Pharmacology Biochemistry & Behavior, Vol. 18, pp. 967–971, 1983. © Ankho International. Printed in the U.S.A.

Effects of Repeated Stress on Pituitary Cyclic AMP, and Plasma Prolactin, Corticosterone and Growth Hormone in Male Rats[1,2]

G. JEAN KANT, BRADFORD N. BUNNELL,* EDWARD H. MOUGEY, LEE L. PENNINGTON AND JAMES L. MEYERHOFF

*Department of Medical Neurosciences, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C. 20307 and *Department of Psychology, University of Georgia, Athens GA, 30602*

Received 3 December 1982

KANT, G. J., B. N. BUNNELL, E. H. MOUGEY, L. L. PENNINGTON AND J. L. MEYERHOFF. *Effects of repeated stress on pituitary cyclic AMP, and plasma prolactin, corticosterone and growth hormone in male rats.* PHARMACOL BIOCHEM BEHAV **18**(6) 967–971, 1983.—The effects of five putative stressors (saline injection, cold exposure, forced running, immobilization, and footshock) on levels of pituitary cyclic AMP, plasma prolactin, corticosterone and growth hormone were examined. In naive rats exposed to 15 min of these stressors for the first time, running, immobilization and footshock increased levels of pituitary cyclic AMP, plasma corticosterone and prolactin and decreased growth hormone, typical of stress response in the rat. Cold exposure only increased corticosterone and saline injection did not affect any measured parameter. In rats chronically exposed to the same stressor (once a day for 15 min) for 10 days immediately prior to the experiment, an attenuated pituitary cyclic AMP and plasma prolactin response was seen upon application of 15 min of that stressor on the day of the experiment, compared to the responses observed in the naive rats.

Pituitary Stress Cyclic AMP Prolactin Corticosterone Habituation Growth Hormone

THE application of a stressor evokes numerous neurochemical, hormonal, behavioral and physiological responses in the rat [4, 5, 7, 15, 16, 17, 26, 31, 33, 40]. Our laboratory has been studying the mechanism by which the brain regulates pituitary hormone release in response to stressors. Pituitary cyclic AMP appears to be involved in the release and synthesis of pituitary hormones. Releasing factors including CRF (corticotropin releasing factor) have been shown to increase levels of pituitary cyclic AMP *in vitro;* and conversely, the addition of cyclic AMP or its analogues increases the release and synthesis of pituitary hormones [1, 9, 18, 19, 20, 32, 35, 41].

We have previously reported that some putative stressors elevate pituitary cyclic AMP *in vivo* and have suggested that pituitary cyclic AMP might be an important link between stress-induced hypothalamic release of neurotransmitters and releasing factors and observed pituitary hormone release [3, 13, 14, 28].

Physiological and hormonal responses to stressors can be attenuated by repeated exposure or habituation to that stressor [6, 8, 10, 27]. If pituitary cyclic AMP response is directly involved in the stress-induced release of hormones, then pituitary cyclic AMP response to stressors should also be diminished in chronically stressed animals. The present study was designed to test this hypothesis.

METHOD

Animals

Male Sprague-Dawley rats (200–250 g) were individually housed in a light and temperature-controlled room with food and water freely available. Lights were on from 0600 to 1800 hours.

Experimental Procedures

Initially rats were divided into two groups. One group consisting of 36 rats was handled each day for 10 days. Han-

[1]In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

[2]The views of the author(s) do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4–3, AR 360-5).

dling included habituating the rats to traversing an open-ended plastic cylinder similar to the microwave sacrifice applicator. The other group consisting of 60 rats was subdivided into 5 groups of 12 rats each. Each group of 12 rats was subjected to one of the 5 stressors tested (see below) each day for 15 min for 10 days immediately preceding the experimental day. These rats were also habituated to traversing the plastic cylinder.

The 5 tested stressors included saline injection, cold exposure, forced running, immobilization and footshock. Saline injected rats received a single daily IP injection of saline (0.5 ml) and were replaced in their home cage. Animals subjected to cold were first sprayed with tap water to wet their fur and then placed in a cage in a 4°C chamber. Forced running was accomplished by placing the rats in an activity wheel (diameter=38 cm) that was driven by a motor. Wheel speed was 8 rpm. Shock was delivered to the floor bars of an operant box. The footshock (0.10 watts, supplied by a constant power generator/scrambler) was delivered on a variable time schedule on the average of once per 30 sec. Shock duration was 5 sec. Immobilization was performed by keeping the rat in the plastic holder (5.7 cm) used to immobilize rats for the microwave sacrifice technique.

On the day of the experiment the 12 rats in each repeatedly stressed group were divided into two groups of 6 each. Six rats were sacrificed immediately upon removal from their home cage approximately 24 hours after their last stress session (chronic controls). The other six rats were subjected to an 11th exposure to the same stressor as on previous 10 days and then sacrificed immediately after the 15 min session (chronic stressed). The handled only "naive" group of rats was divided into 6 groups of 6 rats each. Six rats (naive controls) were sacrificed immediately after removal from their home cage. The other 5 groups were each subjected to one of the 5 stressors for 15 min immediately prior to sacrifice (naive stressed).

Sacrifice and Assay Procedures

Animals were sacrificed by a 5 sec exposure to high power microwave irradiation, a technique which has been shown to eliminate post mortem changes in cyclic AMP [12, 23, 29]. The microwave power generator was a modified Varian PPS-2.5 with an output of 2.5 KW at a frequency of 2450 Megahertz [2,22]. Since brief immobilization is required during the microwave sacrifice procedure rats were placed in a 5.7 cm diameter clear plastic cylinder with a closed conical end which was inserted into the waveguide. A plunger was inserted behind the rat to prevent backward movement.

After microwave sacrifice, the rats were decapitated and the trunk blood was collected in heparinized beakers. The blood was centrifuged and the plasma stored at −20°C until assayed for plasma hormones. The heads were cooled briefly on dry ice and then the pituitaries were dissected, weighed and sonicated in 1 ml of 50 mM sodium acetate buffer, pH 6.2. After centrifugation, the supernatants were stored at −70°C until assayed for cyclic AMP.

Materials for the growth hormone and prolactin assays were provided by the National Institute of Health through the Rat Pituitary Hormone Distribution Program. Rat prolactin and growth hormone were radioiodinated as previously described [21]. Plasma was assayed for corticosterone using an antibody produced in our laboratory in rabbits [30]. Within assay variation was <5% and between assay variation was <12%. Recovery of added corticosterone was >92%.

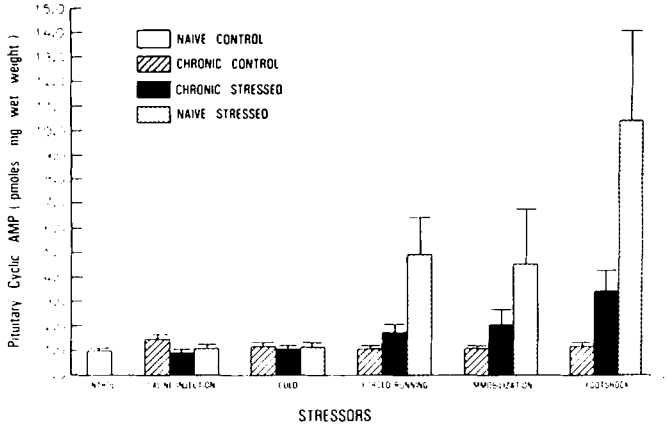

FIG. 1. Effect of stressors on pituitary cyclic AMP. Values represent mean±SEM. N=6. Naive controls were never experimentally stressed. Chronic controls were stressed for 15 min daily for the 10 days preceding the experiment but not stressed during the 24 hr immediately before sacrifice. Chronic stressed animals were stressed for 15 min daily for the 10 days preceding the experiment and again for the 15 min immediately before sacrifice. Naive stressed rats were stressed for the first time during the 15 min immediately before sacrifice. Two-way analysis of variance of the pituitary cyclic AMP data showed significant effects of stressor type, $F(4)=4.68$, $p<0.01$ and treatment group, $F(2)=9.26$, $p<0.001$ and a significant stressor × treatment interaction, $F(8)=2.53$, $p<0.05$. Followup comparisons of the 3 treatment groups by Student's t test found significant differences between the chronic stress and naive stress groups ($p<0.01$).

Cyclic AMP was determined by radioimmunoassay using an antibody developed and characterized in our laboratory [21,38]. A highly specific antiserum was used at a final dilution of 1:400,000. The antiserum exhibited cross-reactivities for ATP and cyclic GMP of less than 0.00007 and 0.14% respectively. The assay data were analyzed by computer [37]. Within assay variation was 7% and between assay variation was 18%. Phosphodiesterase treatment of tissue extracts reduced cyclic AMP to undetectable levels.

RESULTS

In naive rats, 15 min of immobilization, forced running or footshock markedly elevated pituitary cyclic AMP as shown in Fig. 1. Neither cold exposure nor saline injection affected pituitary cyclic AMP levels. The pituitary cyclic AMP response was attenuated in the rats chronically exposed to the stressor. Baseline non-stressed levels of pituitary cyclic AMP were not different in the chronically exposed rats 24 hours following their last session compared to naive control rats. Two-way analysis of variance of the pituitary cyclic AMP data showed significant effects of stressor type, $F(4)=4.68$, $p<0.01$ and treatment group, $F(2)=9.26$, $p<0.001$ and a significant stressor × treatment interaction, $F(8)=2.53$, $p<0.05$. Followup comparisons of the 3 treatment groups by Student's t test found significant differences between the chronic stress and naive stress groups ($p<0.01$).

Plasma corticosterone increased in response to all stressors except saline injection in both chronic and naive stressed groups (Fig. 2). Two-way analysis of variance showed significant effects of stressor, $F(4)=15.29$, $p<0.001$ and treatment, $F(2)=84.6$, $p<0.001$ and a significant stressor × treatment interaction, $F(8)=7.90$, $p<0.001$. The cortico-

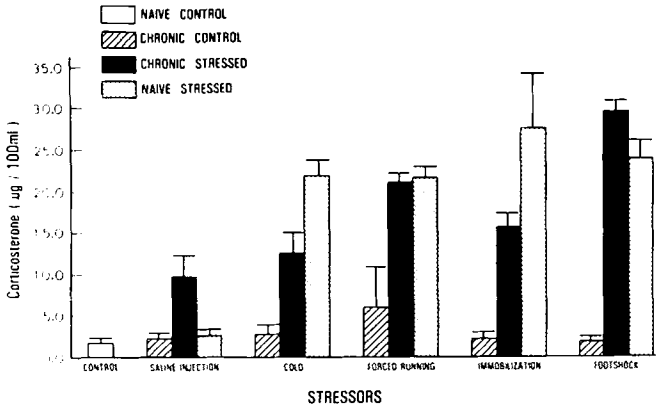

FIG. 2. Effect of stressors on plasma corticosterone. Values represent mean±SEM. N=6. See Fig. 1 legend for group description. Two-way analysis of variance showed significant effects of stressor, $F(4)=15.29$, $p<0.001$ and treatment, $F(2)=84.6$, $p<0.001$ and a significant stressor × treatment interaction, $F(8)=7.90$, $p<0.001$. Comparisons of treatment groups by Student's t test showed significant differences between both chronic and naive stress groups vs. controls ($p<0.05$), but not between the two stress groups.

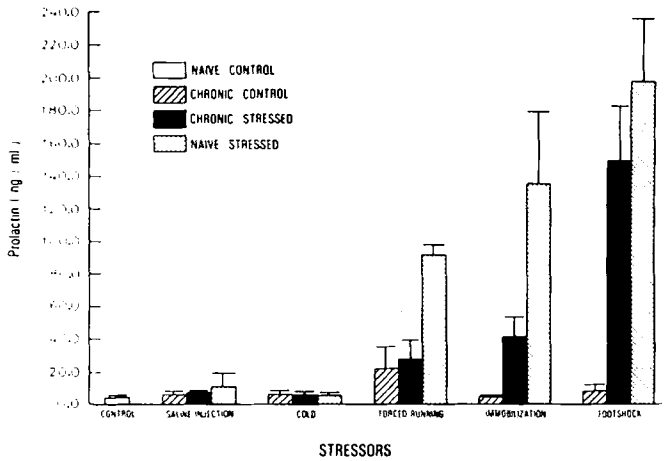

FIG. 3. Effect of stressors on plasma prolactin. Values represent mean±SEM. N=6. See Fig. 1 legend for group description. Two-way analysis of variance showed significant effects of stressor, $F(4)=19.0$, $p<0.001$ and treatment, $F(2)=24.6$, $p<0.001$ and a significant treatment × stressor interaction, $F(8)=5.9$, $p<0.001$. The chronically stressed group was significantly different from both the controls and the naive stressed groups (Student's t-test, $p<0.05$).

sterone response did not habituate; stress response was similar in both naive and chronic groups. Comparisons of treatment groups by Student's t test showed significant differences between both chronic and naive stress groups vs. controls ($p<0.05$), but not between the two stress groups.

Footshock, immobilization, or forced running markedly increased plasma prolactin, while cold and saline injection had no effect (Fig. 3). Two-way analysis of variance showed a significant effects of stressor, $F(4)=19.0$, $p<0.001$ and treatment, F(2)24.6, $p<0.001$ and a significant treatment × stressor interaction, $F(8)=5.9$, $p<0.001$. Habituation of prolactin response to stressors developed in the chronically stressed groups. The chronically stressed group was significantly different from both the controls and the naive stressed groups (Student's t test, $p<0.05$).

Growth hormone levels were decreased following forced running, immobilization and footshock (Fig. 4). Two-way analysis of variance showed significant effects of stressor, $F(4)=2.98$, $p<0.05$ but not of treatment group. No significant interaction was seen.

Correlation analyses on all data revealed positive correlations between pituitary cyclic AMP and prolactin (Spearmans rank coefficient $s_r=0.562$, $p<0.05$), pituitary cyclic AMP and corticosterone ($s_r=0.488$, $p<0.05$) and corticosterone and prolactin ($s_r=0.624$, $p<0.05$). Negative correlations were observed between growth hormone and pituitary cyclic AMP ($s_r=0.138$, $p>0.05$), growth hormone and prolactin ($s_r=-0.150$, $p>0.05$) and growth hormone and corticosterone ($s_r=-0.223$, $p<0.05$).

DISCUSSION

Acute stressors increased levels of pituitary cyclic AMP *in vivo* as we have previously reported [3, 13, 14, 28]. Plasma prolactin and corticosterone were elevated and plasma growth hormone was decreased following acute stress, typical of hormonal stress response in the rat [17, 21, 24, 25, 39]. Footshock, immobilization and forced running applied as described in this report were effective stressors judged by

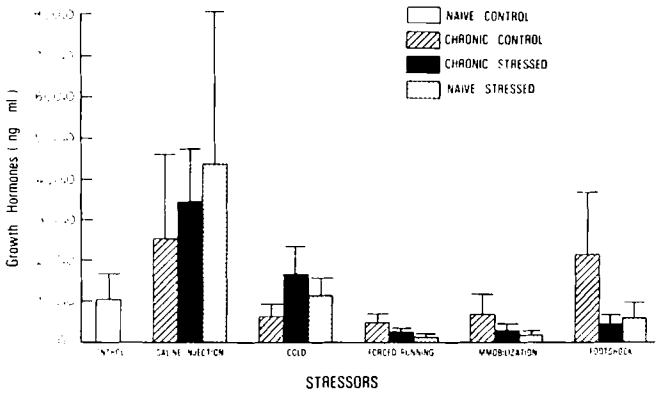

FIG. 4. Effect of stressors on plasma growth hormone. Values represent mean±SEM. N=6. See Fig. 1 legend for group description. Two-way analysis of variance showed significant effects of stressor, $F(4)=2.98$, $p<0.05$ but not of treatment group. No significant interaction was seen.

neuroendocrine responses while cold exposure and saline injection were relatively ineffective.

Chronic exposure to the tested stressors attenuated pituitary cyclic AMP and prolactin response. Corticosterone response to stressors was similar in chronic and naive stress groups. Growth hormone levels were highly variable as has been reported previously [21, 24, 25], obscuring the differences between treatment groups.

Corticosterone response appeared to be sensitive to relatively mild stressors (cold) compared to the other parameters measured and to reach maximal levels after mild stressors. Both these experiments and our previous work support the suggestion that corticosterone is a sensitive indicator of stress or arousal but not an adequate index of the intensity of stress [14]. Possibly, the apparent ceiling on corticosterone response is a reflection of adrenal sensitivity to ACTH. The adrenal gland is most sensitive to ACTH at basal nonstressed concentrations [33]. Longer periods of exposure to

the tested stressors quite possibly would have resulted in measurable corticosterone response habituation as this has been reported to occur following daily 2 hr restraint stress [36]. Thus, the failure of the corticosterone response to habituate in these experiments is more a quantitative than a qualitative feature of the stress response and reflects the limited range of corticosterone response to stress.

On the other hand, the pituitary cyclic AMP and plasma prolactin responses to stressors appear to be sensitive to stressors over a much larger range, and chronic exposure to the tested stressors attenuated both the pituitary cyclic AMP and the prolactin responses, demonstrating habituation. These parameters may be more useful biochemical indices than corticosterone for assessing the intensity of stressors over a wide range.

Repeated exposure to stressors or events results in physiological and behavioral habituation mediated by central nervous system mechanisms [11]. The observed habituation of the pituitary cyclic AMP response to stressors lends support to our hypothesis that pituitary cyclic AMP links stress-induced release of neurotransmitters and/or releasing factors with subsequent pituitary hormonal release. The specific hormones controlled by pituitary cyclic AMP mechanisms, however remain undetermined. The discrepancy between pituitary cyclic AMP and plasma corticosterone habituation does not clearly rule out control of ACTH by pituitary cyclic AMP since adrenal gland responsiveness is a factor in the observed corticosterone values. Growth hormone release has been suggested to be influenced by cyclic AMP in *in vitro* experiments [34] but the variability in growth hormone levels seen in these experiments makes it impossible to draw clear conclusions regarding the relationship between pituitary cyclic AMP and growth hormone release. Interestingly the pituitary cyclic AMP and prolactin responses in the situations tested appear to be similar. However the exact relationship between pituitary cyclic AMP *in vivo* and the release or synthesis of particular hormones remains to be determined.

The pituitary cyclic AMP response to stress might also reflect the feedback effects of pituitary or adrenal hormones upon the pituitary rather than the direct effects of hypothalamic factors. Experiments to distinguish between these alternate possibilities are underway in our laboratories.

ACKNOWLEDGEMENTS

We wish to thank David Collins, Clint Wormley, and Willie Gamble for performing the cyclic nucleotide and hormone assays. We also acknowledge Clyde Kenion, Golden Driver, SP5 Leigh Landman Roberts, and SP4 Terry Eggleston for expert technical assistance in performing the experiment and data analysis and Pat Conners for secretarial support.

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