

Chronic Intermittent Cold Stress Sensitises the Hypothalamic-Pituitary-Adrenal Response to a Novel Acute Stress by Enhancing Noradrenergic Influence in the Rat Paraventricular Nucleus

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Abstract

Chronic intermittent cold stress sensitises activation of the hypothalamic-pituitary-adrenal (HPA) axis by novel acute stress. We have shown that enhanced noradrenergic function in limbic forebrain contributes to HPA sensitisation. In the present study, we investigated whether chronic intermittent cold also induced changes in noradrenergic function in the paraventricular nucleus (PVN), the primary mediator of the HPA stress response. Rats were exposed to chronic intermittent cold (7 days, 6 h per day, 4 °C). On the day after final cold exposure, there were no differences in baseline plasma ACTH, but the peak ACTH response to 30 min of acute immobilisation stress was greater in cold-stressed rats compared to controls. Bilateral microinjection of the α_1 -adrenergic receptor antagonist benoxathian into the PVN reduced acute stress-induced adrenocorticotrophic hormone (ACTH) levels by approximately 25% in controls. Furthermore, in cold-stressed rats, all of the sensitisation of the ACTH response was blocked by benoxathian, to a level comparable to benoxathian-treated controls. In a second study using microdialysis to measure norepinephrine release in the PVN, there were no differences in either baseline or acute stress-induced increases in norepinephrine release in the PVN of cold-stressed rats compared to controls. Thus, in a third study, we tested potential alterations in postsynaptic α_1 -receptor sensitivity after chronic cold stress. Dose-dependent activation of ACTH secretion by microinjection of the α_1 -adrenergic receptor agonist, phenylephrine, into the PVN was significantly enhanced in cold-stressed rats compared to controls. Thus, the sensitised HPA response to acute stress after chronic intermittent cold exposure is at least partly attributable to an enhanced response to α_1 -adrenergic receptor activation in the PVN. Chronic stress-induced plasticity in the acute stress response may be important for stress adaptation, but may also contribute to pathophysiological conditions associated with stress. Thus, understanding the neural mechanisms underlying such adaptations may help us understand the aetiology of such disorders, and contribute to the future development of more effective treatment or prevention strategies.

Stress is a predisposing or exacerbating factor in many pathophysiological conditions, from cardiovascular disease (1), to tumour formation (2). A particularly important link has been identified between stress and psychiatric illness, including depression and anxiety disorders (3–5). The diverse set of behavioural, autonomic and neuroendocrine adaptations evoked by stressful stimuli serve to maintain homeostasis and enhance survival in the face of a threat or challenge, either real or perceived. However, prolonged or excessive activation of the stress response itself can become maladaptive, contributing to the negative health consequences associated with stress (6).

Activation of the hypothalamic-pituitary-adrenal (HPA) axis represents the major neuroendocrine response to acute stress. Neurones in the hypothalamic paraventricular nucleus (PVN) release corticotrophin-releasing hormone (CRH) and other secretagogues into the pituitary portal system to stimulate secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, which in turn elicits release of adrenal corticosteroids (7). In addition to the primary neural circuits mediating this fundamental component of the stress response, other brain systems also play a more widespread modulatory role in coordinating, integrating or modifying the systemic response to stress. One such system is the brain noradrenergic

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system. Stress-induced release of norepinephrine plays an important modulatory function in many brain regions, facilitating synaptic transmission in circuits involved in mediating or regulating specific behavioural and physiological responses evoked by stress (8–10). For example, in previous studies, we have shown that stress-induced release of norepinephrine in extrahypothalamic limbic forebrain regions, including the lateral bed nucleus of the stria terminalis (BSTL) and medial amygdala, facilitates activation of the HPA axis in response to acute immobilisation stress (11, 12).

There is also evidence that norepinephrine exerts effects directly in the PVN to influence HPA reactivity (13–18). The PVN receives noradrenergic innervation from the A1 and especially the A2 noradrenergic cell groups in the medulla, with a lesser input from the locus coeruleus (19, 20). Adrenergic receptors, especially α_1 -adrenergic receptors, are moderately dense in the PVN (21, 22), and we and others have demonstrated the expression of α_1 -adrenergic receptor subtypes specifically in the parvocellular region of PVN containing the CRH- and vasopressin-synthesising output neurones that mediate activation of the HPA axis in response to acute stress (23–26).

Chronic repeated stress can sensitise the subsequent activation of the HPA axis in response to a novel acute stressor (27–31). Enhanced reactivity to acute stress following a period of repeated intermittent stress may represent an important regulatory alteration serving to increase an organism's ability to cope successfully with impending threat, and to respond more efficiently to a persistent challenge. However, excessive HPA reactivity can be detrimental, contributing to the development of stress-related psychopathology (6). Indeed, dysregulation of both noradrenergic and HPA reactivity have been described in psychopathological states associated with chronic stress, including depression and anxiety disorders (32–34). Thus, understanding the mechanisms underlying HPA dysregulation and the role played by norepinephrine may help us understand the aetiology of such disorders, or the long-term regulatory mechanisms underlying their treatment.

We have shown previously that an enhanced facilitatory influence of norepinephrine in the BSTL plays a role in sensitisation of the HPA axis after chronic intermittent cold stress (31). However, it is not known what effect chronic intermittent stress might have on noradrenergic modulatory function in the PVN itself, the primary motor output of the HPA axis. Thus, in the present study, we investigated changes in the modulatory influence of stress-evoked norepinephrine release in the PVN that may contribute to sensitisation of acute HPA stress reactivity following chronic intermittent cold exposure. A combination of neurochemical and pharmacological approaches was used to assess any potential changes in both norepinephrine release and in the response to α_1 -adrenergic receptor activation in the PVN. Portions of this work have been presented previously in abstract form (35).

Materials and methods

Animals

A total of 134 male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA), weighing 225–250 g upon arrival, were used in these experiments. They were

allowed to acclimatise to the animal facility for 7–10 days before use in any experimental procedures. The housing facility was maintained on a 12/12 h light/dark cycle (lights on 07.00 h). Animals were housed initially three per cage, with *ad lib* access to food and water, and then singly housed after any surgical procedure and during the conduct of the experiments. Experiments were conducted between 09.00 h and 12.00 h, during the light portion of the cycle. All animal procedures were conducted in accordance with NIH guidelines, and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Chronic intermittent cold exposure

In each experiment, rats were randomly assigned to either a control or cold condition. During the light phase of the light cycle, rats in the cold condition were transported, in their home cages with food, water and bedding, into a cold room maintained at 4 °C for a period of 6 h. After 6 h, they were returned to the main housing room. This procedure was repeated every day for 7 consecutive days. Rats in the control condition were left undisturbed in the housing facility during this time. Testing took place on the day after the last cold exposure.

Acute immobilisation stress

For acute stress, rats were immobilised by holding them prone on a flat, plastic rack large enough to support their body securely (26 cm × 13 cm) while their limbs were taped gently but securely to the rack with medical adhesive tape. Strips of tape were placed across the body and back of the head to prevent excessive head movements. This procedure took approximately 1 min, and the duration of the stress period was 30 min from that point. At the end of the stress period, the animal was held in place gently while the tape was removed with scissors, and then returned to its home cage.

Experiment 1: microinjection of the α_1 -adrenergic receptor antagonist benoxathian into the PVN to assess changes in noradrenergic modulatory influence on HPA reactivity

Thirty-two rats were used in this experiment. Surgery was conducted 4 days before beginning the 7-day intermittent cold treatment. Animals were anaesthetised with a cocktail of ketamine 43 mg/kg, acepromazine 1.4 mg/kg and xylazine 8.6 mg/kg, given in a volume of 1.0 ml/kg, *i.m.* An indwelling silastic catheter was implanted into the jugular vein, then exteriorised at the back of the neck and loaded with heparinised saline (50 U/ml) to maintain patency. Rats were then placed in a stereotaxic apparatus with the incisor bar set at –3.3 mm, adjusted as necessary to achieve a flat skull, indicated by equal dorsoventral coordinates for lambda and bregma. Two guide cannulae, constructed of 22-gauge stainless steel tubing 14 mm in length, were implanted bilaterally, aimed one at each PVN using the following coordinates relative to bregma, with a 10° lateral angle (18): anteroposterior –1.7 mm; mediolateral \pm 1.8 mm; and dorsoventral –5.3 mm measured from dura. The guide cannulae were targeted to terminate 2 mm dorsal to the PVN, at a level corresponding to plate 25 in the atlas of Paxinos and Watson (36). The cannulae were anchored to the skull with jeweler's screws and dental acrylic, and fitted with 30-gauge obturators to maintain patency. Rats were housed singly for 4 days before beginning the intermittent cold exposure. Control rats were treated identically, but remained in their home cages in the housing room for an equivalent period of time, with no cold exposure.

On the day of the experiment, 1 day following the last cold exposure, animals were transported to the testing room, and the venous catheter was connected via PE tubing to a 1-ml syringe filled with heparin-saline (50 U/ml) for repeated blood sampling. After 90 min of acclimatisation to the test room, the obturators were removed from the guide cannulae and replaced with 30-gauge microinjectors extending 2 mm beyond the tips of the guides, placing them in the PVN. The injectors were connected via PE-10 tubing to a Hamilton syringe mounted on a syringe pump (Instech, Plymouth Meeting, PA, USA). Thirty minutes later, a baseline blood sample (0.4 ml) was drawn. Bilateral microinjections of vehicle (sterile saline), or the α_1 -adrenergic receptor antagonist benoxathian (2.0 nmol) were then made into the PVN in a volume of 0.2 μ l, delivered at a rate of 0.2 μ l/min ($n = 7$ –9 per group). After injections were complete, the cannulae were left in place for

an additional 4 min to allow for diffusion of the drug. A second baseline blood sample was then taken before removing the injectors.

Rats were then subjected to 30 min of immobilisation stress as described above, during which a blood sample was taken after 5 min stress, and another after 30 min, immediately before the animals were released at the end of the stress period. Following the termination of stress, animals were returned to their home cages, and blood samples were taken after 15, 30 and 60 min of poststress recovery. All blood samples were replaced immediately by infusing an equivalent volume of sterile saline. In previous experiments, we have determined that this repeated sampling and replacement procedure does not affect plasma ACTH measures (11, 12). Blood was collected into tubes containing 10 µl of 1.0 M EDTA. Plasma was separated by centrifugation at 12 000 r.p.m. for 5 min at 4 °C, and stored at -80 °C until assayed. Plasma ACTH levels were determined in 200 µl samples by radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). The detection limit of the assay was 15 pg/ml; intra-assay and interassay coefficients of variation were 9% and 11%, respectively.

For analysis, baseline ACTH levels were first compared in rats exposed to chronic intermittent cold and control rats using a t-test. Responses to acute stress were then analysed by three-way ANOVA with repeated measures, with Cold Treatment and Drug as the between-subject variables and Time as the within-subject variable. *Post-hoc* comparisons were conducted using the Newman-Keuls test to identify any effects of chronic cold and acute drug treatment on ACTH secretion at each time point, including the response to stress. $P < 0.05$ was considered statistically significant in all analyses. Placement of the microinjection cannulae were determined histologically. All cases in which injection sites were located outside the PVN were eliminated *a priori* from further analyses, and were not included in the total number of animals reported. This resulted in the elimination of six cases.

Experiment 2: stress-induced norepinephrine release in the PVN

Twelve animals were used for this experiment. Rats were anaesthetised and placed in a stereotaxic apparatus as above. A microdialysis guide cannula assembly (CMA/12; CMA Microdialysis, North Chelmsford, MA, USA) was implanted to terminate 0.2 mm above PVN, using the following coordinates relative to bregma, with a 10° lateral angle: anteroposterior -1.7, mediolateral +1.8 mm, and dorsoventral -7.1 mm from dura. The guide cannula was anchored to the skull, and an obturator inserted to maintain patency. Rats were then singly housed, and chronic intermittent cold treatment was begun 3–4 days after surgery.

On the day of the experiment, rats in the control or chronic intermittent cold conditions ($n = 6$ each) were transported to the testing room. The obturator was removed and replaced with a microdialysis probe (CMA/12), with a molecular weight cutoff of 20 kDa and 1 mm of active membrane. The probe extended 1 mm beyond the tip of the guide cannula, centring the active membrane along the lateral aspect of the parvocellular PVN, to avoid excessive damage to the nucleus proper. Animals were placed in a dialysis chamber, a plexiglas cage lined with bedding, identical to their home cage with the lid modified to accommodate the perfusion line. Artificial cerebrospinal fluid (147 mM NaCl, 2.5 mM KCl, 1.3 mM CaCl₂, 0.9 mM MgCl₂, pH 7.4) was perfused through the dialysis probe at a rate of 1.0 µl/min. Dialysate sample collection began after a 2-h equilibration period. Each sample period was 30 min, and samples were collected into tubes containing 2.5 µl of stabilising solution (0.2 µM EDTA, 0.2 µM ascorbic acid, 0.2 M perchloric acid) and immediately placed on ice. After three baseline samples were collected, animals were subjected to 30 min of acute immobilisation stress as described above, during which time one dialysate sample was collected. The rats were then released from immobilisation and returned to the dialysis chamber, where three poststress recovery samples were collected. Following all experiments, placement of the dialysis probe was determined histologically. Four cases in which the active portion of the probe was found to be located outside the PVN were eliminated *a priori* from further analyses, and were not included in the total number of animals reported (16 rats prepared for a final $n = 12$).

The amount of norepinephrine in the microdialysate samples was measured by high-performance liquid chromatography with coulometric detection (Coulochem2, ESA Inc., East Chelmsford, MA, USA). The mobile phase consisted of 60 mM sodium phosphate, 75 µM EDTA, 1.5 mM sodium 1-octanesulphonic acid and 6% methanol, pH 4.7, with a flow rate of 0.6 ml/min. Under these conditions, norepinephrine had a retention time

of approximately 7.6 min. Norepinephrine was quantified against a calibration curve run daily, ranging from 0.5 to 25 pg. The detection limit for norepinephrine was approximately 0.5 pg. Mean baseline norepinephrine levels were first compared using a t-test. Acute stress-induced changes in norepinephrine levels were then analysed using two-way ANOVA with repeated measures, followed by *post-hoc* analyses with the Newman-Keuls test to identify the source of any significant effects indicated by ANOVA. $P < 0.05$ was considered statistically significant in all analyses.

Experiment 3: microinjection of the α_1 -adrenergic receptor agonist phenylephrine into the PVN to assess changes in HPA sensitivity to adrenergic receptor activation

This experiment was conducted essentially as in experiment 1, but with no administration of acute immobilisation stress. Sixty-five rats were prepared surgically and assigned to the chronic intermittent cold stress or control treatments as above. On the day of the experiment, rats were transported to the testing room, the venous catheter was connected, and they were allowed to acclimatise for 90 min, at which time the microinjectors were inserted. Thirty minutes later, a baseline blood sample was taken. One minute after the baseline sample, phenylephrine was microinjected bilaterally into the PVN at one of four doses: 2, 10, 20 or 50 nmol ($n = 6-10$ per group), delivered in a volume of 0.2 µl/side at a rate of 0.2 µl/min. The cannulae were left in place, and subsequent blood samples were taken at 5, 10, 15, 30, and 60 min after microinjection. Plasma ACTH concentrations were measured as in experiment 1. Baseline ACTH levels were first compared using a t-test. Responses to phenylephrine were then analysed by three-way ANOVA with repeated measures, with Cold Treatment and Drug as between-subject variables and Time as the within-subject variable. *Post-hoc* comparisons were conducted using the Newman-Keuls test, with $P < 0.05$ considered statistically significant. Ten cases in which injection sites were located outside the PVN were eliminated *a priori* from further analyses, and were not included in the total number of animals reported.

Control microinjections

In a separate set of limited control experiments, 25 additional rats were used to verify that the effects of benoxathian and phenylephrine microinjections were specific to PVN. In the first control study, stress-induced ACTH responses were measured after purposely misplaced microinjections of benoxathian (2.0 nmol) 1 mm above PVN along the same cannula approach, in ventral thalamus, and compared to rats receiving 2 nmol benoxathian or vehicle into the PVN as in experiment 1 ($n = 4$ per group). In a second control experiment, ACTH responses of five rats receiving phenylephrine microinjections (50 nmol) purposely misplaced 1 mm above the PVN were compared to the responses of control rats receiving 50 nmol phenylephrine in the PVN in experiment 3 above. Finally, to determine any potential baseline effects of benoxathian, plasma ACTH was compared in rats receiving microinjections into the PVN of vehicle or benoxathian (2.0 nmol, $n = 4$ per group), but not exposed to acute immobilisation stress.

Results

Experiment 1: microinjection of the α_1 -adrenergic receptor antagonist benoxathian into the PVN to assess changes in noradrenergic modulatory influence on HPA reactivity

There were no differences in baseline plasma ACTH levels between animals exposed to chronic intermittent cold and controls, either before or after drug or vehicle microinjections into the PVN ($t_{30} = 0.34$, $P = 0.73$). Figure 1(A) shows a representative micrograph with microinjection sites localised bilaterally in the PVN. Microinjection itself did not alter baseline ACTH (pre versus postdrug baseline: $t_{31} = 1.26$, $P = 0.21$) (Fig. 2). After benoxathian microinjections, ANOVA revealed significant main effects of Drug ($F_{1,28} = 9.35$, $P < 0.01$) and Time ($F_{6,168} = 125.55$, $P < 0.00001$), and a significant Drug–Time interaction ($F_{6,168} = 4.53$, $P < 0.001$).

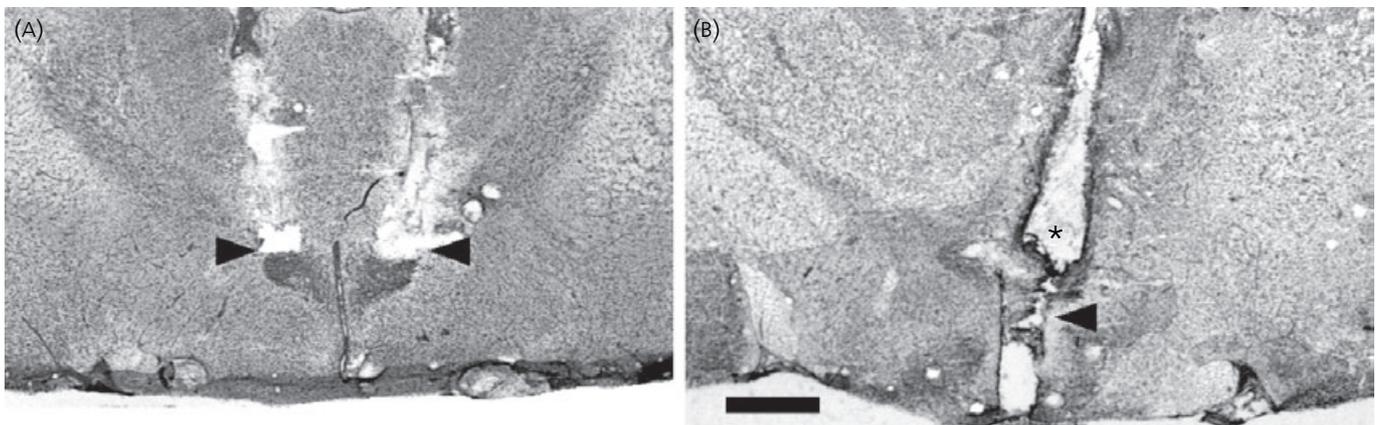


FIG. 1. Representative micrographs of cresyl-stained sections corresponding to plate 28 in the atlas of Paxinos and Watson (36), illustrating histological localisation of microinjection sites and microdialysis probe tracks in the paraventricular nucleus (PVN). (A) Microinjection sites, indicated by cannulae tips (arrowheads), located bilaterally just dorsal to parvocellular PVN, as adapted from (18). (B) Microdialysis probe track (arrowhead) located along the lateral aspect of parvocellular PVN. The guide cannula track (asterisk) terminates just dorsal to the target. Scale bar = 1 mm in both panels.

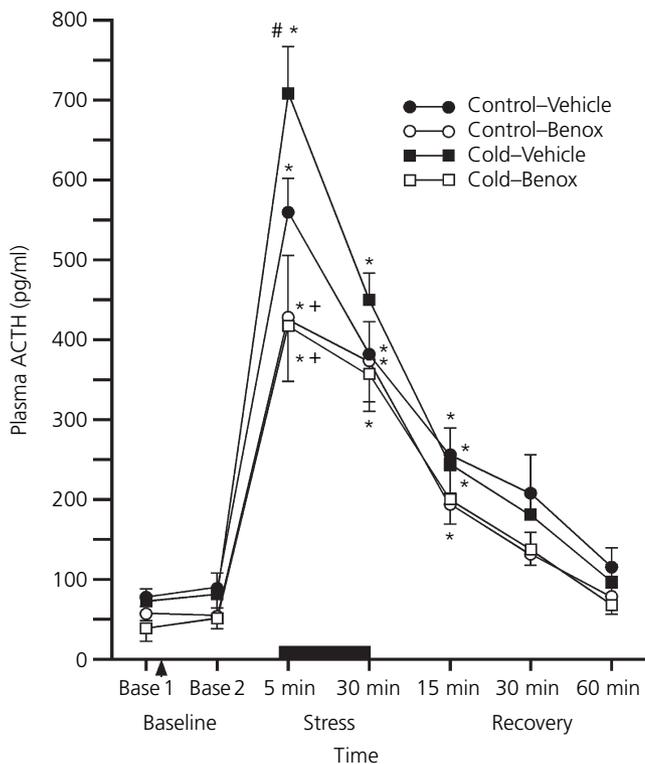


FIG. 2. Effects of bilateral administration of the α_1 -adrenergic receptor antagonist benoxathian (2 nmol) into the paraventricular nucleus (PVN) on the hypothalamic-pituitary-adrenal response to acute stress in control and cold-sensitised rats. There were no baseline differences in plasma adrenocorticotrophic hormone (ACTH) between groups, and antagonist administration alone did not affect baseline. Acute immobilisation stress significantly increased plasma ACTH concentrations measured at 5 min and 30 min after stress onset in all groups. Benoxathian attenuated the acute immobilisation stress-induced ACTH response in both control and cold-sensitised rats. The response after benoxathian in cold-stressed rats was comparable to that in control rats. Data are expressed as mean \pm SEM. * $P < 0.05$ compared to baseline; # $P < 0.05$ compared to noncold-stressed control rats, indicating sensitisation; + $P < 0.05$ compared to vehicle-treated rats in the same chronic stress condition.

Post-hoc analyses indicated that stress significantly increased plasma ACTH concentrations in all treatment groups, returning to baseline after 30 min poststress recovery (Fig. 2). Consistent with previous observations (31), the peak ACTH response at the 5-min time point was greater in cold-stressed rats than in controls ($P < 0.001$) (Fig. 2). Also consistent with previous results, *post-hoc* comparisons showed that α_1 -adrenergic receptor blockade by benoxathian microinjection in the PVN significantly attenuated the ACTH response to stress at the 5-min time point in both cold-stressed (41% reduction in peak ACTH) and control rats (23% reduction). Furthermore, the ACTH response to acute immobilisation stress following benoxathian administration was essentially identical in cold-stressed and control rats, indicating that the enhanced response seen after chronic intermittent cold exposure was completely blocked by α_1 -adrenergic receptor antagonism in the PVN.

Experiment 2: stress-induced norepinephrine release in the PVN

Figure 1(B) shows a representative micrograph with the microdialysis probe track localised in the PVN. There was no significant difference in mean baseline norepinephrine levels measured in microdialysate samples collected in the PVN of cold-stressed rats (1.55 ± 0.12 pg/sample, mean \pm SEM) and control rats (1.15 ± 0.18 pg/sample; $t_{10} = 1.85$, $P = 0.094$) (Fig. 3). ANOVA indicated a significant main effect of time ($F_{6,36} = 14.67$, $P < 0.00001$), but no difference between groups ($F_{1,6} = 1.04$, $P = 0.35$). *Post-hoc* analyses showed that 30 min of acute immobilisation stress induced a significant and comparable two-fold increase in extracellular norepinephrine levels in the PVN of both cold-stressed rats (to 3.17 ± 0.58 pg/sample) and control rats (to 2.22 ± 0.33 pg/sample). Figure 3 portrays these data with each value expressed as a percent of the mean baseline calculated for each rat.

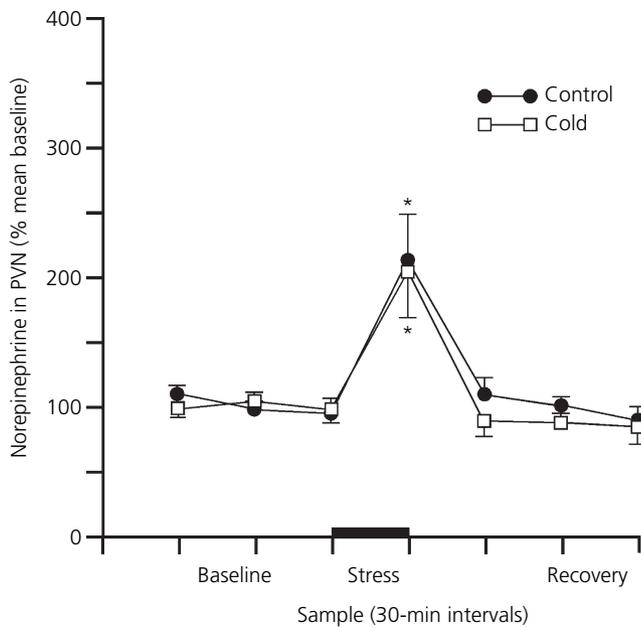


FIG. 3. Acute immobilisation stress (30 min) induced a similar increase in norepinephrine release in the paraventricular nucleus (PVN) of control rats and rats previously exposed to chronic cold stress. There were no significant differences in baseline norepinephrine levels in microdialysate samples collected in the PVN of cold-stressed rats (1.55 ± 0.12 pg/sample, mean \pm SEM) and control rats (1.15 ± 0.18 pg/sample). Data are presented as a percent of the mean baseline level (mean \pm SEM) calculated for each rat. Immobilisation stress induced an approximate two-fold increase in extracellular norepinephrine levels in both groups, returning to baseline by 30 min after stress termination. * $P < 0.05$ for both groups compared to their respective baselines.

Experiment 3: microinjection of the α_1 -adrenergic receptor agonist phenylephrine into the PVN to assess changes in HPA sensitivity to adrenergic receptor activation

As in experiment 1, there was no difference in baseline ACTH levels between cold-stressed rats and controls ($t_{63} = 1.11$, $P = 0.27$). Three-way ANOVA revealed significant main effects of Cold ($F_{1,47} = 23.84$, $P < 0.0001$), Drug ($F_{3,47} = 9.48$, $P < 0.0001$) and Time ($F_{5,235} = 51.53$, $P < 0.00001$); significant Cold \times Time ($F_{5,235} = 11.82$, $P < 0.00001$) and Drug \times Time interactions ($F_{15,235} = 7.06$, $P < 0.00001$); and a significant three-way Cold \times Drug \times Time interaction ($F_{15,235} = 1.73$, $P < 0.05$).

Post-hoc analyses indicated that there was a dose-dependent increase in ACTH secretion induced 5–30 min after microinjection of phenylephrine into PVN, and also that this effect was significantly enhanced in rats exposed to chronic intermittent cold stress compared to control rats (Fig. 4). In control rats, only the 50 nmol dose of phenylephrine induced an ACTH response that reached significance relative to predrug baseline. By contrast, in rats exposed to chronic intermittent cold stress, ACTH was significantly elevated after 10, 20 or 50 nmol phenylephrine. Moreover, the ACTH response to each of these doses was significantly greater in cold-stressed rats compared to control rats receiving the same doses (Fig. 4). Microinjection of 2 nmol phenylephrine did not increase ACTH significantly above baseline levels in either group.

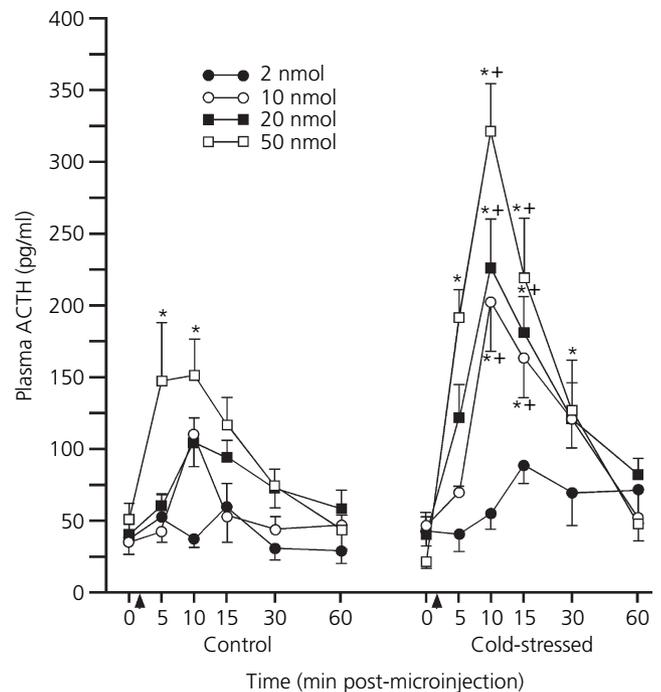


FIG. 4. Changes in the adrenocorticotrophic hormone (ACTH) response to bilateral administration of the α_1 -adrenergic receptor agonist phenylephrine into the paraventricular nucleus after previous exposure to chronic intermittent cold stress. Phenylephrine (2–50 nmol/0.2 μ l/site) stimulated ACTH secretion in a dose-dependent manner, with a maximal effect seen 10 min after microinjection, and returning to baseline gradually by 60 min after drug administration. The ACTH responses to phenylephrine were significantly enhanced at the 10, 20 and 50 nmol doses in cold-stressed rats compared to control rats. Data are expressed as mean \pm SEM. * $P < 0.05$ compared to baseline; + $P < 0.05$ compared to noncold-stressed control rats at the same dose and time point.

Control microinjections

In the first control study, microinjections of the α_1 -antagonist benoxathian (2 nmol) purposely misplaced into a site 1 mm above PVN had no effect on acute stress-induced activation of ACTH secretion, with the response being no different from that seen after vehicle microinjections made into PVN. By contrast, as in experiment 1, benoxathian microinjections into the PVN significantly attenuated the ACTH response (Fig. 5A). In the second control study, purposely misplaced microinjections of phenylephrine (50 nmol) made 1 mm above PVN had no significant effect on plasma ACTH, and this was significantly different from the response elicited by 50 nmol phenylephrine microinjected into the PVN of the control rats in experiment 3 ($P < 0.01$) (Fig. 5B). After the misplaced phenylephrine injections, there did appear to be a slight delayed rise in ACTH, albeit nonsignificant, perhaps reflecting diffusion of the drug to PVN (Fig. 5B), although this was not explored further. Finally, neither vehicle nor 2 nmol benoxathian microinjected into the PVN in the absence of acute immobilisation stress had any effect on plasma ACTH levels (Fig. 5C).

Discussion

In the present study, activation of the rat HPA axis by an acute stressor was enhanced following a period of chronic

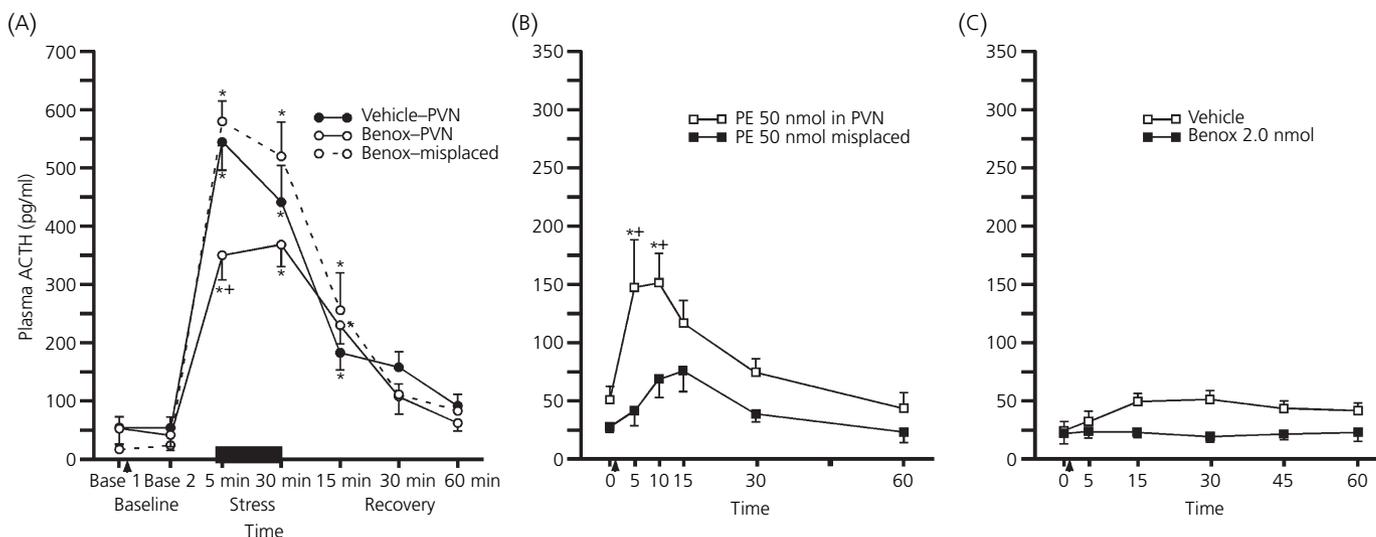


Fig. 5. Results of control experiments. (A) Microinjections of the α_1 -adrenergic receptor antagonist benoxathian (2 nmol) purposely misplaced 1 mm above the paraventricular nucleus (PVN) had no effect on stress-induced activation of adrenocorticotrophic hormone (ACTH) secretion, being comparable to vehicle microinjections in the PVN, and in contrast to the significant attenuation seen after benoxathian microinjections in the PVN. * $P < 0.05$ compared to baseline; + $P < 0.05$ compared to both other groups. (B) Similarly, microinjections of the α_1 -adrenergic receptor agonist phenylephrine (50 nmol) purposely misplaced 1 mm above PVN had no effect on plasma ACTH. * $P < 0.05$ compared to baseline; + $P < 0.05$ phenylephrine microinjections in the PVN compared to misplaced injections. (C) Bilateral microinjections of either vehicle or benoxathian (2 nmol) into the PVN had no effect on plasma ACTH in the absence of acute stress. Data in all panels are expressed as mean \pm SEM.

intermittent cold stress exposure, consistent with previous reports from our laboratory and other studies (31, 37). The mechanisms by which chronic repeated stress may induce such plasticity in the acute response to stress may be an important component in the successful anticipation of, and adaptation to future stress. Of equal importance, such regulatory changes may also contribute to the detrimental consequences of chronic stress, or to the many pathophysiological disorders associated with dysregulation of the HPA axis. However, the mechanisms underlying this HPA sensitisation have not been well understood.

The results of the present study suggest that alterations in the stress-activated modulatory function of norepinephrine may play a role in sensitising the HPA response to acute stress following a period of chronic intermittent stress. The PVN is innervated by noradrenergic afferents arising primarily from the A1 and A2 cell groups in the medulla oblongata, with a minor input from the A6 cells in the locus coeruleus (19, 20). These ascending noradrenergic pathways have been shown to be important in activation of the HPA axis in response to a number of different stressors (14, 16, 17, 38, 39). Most studies have indicated that norepinephrine exerts a facilitatory influence in the PVN on acute activation of the HPA axis, and that this effect is mediated primarily by activation of α_1 -adrenergic receptors (15, 16, 40). Electrical stimulation of the ventral noradrenergic bundle increased secretion of CRH, whereas mechanical or neurotoxic lesions of the same pathway inhibited stress-induced activation of CRH and vasopressin mRNA expression in the PVN, and also inhibited stress-induced release of ACTH (39, 41, 42). Lesions of the locus coeruleus have also been shown to attenuate the ACTH response to restraint stress (43). By contrast, microinjection of norepinephrine directly into the PVN stimulated expression of Fos and CRH mRNA in the PVN, induced

POMC mRNA expression in the anterior pituitary, and increased ACTH and corticosterone concentrations in plasma (18, 44, 45).

Thus, norepinephrine acts in the PVN to facilitate HPA activation. However, this facilitatory influence is modulatory in nature; whereas norepinephrine acts in the PVN, and elsewhere, to enhance the HPA response to stress, norepinephrine does not appear to be necessary for the response to occur (7, 46, 47). For example, depletion of norepinephrine in the PVN by 6-OHDA lesions, or by transection of the ascending projections from the medulla did not affect plasma corticosterone responses, or the induction of Fos expression in the PVN by stressors such as footshock, interleukin-1 administration, or restraint stress (46–48). The results of the present study are consistent with such a modulatory influence of norepinephrine because the ACTH response to acute immobilisation stress was attenuated after benoxathian microinjections in the PVN, but it was not abolished completely.

Nonetheless, essentially all of the enhancement of the ACTH response to acute stress seen following chronic intermittent cold exposure was blocked by α_1 -adrenergic receptor blockade in the PVN because the response after benoxathian was identical in control and cold-stressed rats. This suggests that increased noradrenergic modulatory function played a major part in the sensitisation of the HPA response. This finding is similar to a previous observation that we have reported regarding noradrenergic modulatory influence in the lateral bed nucleus of the stria terminalis (31), an extrahypothalamic forebrain region which also regulates activation of the HPA axis, and in which a facilitatory effect of norepinephrine has also been demonstrated (11).

The results of the present study are consistent with those of previous studies showing sensitisation of both the HPA axis

and brain noradrenergic activity following chronic cold stress (37, 49–51). An increase in acute stress-induced activation of the HPA axis, and of Fos expression in locus coeruleus, and other stress-related forebrain regions including the PVN, amygdala and the paraventricular thalamic nucleus, were observed after exposure to chronic intermittent cold stress using a protocol very similar to the one we employed in the current study (37). In other studies, an increase in activity and excitability of locus coeruleus noradrenergic neurones, and in extracellular norepinephrine levels measured in the medial prefrontal cortex, were found to be enhanced after a longer and more severe regimen of continuous cold exposure (49–52). Sensitisation of the HPA axis following chronic intermittent cold exposure, similar to that obtained in the present study, was observed in both Sprague-Dawley rats and Wistar-Kyoto rats, a strain that is especially vulnerable to the detrimental physiological effects of stress (31). Also consistent with the present study, there was no change in the acute stress-induced release of norepinephrine in the lateral bed nucleus of the stria terminalis of Sprague-Dawley rats. However, acute stress-induced norepinephrine release was significantly enhanced in the Wistar-Kyoto rats, which exhibit a deficient brain noradrenergic response to acute stress under basal conditions (i.e. with no previous exposure to chronic intermittent cold stress) (31, 53).

Similarly, in the present study, there was only a slight but nonsignificant elevation in basal extracellular norepinephrine levels measured in the PVN of cold-stressed Sprague-Dawley rats, but no change in the magnitude of acute stress-induced increase in norepinephrine release. Previous studies have shown that the duration, nature and severity of chronic stress are important factors in determining whether a detectable change in basal or evoked norepinephrine activity may be induced (50). For example, in studies using a more severe form of cold stress, it was shown that 2 weeks of continuous cold exposure potentiated tail-shock-evoked release of norepinephrine in the prefrontal cortex of Sprague-Dawley rats, but that 1 week of continuous exposure was insufficient, and also that continuous cold exposure enhanced stress-induced norepinephrine release, but intermittent cold exposure over a similar time frame did not (50, 54, 55). Thus, although it is possible that, in some contexts, part of the enhanced noradrenergic modulatory function observed following chronic intermittent cold exposure could be attributable to an increase in acute stress-induced release of norepinephrine, the lack of any such increase in the present study in the face of enhanced noradrenergic facilitation of the HPA axis suggests that some other mechanism(s) must be at work. Nonetheless, any such increase in norepinephrine release that might occur under more severe chronic stress conditions would be expected to work synergistically with the increase in adrenergic receptor responsiveness observed in the present study, further enhancing acute stress-induced activation of the HPA axis.

Expression of α_1 -adrenergic receptor binding sites and α_1 -receptor subtype-specific mRNA expression have both been described in parvocellular PVN, in subpopulations of cells that participate in stress-induced activation of the HPA axis (21, 22, 24–26, 56, 57). In addition, pharmacological studies have indicated that facilitation of HPA activity by norepi-

nephrine in the PVN is mediated primarily by α_1 -adrenergic receptors (15, 16, 40). Therefore, we investigated potential changes in the sensitivity of the HPA response to α_1 -adrenergic agonist administration directly into the PVN following chronic intermittent cold exposure, and found this response to be enhanced dramatically. Such an increase in α_1 -adrenergic receptor response sensitivity in the PVN following chronic intermittent cold exposure may be the result of an increase in postsynaptic receptor number, an increase in receptor affinity, or a facilitation of receptor-activated signal transduction processes.

However, there is surprisingly little evidence regarding the potential regulation of α_1 -receptor expression or function with chronic stress *in vivo*. In one study, chronic unpredictable stress did not change α_1 -receptor binding density in the limbic system (58). On the other hand, pharmacological studies have suggested that chronic elevations in noradrenergic neurotransmission (e.g. by chronic treatment with norepinephrine reuptake blockers) can facilitate subsequent behavioural responses to α_1 -agonist administration (59), and increase agonist affinity for brain α_1 -receptor binding sites (60). Although there is by no means a consensus, studies performed *in vitro* have also suggested that α_1 -receptor expression or sensitivity can be regulated by agonist exposure. Chronic treatment of renal epithelial cells with agonist decreased α_1 -receptor number (61). By contrast, α_1 -receptor-mediated signal transduction and induction of cell growth increased with chronic exposure of cultured neonatal rat cardiac myocytes to norepinephrine (62). Some of this apparent discrepancy may be explained by observations suggesting that the different α_1 -receptor subtypes may be differentially regulated by agonist exposure. Chronic treatment of cardiac myocytes with α_1 -receptor agonists increased α_{1A} -, but decreased α_{1B} -receptor mRNA expression (63). Similarly, chronic exposure to norepinephrine increased α_{1A} - (formerly α_{1C}), but decreased α_{1B} - and α_{1D} -receptor mRNA expression (62). These responses were accompanied by corresponding changes in receptor protein and binding site expression, and were attributed to an α_1 -receptor-mediated mechanism (62). Incubation of cells stably transfected with α_1 -adrenergic receptor subtypes with phenylephrine for 24 h down-regulated α_{1A} - and α_{1B} -receptors, but α_{1D} -receptors were increased. Similarly, agonist-stimulated inositol phosphate formation was reduced in cells transfected with α_{1A} - or α_{1B} -, but not α_{1D} -receptors (64). Further evidence also indicates that the α_1 -receptor subtypes differ in the extent to which agonist exposure induces receptor internalisation and, moreover, they differ in the degree to which these agonist-mediated regulatory processes are desensitised by previous agonist exposure (65). Thus, to the extent that stress-induced agonist exposure may be a component of the regulatory plasticity invoked by chronic intermittent cold stress, it is possible that changes in the sensitivity of α_1 -adrenergic receptors, and consequent changes in the modulatory influence of norepinephrine, may be regulated in a region- and receptor subtype-specific fashion, and may account in part for the sensitised reactivity of the HPA axis.

Regulation and modulation of the HPA axis and the systemic response to stress is a necessarily complex process, involving a number of interacting, convergent and sometimes

redundant systems (7, 28, 37). Thus, in addition to the PVN, it is possible that noradrenergic activity in other extrahypothalamic brain regions may also contribute to sensitisation of the HPA axis after exposure to chronic cold stress. For example, we have shown previously that norepinephrine acts in the lateral bed nucleus of the stria terminalis to facilitate acute stress-induced activation of the HPA axis (10–12), and that this influence is increased following chronic cold stress (31). In addition, other brain circuits and systems that modulate the HPA axis are also affected by exposure to chronic cold stress, including the amygdala and the paraventricular thalamic nucleus, amongst others (37), and these too may participate in long-term plasticity in the response to acute stress. Many of these regions also receive noradrenergic input, suggesting multiple mechanisms by which norepinephrine, as well as other modulatory transmitters, may all contribute to the long-term adaptation and regulation of the HPA axis in the face of chronic stress.

In conclusion, long-term regulation and plasticity of the stress response is an important component of coping and adaptation in the face of severe, chronic or repeated stress, invoked in anticipation of subsequent challenges (3). However, enhanced or prolonged activity of normally adaptive stress response systems may become detrimental over time, and may be associated with a number of pathophysiological conditions related to chronic stress (6). Dysregulation of both the HPA axis and the brain noradrenergic system have been described in association with a number of neuropsychiatric disorders related to chronic stress, including depression, post-traumatic stress disorder and other anxiety disorders (32–34, 66–68). Thus, understanding the processes by which these systems are dysregulated may help us to understand better the aetiology of such disorders and their relation to stress, or to illuminate the regulatory mechanisms underlying their successful treatment.

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