a maximal

expression can be expected [22,28]. The difference may be caused by a different function of these transcription modulators. Fos is a transcription enhancer that induces an increase in gene transcription which is dependent on the perception of the stimulus [7,18]. P-CREB is a transcription factor that already plays an important role in the basal expression of genes and a relatively mild stimulus can induce a strong increase in the phosphorylation of CREB [11,18]. Interestingly, the preshock experience sensitizes the shock prod induced expression of CRH-ir in the PVH and the CeA, while no sensitized P-CREB-ir response was found in these brain areas. This indicates that an enhanced phosphorylation of the transcriptional activator CREB, which can bind to the CRE site in the CRH promoter [45], is not necessary for a sensitized CRH-ir response.

We found that the shock prod stimulus increased the number of P-CREB-ir neurons in the LC of both the preshock and control rats in the same magnitude. To our knowledge the acute effects of stress on the number of P-CREB-ir neurons in the LC was not studied before, despite the fact that P-CREB is involved in the transcription of the tyrosine hydroxylase gene [27], the rate limiting enzyme for the production of noradrenaline in the LC. These noradrenergic neurons play an important role in the stimulus-induced activation of a wide range of brain areas including the amygdala, the hypothalamus and brainstem areas [14].

The electrified prod induced an increase in P-CREB in the PVH and the LC, but not in the BNST, the basolateral amygdala and the CeA. In contrast, a stressful experience like foot shocks induces an increase in Fos-ir in all the above mentioned brain areas [28]. The difference between those experiments may be caused by the intensity of the stressor, a foot shock session being more aversive than an electrified prod inserted in the home cage or, as for the sensitization component, by a different functional role of the two transcription factors.

Acknowledgements

A.W. Bruijnzeel was supported by Solvay Pharmaceuticals, Weesp, The Netherlands.

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