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Differential Responsivity of the Hypothalamic-Pituitary-Adrenal Axis to Glucocorticoid Negative-Feedback and Corticotropin Releasing Hormone in Rats Undergoing Morphine Withdrawal: Possible Mechanisms Involved in Facilitated and Attenuated Stress Responses

H. Houshyar,* M. D. Galigniana,* W. B. Pratt* and J. H. Woods*†
Departments of *Pharmacology and †Psychology, University of Michigan, Ann Arbor, MI, USA.

Key words: restraint stress, morphine, dexamethasone, CRH, glucocorticoid receptor.

Abstract

Chronic morphine treatment produces profound and long-lasting changes in the pituitary-adrenal responses to stressful stimuli. The purpose of the present study was to explore the mechanisms involved in these altered stress responses. Chronic morphine administration increased basal plasma concentrations of corticosterone and adrenocorticotropic hormone (ACTH), which peaked at 36 h after the final morphine injection and returned to normal levels within 84-h. Whole brain glucocorticoid receptor protein expression was reduced (approximately 70%) in morphine-treated rats 4-h after the final morphine injection and these levels recovered within 16-h. Twelve hours following morphine withdrawal, rats displayed normal ACTH, but potentiated and prolonged corticosterone responses to restraint stress. Both the ACTH and corticosterone responses to restraint in acutely withdrawn rats were insensitive to dexamethasone. Furthermore, acutely withdrawn rats displayed reduced ACTH but prolonged corticosterone responses to peripheral corticotropin releasing hormone (CRH) administration. These findings suggest that the normal ACTH and enhanced corticosterone responses to stress in acutely withdrawn rats involved decreased sensitivity of negative-feedback systems to glucocorticoids, reduced pituitary responsivity to CRH, and enhanced sensitivity of the adrenals to ACTH. Eight days following morphine withdrawal, rats displayed dramatically reduced ACTH, but normal corticosterone responses to restraint stress. These rats displayed enhanced sensitivity to dexamethasone and normal pituitary-adrenal responses to CRH. These data suggest that the reduced ACTH responses to stress in 8-day withdrawal rats involved increased sensitivity of negative-feedback systems to glucocorticoids as well as reduced CRH and/or AVP function in response to stress. Taken together, the results of this study illustrate some of the mechanisms mediating altered stress responsivity in rats that have received chronic morphine treatment.

Epidemiological and clinical studies indicate that stress is related to the abuse of opioids in humans (1–3). One of the principal biological responses to stress is the activation of the hypothalamic-pituitary-adrenal (HPA) axis and it has been proposed that an atypical responsivity of the HPA axis to stressors may contribute to drug abuse in humans (4, 5). Indeed, laboratory studies have shown that chronic morphine treatment modifies the HPA responses to stress (6–10) and that stress-induced alterations of the reinforcing effect of morphine involve the HPA axis (11, 12).

The effects of acute morphine treatment and acute stress exposure on HPA activity are similar in rodents. Both stimuli increase the release of corticotropin releasing hormone (CRH), which in turn increases adrenocorticotropic hormone (ACTH) and corticosterone secretion (6, 8, 13–16). Chronic morphine treatment and chronic exposure to a homotypic

Correspondence to: Hani Houshyar, Department of Physiology, University of California, 513 Parnassus Avenue, Room HSW 750, San Francisco, CA 94143, USA (e-mail: hani@itsa.ucsf.edu).

stressor also have similar effects on the HPA axis, both resulting in decreased hormonal responses to these stimuli with repeated exposure (6, 8, 17–19). More recently, we have shown that not only the hormonal, but also the physiological effects of chronic morphine treatment are comparable to those observed with chronic stress (20). Chronic morphine treatment reduced body weight, induced thymus involution, as well as adrenal gland hypertrophy, and elevated basal corticosterone concentrations, which are all characteristic responses associated with chronic stress exposure (20, 21).

We also showed that despite the negative-feedback effects of increased basal corticosterone concentrations, rats undergoing acute (12-h) morphine withdrawal displayed a potentiated and prolonged corticosterone response to restraint (20). This enhanced adrenal response to stress in acutely withdrawn rats is similar to the well characterized facilitated HPA responses to an acute, novel stressor in chronically stressed rats (21–24). The exact neurocircuitries involved in the facilitated responses to an acute, novel stressor in chronically stressed rats have not been clarified yet. However, a number of physiological changes associated with chronic stress that might be involved in facilitation include: (i) enhanced activity of the paraventricular thalamic nucleus (24); (ii) enhanced activation of CRH neurones in the paraventricular (PVN) nucleus of the hypothalamus, in particular those coexpressing arginine vasopressin (AVP) (25); (iii) reduced pituitary CRH binding sites, but increased pituitary ACTH content and pituitary responsivity to CRH (25–29); (iv) increased adrenal sensitivity to ACTH (21, 29); and (v) reduced hippocampal glucocorticoid receptor (GR) expression accompanied with decreased sensitivity to the negative-feedback effects of glucocorticoids (22, 29–33).

In contrast to these facilitated HPA responses, chronic exposure to some stressors has been shown to result in attenuated HPA responses to an acute, novel stressor. Chronic exposure to social stress, social isolation and postnatal handling have been shown to reduce the HPA responses to a subsequent novel stressor (34–37). Recently, we have shown that rats undergoing prolonged morphine withdrawal (8-day or 16-day) display a similar reduction in the pituitary-adrenal responses to restraint stress (20). Although the mechanisms mediating reduced HPA responsiveness in chronically stressed rats are currently unclear, two of the primary physiological changes associated with this phenomenon include reduced PVN CRH mRNA expression and CRH release in response to stress (35, 37, 38), and increased hippocampal GR expression associated with enhanced efficiency of negative-feedback systems (34).

It remains to be determined if some of the physiological changes associated with chronic stress exposure are also involved in the hyper- and hypo-responsiveness of the HPA axis in rats undergoing acute and chronic morphine withdrawal, respectively. Therefore, the aim of the present study was to evaluate some of the potential mechanisms mediating the facilitated and attenuated HPA responses to an acute, novel stressor in acute and extended withdrawal in rats. For this purpose, in acutely and chronically withdrawn rats, we examined: (i) changes in basal plasma concentrations of ACTH and corticosterone with chronic morphine treatment to determine the time course of morphine-induced changes in basal HPA activity; (ii) the expression of whole brain GR protein to determine if altered HPA activity in morphine-treated rats may be related to changes in the expression of this receptor; (iii) the effect of dexamethasone treatment on the ACTH and corticosterone responses to restraint stress to determine the sensitivity of negative-feedback systems to glucocorticoids; and (iv) the ACTH and corticosterone responses to peripheral CRH administration to determine the sensitivity of pituitary corticotrophs to this secretogogue.

Materials and methods

Subjects

Male Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, IN, USA), weighing 250–350 g at the beginning of experiment, were used. Rats were housed singly in Plexiglass cages in a room maintained at 20°C, 40–50% humidity with a 12-h/12-h light/dark cycle (light from 07.00 h to 19.00 h) and with free access to food and water. Experiments were initiated between 07.00 h and 07.30 h and each experimental group consisted of 5–10 rats. All experimental procedures were performed according to the Guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Health Council (Department of Health, Education and Welfare), and were approved by the University of Michigan Committee on the Use and Care of Animals.

Morphine dependence and withdrawal

On the basis of previous studies, morphine dependency was induced by subcutaneous injections of morphine (Mallinickrodt Inc., St Louis, MO, USA) twice daily at 07.00 h and 19.00 h for 16 consecutive days (20, 39, 40). During the first 10 days, the dose of morphine was increased by 10 mg/kg per injection each day from 10 to 100 mg/kg per injection, and during the last 6 days rats were maintained on 100 mg/kg per injection (Fig. 1). As a control, nondependent rats received twice daily s.c. injections of saline for 16 consecutive days. There were four experimental groups: (i) nondependent; (ii) morphine-dependent; (iii) 12-h spontaneous morphine withdrawal; and (iv) 8-day spontaneous morphine withdrawal. Experiments in nondependent, morphine-dependent, and 12-h withdrawal rats were carried out on day 17. Experiments in the 8-day withdrawal group were carried out on day 24, after 16 days of treatment with morphine followed by 7 days of treatment with saline. On the final day of the experiment, nondependent, 12-h withdrawal and 8-day withdrawal rats received an injection of saline, whereas morphine-dependent rats received an injection of morphine (100 mg/kg, s.c.). Cessation of chronic morphine treatment induced a number of behavioural and physical withdrawal symptoms, including piloerection, ptosis, lacrimation, rhinorrhea, diarrhoea and body weight loss.

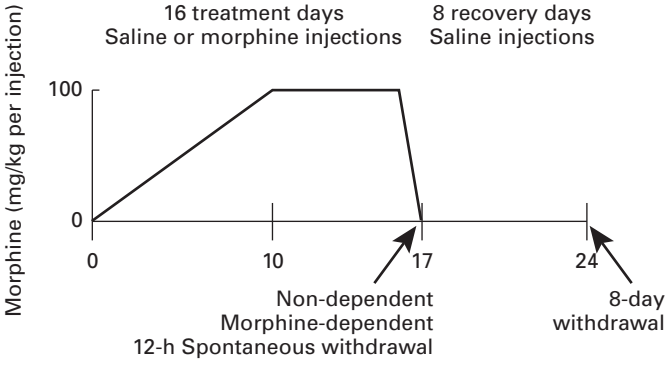

FIG. 1. Timeline of experimental procedure.

Blood sampling and assays

The tip of the tail of each rat (approximately 1 mm) was nicked with a scalpel no. 10 blade and approximately 240 µl of blood was collected using heparinized microcapillary pipettes (Fisher Scientific, Pittsburgh, PA, USA). During the blood collection procedure, which lasted less than 2 min, rats were lightly wrapped in a piece of cloth. This tail nick method allowed rapid and repeated blood collection without causing either behavioural or hormonal distress to the rats as evidenced by basal pituitary-adrenal hormone levels (Fig. 2A,D and Fig. 3A,D), which are within the range previously reported using either tail nick or decapitation methods (8, 23, 41, 42). Blood samples were immediately placed on ice until centrifuged at 4000 r.p.m. for 5 min at 4°C. Thereafter, the plasma was pipetted into 1 ml Cryovials (Fisher Scientific) and stored at −80°C until analysis. ACTH and corticosterone concentrations were determined using commercially available RIA kits purchased from Nichols Institute Diagnostics (San Juan Capistrano, CA, USA) and Diagnostic Products Corporation (Los Angeles, CA, USA), respectively.

Daily basal pituitary-adrenal activity

We have previously shown that basal ACTH and corticosterone concentrations in rats repeatedly treated with saline remain at low levels, whereas basal corticosterone concentrations are markedly elevated in rats chronically treated with morphine (20). Therefore, in the present study, daily basal ACTH and corticosterone concentrations were determined only in morphine-treated rats. On the initial experimental day, the tip of the tail of each rat was nicked, and blood was collected to demonstrate basal hormone levels in nontreated rats. Immediately thereafter, rats received their first morphine injection. On subsequent days, the clot from the tail nick incision was removed and blood was collected daily at approximately 07.00 h immediately prior to morphine (days 1–16) or saline (days 17–24) administration. Daily blood collection did not alter pituitary-adrenal activity in morphine-treated rats as evidenced by similar hormone levels on experimental day 17 in rats exposed to daily blood collection (Fig. 2) and rats naïve to this procedure (Fig. 3B,E and Fig. 4B,E).

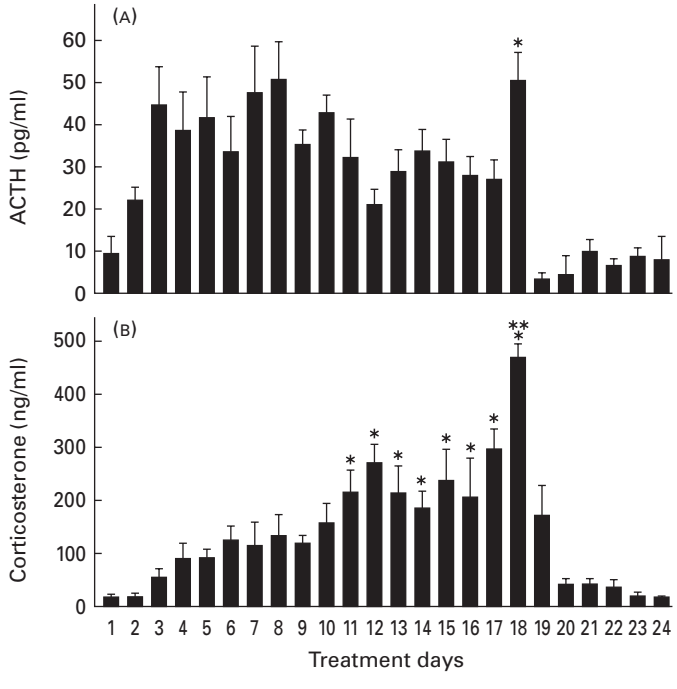

FIG. 2. Basal plasma adrenocorticotropic hormone and corticosterone concentrations of rats chronically treated with morphine. Rats received twice daily injections of increasing doses of morphine (10–100 mg/kg per injection, s.c.) for the initial 16 days followed by 8 days of twice daily administration of saline. Blood was collected daily at approximately 07.00 h, immediately before morphine (days 1–16) or saline (days 17–24) administration. Data are means and SEM (n = 5–10). *P < 0.05 versus Day 1, **P < 0.05 versus all other treatment days.

Pituitary-adrenal activity in the absence of restraint stress

Plasma ACTH and corticosterone concentrations on the final experimental day were determined in nondependent, morphine-dependent, 12-h withdrawal and 8-day withdrawal rats. On this day, after the initial tail nick for

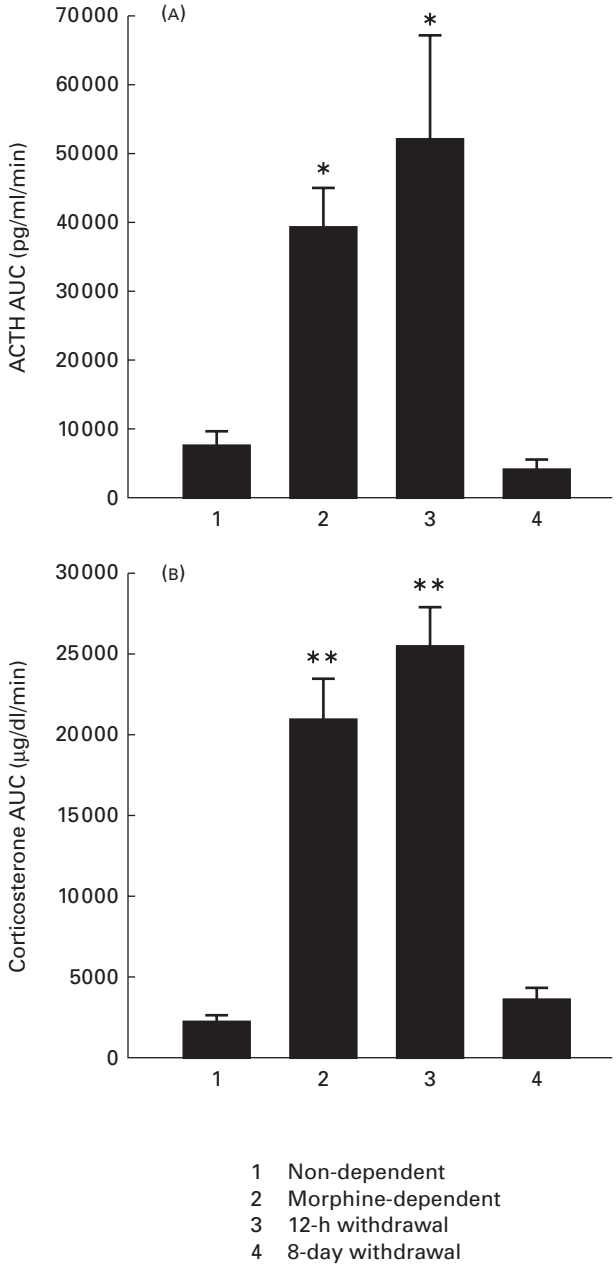

FIG. 3. Total cumulative release (area under curve, AUC) of plasma adrenocorticotropic hormone (ACTH) and corticosterone in the absence of restraint in vehicle- and morphine-treated rats. (A,B) Showing total plasma ACTH and corticosterone AUC in: (i) nondependent; (ii) morphine-dependent; (iii) 12-h withdrawal and (iv) 8-day withdrawal rats. AUC data reflect plasma ACTH and corticosterone release during a 4-h period after administration of saline (groups 1, 3 and 4) or morphine (group 2). Data are means and SEM (n = 5–6). *P < 0.01 and **P < 0.001 versus nondependent and 8-day withdrawal groups.

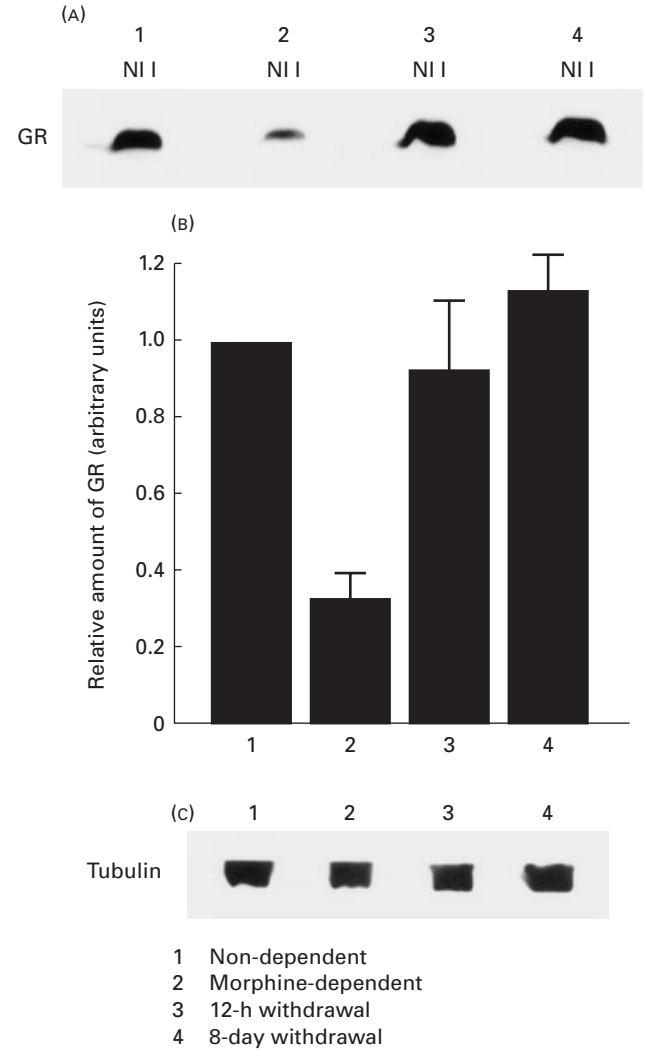

FIG. 4. Effect of chronic morphine treatment on glucocorticoid receptor protein levels in the whole brain. Brains were collected 4 h after the final administration of saline (nondependent, 12-h withdrawal and 8-day withdrawal groups) or morphine (morphine-dependent rats). (A) Showing a representative Western blot illustrating immunoreactive glucocorticoid receptor (GR) from whole brain extracts in nondependent (lane 1), morphine-dependent (lane 2), 12-h withdrawal (lane 3) and 8-day withdrawal (lane 4) rats. (B) Showing a quantitative densitometric analysis of immunoreactive GR protein levels. (C) Showing a representative Western blot illustrating α-tubulin levels in the extracts. Data are means and SEM (n = 5).

determination of basal hormone concentrations, rats received an injection of morphine (morphine-dependent) or saline (nondependent, 12-h or 8-day withdrawal). Thereafter, the same tail nick incision was used for repeated blood collection 15, 30, 60, 90, 120 and 240 min after morphine/saline administration. Plasma hormone concentrations during this period are depicted as total area under curve (AUC) (Fig. 3). At the end of this blood collection period, rats in the four treatment groups were decapitated and whole brains were removed for determination of GR protein expression in the brain.

Brain preparation and extraction of GR from nuclei

Methods used to prepare rat brain for extraction of total GR were modified based on previous publications (43, 44). Rat brain was homogenized in one volume of HEV buffer (10 mM Hepes, 1 mM EDTA, 20 mM sodium orthovanadate, pH 7.5) with an Ultraturrax tissue homogenizer. The homogenate was then incubated on ice for 15 min with 0.05% SDS and 1.0% Triton X-100. Thereafter, 200 U/ml of DNAase I (Boehringer-Mannheim, Mannheim, Germany) was added to the medium and incubated for 15 min on ice. Finally, NaCl (final concentration equal to 0.5 M) was added, and the incubation on ice was continued for an additional 30 min. The homogenates were then centrifuged at 100 000 ***g*** for 30 min at 4°C, the pellet was discarded, and the supernatant was used to immunopurify the GR.

GR immunoprecipitation and Western blotting

The GR was immunoadsorbed from 300 µl aliquots of lysate by rotation for 3 h at 4°C with 10 µl BuGR2 mouse monocolonal antibody (Affinity Bioreagents, Golden, CO, USA) prebound to 80 µl of 20% (w/v) protein A-Sepharose (Sigma, St Louis, MO, USA). Non-immune mouse IgG (Sigma) was used as a control for nonspecifically adsorbed proteins. The immunopellets were washed five times with 1 ml of a buffer containing 20 mM N-tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid, 50 mM NaCl, 4 mM EDTA, 10% glycerol, 0.02% Nonidet P-40, at pH 7.6. Pellets were boiled in 80 µl of SDS sample buffer with 10% (v/v) β-mercaptoethanol, and proteins were resolved in a 9% polyacrilamide/SDS gel. Proteins were finally electrotransferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) and probed for the GR with 2 µg/ml of BuGR2 antibody. The immunoreactive protein was visualized after incubation with horseradish peroxidase-conjugated goat antimouse counter antibody (Sigma). An autoradiograph was obtained by reincubation of the membrane with ^{125}I-labelled counter antibody (DuPont/NEN, Boston, MA, USA).

Sensitivity to dexamethasone

The pituitary-adrenal responses to restraint stress were examined in nondependent, 12-h withdrawal, and 8-day withdrawal rats pretreated with dexamethasone. After basal blood collection, rats received an injection of dexamethasone (0.001, 0.01, 0.1 and 1.0 mg/kg, s.c.) or vehicle. Two hours following dexamethasone/vehicle administration, restraint stress was initiated. Rats were restrained for 4-h in their home cages by being placed in Plexiglass restraint tubes (internal diameter 6 cm, Fisher Scientific) provided with numerous air holes to facilitate breathing and heat dissipation. The same tail nick incision was used for repeated blood collection at 15, 30, 60, 90, 120 and 240 min after initiation of restraint. Dexamethasone (Gensia Pharmaceuticals; Irvine, CA, USA) was purchased in 10 mg/ml vials and diluted with saline to the desired concentrations.

Responsivity to corticotropin-releasing hormone

The pituitary-adrenal responses to peripheral CRH administration were examined in nondependent, 12-h withdrawal and 8-day withdrawal rats. Rats were habituated to the i.p. injection procedure by receiving once daily injections of saline i.p. at approximately 07.00 h for seven consecutive days prior to the experimental day. After basal blood collection, rats received an injection of CRH (1 or 10 µg/kg, i.p.) or vehicle. The same tail nick incision was used for repeated blood collection 15, 30, 60, 90, 120 and 240 min after CRH/vehicle administration. CRH was a generous gift from J. E. Rivier (Salk Institute; La Jolla, CA, USA).

Data analysis

Plasma ACTH and corticosterone concentrations are shown in raw form as well as AUC to facilitate comparisons of hormone levels among the various treatment groups. Both normalized AUC (response above basal hormone concentrations) and total AUC (response including basal hormone concentrations) are shown to account for marked differences in basal hormone concentrations among the various treatment groups. Normalized AUC values are an estimate of ACTH and corticosterone release relative to basal levels, whereas total AUC values are an estimate of total ACTH and corticosterone release, including basal concentrations of these hormones. AUC values were calculated using the trapezoidal rule (45). For the Western blotting studies, the autoradiographs were scanned and bands were semiquantified by densitometry.

Statistical analysis

All data are presented as mean ± SEM. For AUC values, a square root transformation was utilized to achieve homogeneity of variances. One or two-way repeated measures ANOVA and post-hoc pairwise comparisons using

the Tukey HSD test of significance ($P<0.05$) were carried out using Statistica (v. 5.0; Statsoft, Tulsa, OK, USA).

Results

Changes in basal plasma ACTH and corticosterone concentrations in rats chronically treated with morphine (Fig. 2)

Chronic morphine treatment significantly altered basal plasma ACTH and corticosterone concentrations across the 24-day observation period (Fig. 2A,B; both $P<0.0001$). Three days after initiation of chronic morphine treatment, basal plasma concentrations of ACTH and corticosterone had increased approximately four- and three-fold, respectively (ACTH: 9.6 ± 3.9 pg/ml on day 1 versus 44.9 ± 9.2 pg/ml on day 3; corticosterone: 17.2 ± 3.5 ng/ml on day 1 versus 55.5 ± 15.5 ng/ml on day 3). Basal concentrations of ACTH and corticosterone remained elevated throughout the 16 days of morphine treatment, and peaked on day 18, 36 h after the final morphine injection. Plasma ACTH and corticosterone concentrations returned to normal values within 2 days and 3 days after the last morphine injection, respectively.

Importantly, daily blood collection did not alter basal ACTH and corticosterone levels in morphine-treated rats, since hormone levels on day 17 in morphine-treated rats exposed to daily blood collection (Fig. 2) are similar to those observed in morphine-treated rats naïve to this procedure (Fig. 5B,E and Fig. 6B,E).

Total ACTH and corticosterone release in vehicle- and morphine-treated rats in the absence of restraint stress (Fig. 3)

Total ACTH and corticosterone AUC in the absence of restraint stress were significantly different among non-dependent, morphine-dependent, 12-h withdrawal and 8-day withdrawal groups (Fig. 3A,B; both $P<0.0001$). In morphine-dependent rats, during the 4-h after morphine (100 mg/kg) administration, total ACTH and corticosterone release were markedly elevated compared to nondependent rats that had received an injection of saline. Similarly, 12-h withdrawal rats displayed increased total ACTH and corticosterone release compared to nondependent rats. Furthermore, total ACTH and corticosterone release were not significantly different between morphine-dependent rats that had received an injection of morphine (morphine-dependent) and those that had received an injection of saline (12-h withdrawal). Eight days after their last morphine injection, total ACTH and corticosterone release in morphine-treated rats were similar to those observed in nondependent rats.

Effect of chronic morphine treatment on GR protein levels in the rat brain (Fig. 4)

The monoclonal antibody used in the present study recognized a prominent band at approximately 94 kDa in all brain preparations, which is consistent with the estimated molecular mass for rat GR (46). The intensity of the GR band was markedly different among the nondependent, morphine-dependent, 12-h withdrawal and 8-day withdrawal groups (Fig. 4A). In morphine-dependent rats that had received their last morphine injection 4-h earlier, quantitative analysis demonstrated a significant decrease (approximately 70%) of immunoreactive GR compared to nondependent rats that had received a saline injection 4-h earlier (Fig. 4B; $P<0.01$). Brain GR levels were not significantly different between 12-h withdrawal, 8-day withdrawal and non-dependent rats. Aliquots of brain extracts were immunoblotted for α-tubulin to show that equivalent amounts of extract were immunoadsorbed for GR (Fig. 4C).

Effect of dexamethasone on ACTH and corticosterone responses to restraint in vehicle- and morphine-treated rats (Fig. 5)

Pretreatment with dexamethasone significantly and dose-dependently reduced the ACTH and corticosterone responses to 4-h restraint in nondependent rats (Fig. 5A,D; both $P<0.001$).

Twelve hours after their last morphine injection, basal ACTH and corticosterone concentrations in morphine-treated rats were significantly higher than in vehicle-treated rats (Fig. 5B,E; both $P<0.0001$). The ACTH response to 4-h restraint in rats undergoing 12-h withdrawal was similar to the response observed in nondependent rats. However, the corticosterone response to restraint was significantly potentiated and prolonged in 12-h withdrawal rats compared to nondependent rats ($P<0.0001$). In 12-h withdrawal rats, restraint-induced increases in ACTH and corticosterone secretion were not significantly affected by dexamethasone (0.01–1.0 mg/kg) treatment. Furthermore, the ACTH and corticosterone responses to restraint in 12-h withdrawal rats treated with dexamethasone were significantly elevated compared to similarly treated nondependent rats (both $P<0.0001$).

Eight days after their last morphine injection, basal plasma concentrations of ACTH and corticosterone in morphine-treated rats were similar to those observed in nondependent rats (Fig. 5C,F). Despite recovery of normal basal pituitary-adrenal activity, these rats displayed dramatically reduced ACTH ($P<0.05$), but normal corticosterone responses to 4-h restraint stress compared to nondependent rats. Pretreatment with dexamethasone significantly reduced the ACTH ($P<0.05$) and corticosterone ($P<0.01$) responses to restraint in 8-day withdrawal rats. Furthermore, the ACTH response to restraint in 8-day withdrawal rats treated with dexamethasone was significantly reduced compared to similarly treated nondependent rats ($P<0.0001$), whereas the corticosterone response to restraint was similar between these two treatment groups.

Effect of dexamethasone pretreatment on cumulative release (AUC) of plasma ACTH and corticosterone in response to 4-hr restraint in vehicle- and morphine-treated rats (Table 1)

Because basal ACTH and corticosterone varied markedly among nondependent, 12-h withdrawal, and 8-day

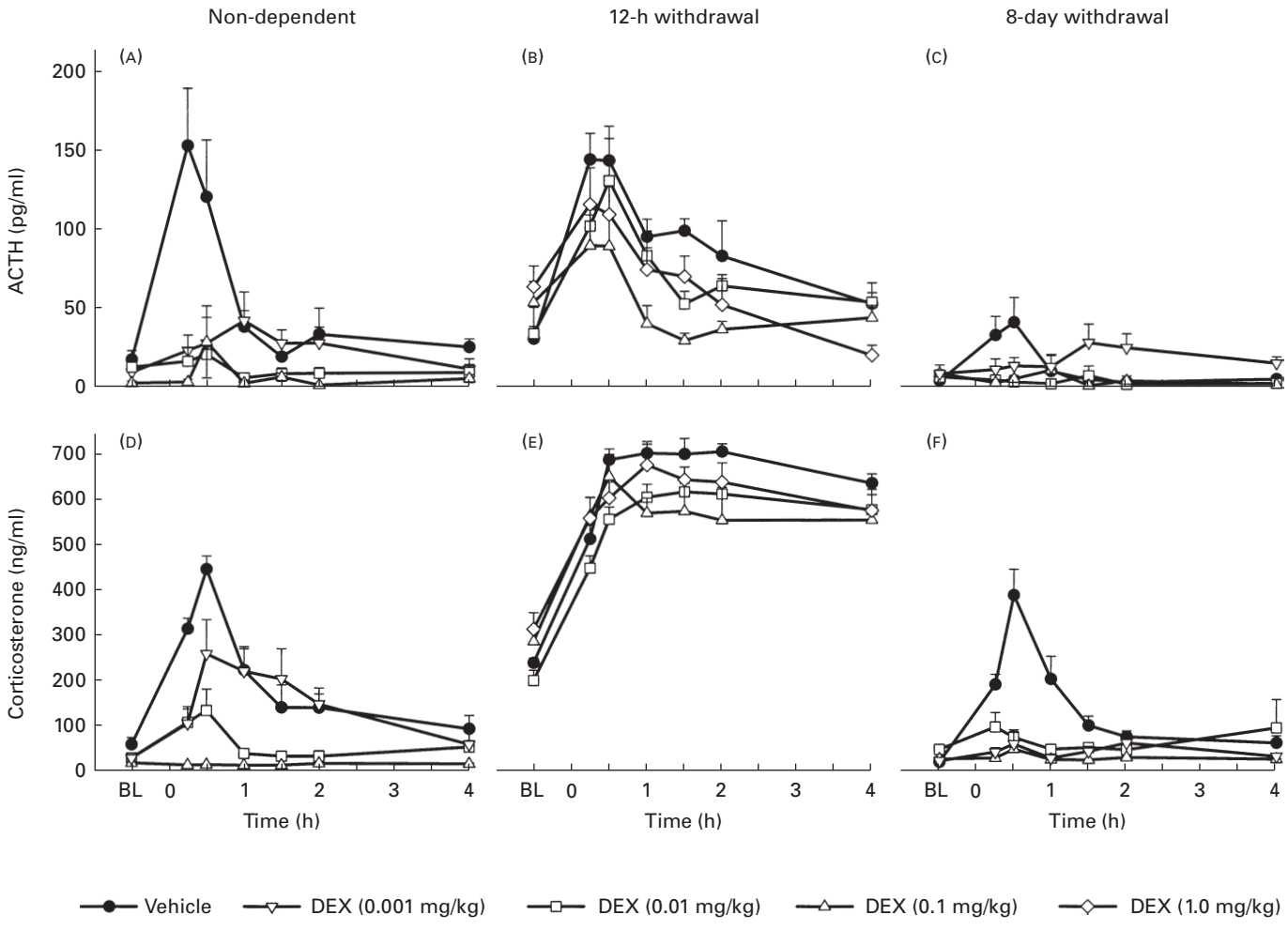

FIG. 5. Effect of dexamethasone on adrenocorticotropic hormone (ACTH) and corticosterone responses to restraint in vehicle- and morphine-treated rats. Blood was collected before dexamethasone (0, 0.001, 0.01, 0.1, 1.0 mg/kg, s.c.) administration to determine basal (BL) hormone concentrations. Restraint stress was initiated 2 h after dexamethasone or vehicle injection. (A,B,C) Showing ACTH responses to 4-h restraint in nondependent, 12-h withdrawal and 8-day withdrawal rats, respectively. (D,E,F) Showing plasma corticosterone responses to 4-h restraint in the same treatment groups. Data are means and SEM (n = 5–6).

withdrawal rats, the data were reanalysed and expressed as normalized and total AUC to facilitate comparisons among the three treatment groups (Table 1). The effect of dexamethasone pretreatment on normalized and total ACTH and corticosterone concentrations varied markedly among the three treatment groups (all $P < 0.0001$). Post-hoc analysis revealed that in nondependent rats, pretreatment with dexamethasone (0.01–0.1 mg/kg) significantly reduced normalized and total ACTH and corticosterone release in response to 4-h restraint. In 12-h withdrawal rats, normalized and total ACTH release in response to restraint were similar to that observed in nondependent rats. However, both normalized and total corticosterone release in response to restraint were markedly elevated in 12-h withdrawal rats compared to nondependent rats. Furthermore, higher doses of dexamethasone (0.1–1.0 mg/kg) were necessary to reduce normalized ACTH release in response to restraint in 12-h withdrawal rats. Additionally, dexamethasone up to a dose of 1 mg/kg did not affect total ACTH, normalized corticosterone or total corticosterone release in response to restraint in rats undergoing 12-h morphine withdrawal. In 8-day withdrawal rats, total ACTH release in response to 4-h restraint was markedly reduced compared to nondependent rats. Moreover, pretreatment with dexamethasone did not affect normalized and total ACTH release in rats undergoing 8-day morphine withdrawal. However, in 8-day withdrawal rats, lower doses of dexamethasone (0.001–0.1 mg/kg) significantly reduced normalized and total corticosterone release in response to restraint.

Effect of peripheral CRH administration on ACTH and corticosterone concentrations in vehicle- and morphine-treated rats (Fig. 6)

In nondependent rats, i.p. administration of CRH significantly and dose-dependently increased plasma ACTH

TABLE 1. Effect of Dexamethasone Pretreatment on Cumulative Release (Area Under Curve, AUC) of Plasma Corticosterone and Adrenocorticotropic Hormone (ACTH) in Response to 4-h Restraint in Vehicle- and Morphine-Treated Rats.

	ACTH (pg/min/dl)		Corticosterone (ng/min/ml)	
Treatment	Normalized AUC	Total AUC	Normalized AUC	Total AUC
Non-dependent				
Restraint + vehicle	25446 ± 8748	35036 ± 8746	91850 ± 12311	122080 ± 15956
+ Dex 0.001	9621 ± 3816[a]	14595 ± 3438	73551 ± 17909	88135 ± 17729
+ Dex 0.01	763 ± 1508[a]	6223 ± 2284[a]	19812 ± 12015[a]	33611 ± 10940[a]
+ Dex 0.1	864 ± 859[a]	2021 ± 369[a]	−1979 ± 1752[a]	5763 ± 410[a]
12-h withdrawal				
Restraint + vehicle	41206 ± 2607	55182 ± 4407	213709 ± 10619[b]	347178 ± 8664[b]
+ Dex 0.01	25653 ± 9549[b]	44225 ± 2414[b]	194730 ± 14346[b]	298358 ± 12985[b]
+ Dex 0.1	1049 ± 3317[a]	30670 ± 4907[b]	150842 ± 18459[b]	307609 ± 21512[b]
+ Dex 1.0	6721 ± 7277[a]	41861 ± 6438[c]	151623 ± 16888[c]	323175 ± 15970[c]
8-day withdrawal				
Restraint + vehicle	6730 ± 1793	8451 ± 2711[b]	82609 ± 10877	91189 ± 11242
+ Dex 0.001	4533 ± 2613	8792 ± 3457	11554 ± 7216[a,b]	21285 ± 5867[a,b]
+ Dex 0.01	−1552 ± 2291	1557 ± 654	9662 ± 6695[a]	32893 ± 7491[a]
+ Dex 0.1	−3127 ± 2833	1248 ± 618	2256 ± 2131[a]	14284 ± 2409[a]

[a]$P < 0.05$ compared with vehicle pretreatment within the same treatment group. [b]$P < 0.05$ compared to the same dexamethasone pretreatment in the nondependent group. [c]$P < 0.05$ compared to dexamethasone (0.1 mg/kg) pretreatment in the nondependent group.

and corticosterone concentrations compared to vehicle control (Fig. 6A,C; both $P < 0.0001$).

In 12-h withdrawal rats, CRH administration also significantly increased plasma ACTH ($P < 0.001$) and corticosterone ($P < 0.05$) concentrations (Fig. 6B,E). However, in these rats, CRH only at the dose of 10 μg/kg significantly increased both ACTH ($P < 0.001$) and corticosterone ($P < 0.05$) concentrations. Furthermore, the ACTH responses to CRH were significantly reduced in 12-h withdrawal rats compared to nondependent rats (treatment × time, $P < 0.01$). In contrast, the corticosterone responses to CRH were significantly increased in 12-h withdrawal rats (treatment × time, $P < 0.01$).

In 8-day withdrawal rats, CRH administration also significantly increased plasma ACTH ($P < 0.0001$) and corticosterone ($P < 0.001$) concentrations (Fig. 6B,D). In these rats, peripheral CRH administration at doses of 1 μg/kg and 10 μg/kg dose-dependently increased plasma ACTH ($P < 0.05$ and $P < 0.001$, respectively) and corticosterone ($P < 0.05$ and $P < 0.001$, respectively) concentrations. The ACTH and corticosterone responses to CRH were similar between 8-day withdrawal and nondependent rats.

Cumulative release (AUC) of plasma ACTH and corticosterone in response to peripheral CRH administration in vehicle- and morphine-treated rats (Table 2)

The effect of peripheral CRH administration on normalized and total ACTH and corticosterone release was significantly different among the three treatment groups (all $P < 0.0001$). Post-hoc analysis revealed that in nondependent rats, peripheral CRH (1–10 μg/kg) administration significantly elevated both normalized and total ACTH as well as corticosterone release. However, in 12-h withdrawal rats, only the higher dose of CRH (10 μg/kg) significantly increased normalized or total ACTH release. Moreover, in 12-h withdrawal rats, neither 1 μg/kg nor 10 μg/kg CRH affected total corticosterone release, whereas both doses of CRH significantly elevated normalized corticosterone release. Rats undergoing 8-day morphine withdrawal displayed similar increases in ACTH and corticosterone release in response to peripheral CRH administration compared to nondependent rats.

Discussion

The results of the present study support and extend our previous observations (20) that chronic morphine treatment has paradoxical effects on the pituitary-adrenal axis, resulting in facilitated responses to restraint in rats undergoing acute (12-h) morphine withdrawal and attenuated responses in rats undergoing chronic (8-day) morphine withdrawal. In the present study, we also demonstrated that chronic morphine treatment resulted in persistent elevation of basal corticosterone secretion, whereas ACTH secretion was only slightly increased in morphine-dependent rats. Chronic corticosterone hypersecretion in morphine-dependent rats was associated with a marked reduction of whole brain GR protein levels, which recovered rapidly after the final morphine injection. Furthermore, the normal ACTH and exaggerated corticosterone responses to restraint in rats undergoing acute withdrawal were associated with reduced sensitivity of negative-feedback systems to glucocorticoids, reduced pituitary responsitivity to CRH, and enhanced adrenal sensitivity to ACTH. On the other hand, the reduced ACTH responses to restraint in rats undergoing chronic withdrawal were associated with enhanced sensitivity of negative-feedback systems to circulating glucocorticoids, but normal pituitary responsivity to CRH.

In the present study, we demonstrated that chronic morphine treatment resulted in persistent elevation of basal

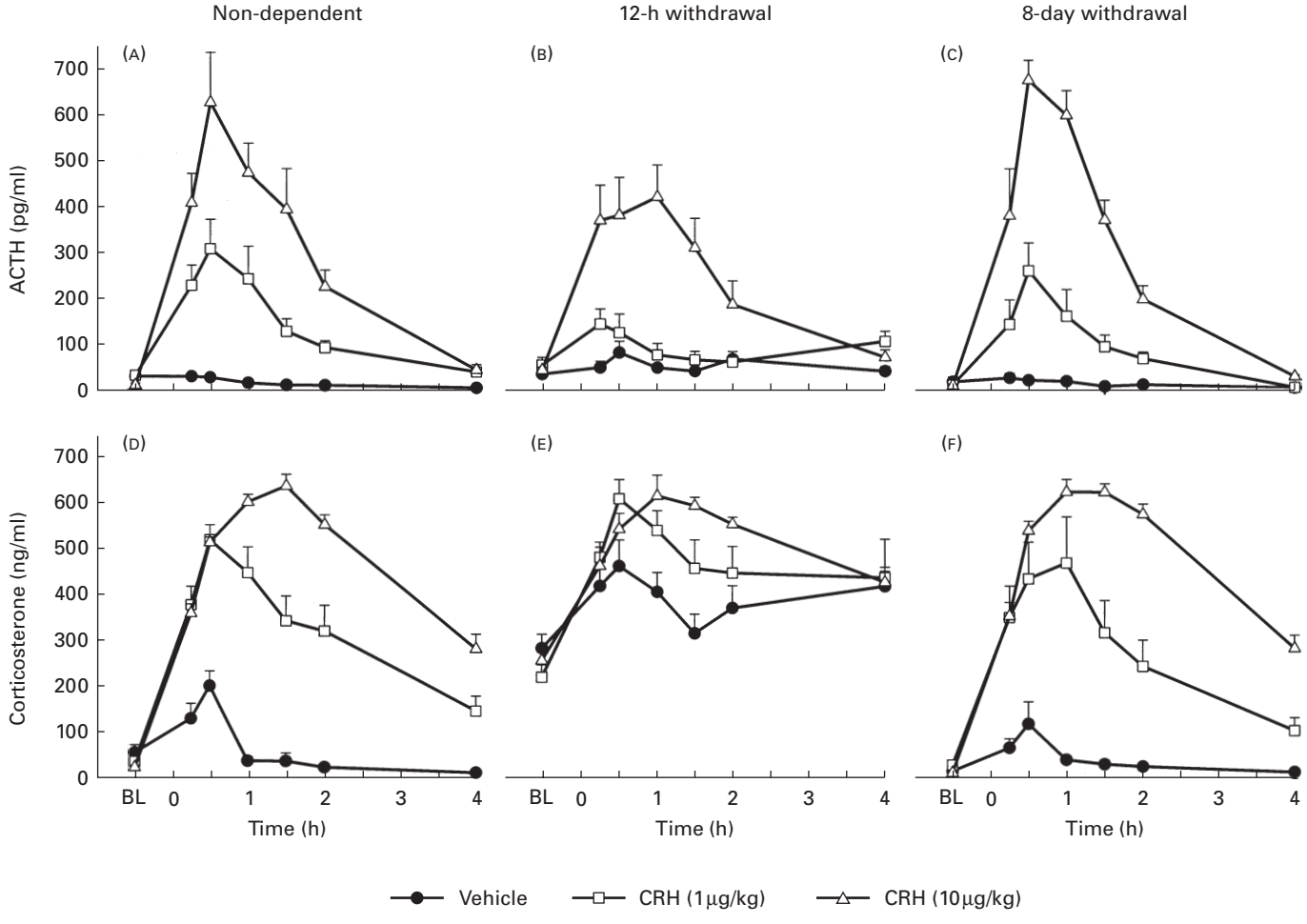

FIG. 6. Adrenocorticotropic hormone (ACTH) and corticosterone responses to peripheral corticotropin releasing hormone (CRH) administration in vehicle- and morphine-treated rats. Blood was collected before CRH (0, 1, 10 μg/kg, i.p.) administration to determine basal (BL) hormone concentrations. (A,B,C) Showing plasma ACTH responses to peripheral CRH administration in nondependent, 12-h withdrawal and 8-day withdrawal rats, respectively. (D,E,F) Showing plasma corticosterone responses to exogenous CRH administration in the same treatment groups. Data are means and SEM (n = 5–6).

TABLE 2. Cumulative Release (Area Under Curve, AUC) of Plasma Corticosterone and Adrenocorticotropic Hormone (ACTH) in Response to Peripheral Corticotropin Releasing Hormone (CRH) Administration in Vehicle- and Morphine-Treated Rats.

	ACTH (pg/min/dl)		Corticosterone (ng/min/ml)	
Treatment	Normalized AUC	Total AUC	Normalized AUC	Total AUC
Non-dependent				
CRH vehicle	−7373 ± 3746	9736 ± 1516	11211 ± 5878	42017 ± 3694
CRH (1 μg/kg)	57057 ± 1578[a]	96137 ± 20051[a]	129427 ± 15834[a]	193707 ± 20871[a]
CRH (10 μg/kg)	126944 ± 20865[a]	169023 ± 26276[a]	178524 ± 4761[a]	258751 ± 6707[a]
12-h withdrawal				
CRH vehicle	6977 ± 1527	26878 ± 4766	38721 ± 11533	217826 ± 7869[b]
CRH (1 μg/kg)	18742 ± 4095	52477 ± 9097	98036 ± 13750[a]	256848 ± 20042
CRH (10 μg/kg)	105209 ± 17386[a]	157801 ± 25316[a]	105027 ± 8340[a]	287457 ± 13696
8-day withdrawal				
CRH vehicle	1535 ± 3683	9366 ± 2711	13376 ± 4923	25334 ± 5422
CRH (1 μg/kg)	47860 ± 11417[a]	67308 ± 14560[a]	119364 ± 2578[a]	173201 ± 34686[a]
CRH (10 μg/kg)	155855 ± 10947[a]	207285 ± 14325[a]	185403 ± 6642[a]	263247 ± 9551[a]

[a]$P < 0.05$ compared to vehicle treatment within the same treatment group. [b]$P < 0.05$ compared to the same CRH treatment in the nondependent group.

plasma corticosterone concentrations to approximately 10 times the levels observed in vehicle-treated rats. However, other studies have shown that basal corticosterone concentrations are normal in morphine-dependent rats (8, 9). This discrepancy is likely due to procedural differences in the induction of morphine dependence. Since basal corticosterone concentrations are also increased in chronically stressed rats (21, 32, 36), our results suggest that chronic morphine dependence under our conditions is a chronic stressor. Despite the marked increase of basal corticosterone secretion, basal ACTH concentration was only slightly increased in morphine-treated rats. This finding is consistent with previous observations that basal ACTH concentration is generally normal in chronically stressed rats (21, 32). Nonetheless, we have previously shown that chronic morphine treatment results in adrenal hypertrophy and thymus involution, which are both classic signs of chronic ACTH hypersecretion and chronic stress exposure (20, 21).

HPA axis activity in rats is very tightly regulated by the negative-feedback effects of circulating corticosterone (47). Corticosterone regulates HPA axis activity by interacting with two steroid receptors: mineralocorticoid (MR) and glucocorticoid (GR) receptors. Because of the higher affinity of MR for corticosterone, it has been suggested that MR modulate HPA activity under trough basal conditions, whereas GR play a primary role in the regulation of the diurnal peak of the circadian rhythm as well as the HPA responses to stress (16, 21, 48). In the present study, we demonstrated a marked downregulation of whole brain GR protein levels in morphine-treated rats. Whole brain GR levels were dramatically reduced 4-h after the final morphine injection, but had recovered within 16-h after termination of chronic morphine treatment. Using the ligand binding technique, Budziszewska *et al.* (40) similarly reported a reduction of hippocampal GR, but not MR, density in morphine-dependent rats that were adrenalectomized. The technique of receptor extraction with DNAase digestion used in present study has the advantage of detecting all forms of GR, in both nuclear and cytosolic compartments of cells in nonadrenalectomized rats (43, 44). However, using this technique, we determined GR protein levels within the whole brain rather than specific anatomical loci. Examination of GR expression within the whole brain may have concealed changes in GR within specific brain regions 16-h after the final morphine injection. Indeed, previous studies have shown that although chronic elevation of corticosterone concentration by either repeated stress or exogenous corticosterone administration reduces GR expression in the brain, this effect tends to be restricted to the hippocampus (30, 49). Nonetheless, the marked downregulation of whole brain GR in morphine-dependent rats closely resembles GR downregulation previously reported in chronically stressed rats and is likely an adaptive response to prolonged corticosterone hypersecretion.

Despite the negative-feedback effects of elevated basal corticosterone concentrations, morphine-treated rats displayed potentiated and prolonged corticosterone responses to restraint stress while undergoing acute morphine withdrawal. This response is similar to the facilitated HPA responses to an acute, novel stressor in chronically stressed rats (21–24). Furthermore, the hormonal responses to restraint in acutely withdrawn rats were insensitive to the negative-feedback effects of the synthetic glucocorticoid dexamethasone. Dexamethasone is commonly used to examine the integrity of negative-feedback systems (50–53). However, the primary site of action for dexamethasone is the pituitary (54). This could explain the discrepancy that despite reduced sensitivity to the negative-feedback effects of dexamethasone, rats undergoing 12-h withdrawal displayed normal whole brain GR protein expression. Alternatively, as discussed previously, it is possible that GR expression is reduced within some brain regions in acutely withdrawn rats, but that this effect is diluted by examination of the whole brain. With respect to dexamethasone-sensitivity, rats chronically treated with morphine again closely resemble chronically stressed rats, since rats chronically exposed to stress also display reduced sensitivity to negative-feedback effects of glucocorticoids (21, 22, 29, 31, 32). Thus, the facilitated corticosterone responses to restraint stress in acutely withdrawn rats are at least in part mediated by impaired negative-feedback systems.

Although acutely withdrawn rats displayed an exaggerated corticosterone response to restraint stress, the ACTH response to restraint was normal in these animals. This discrepancy between the ACTH and corticosterone responses to restraint in acutely withdrawn rats suggests that the adrenals of these rats are more sensitive to ACTH. Indeed, we have previously reported that acutely withdrawn rats display markedly increased adrenal gland weights (20). Moreover, our observation that the corticosterone responses to peripheral CRH administration were also exaggerated in acutely withdrawn rats, despite reduced ACTH responses to this hormone, also suggests enhanced adrenal sensitivity to ACTH in these rats. In this respect, rats chronically treated with morphine also resemble chronically stressed rats, since others have shown that the adrenals of chronically stressed rats are also enlarged and hyperresponsive to ACTH (21, 29).

The reduced ACTH responses to peripheral CRH administration in acutely withdrawn rats also suggest reduced pituitary sensitivity to CRH. Previously, it has been shown that chronic stress exposure and chronic CRH administration result in downregulation and desensitization of CRH receptors in the rat pituitary and brain (27, 28, 55). Recently, Iredale *et al.* (56) also showed that CRH-type I receptor expression is reduced in morphine-dependent rats undergoing naltrexone-precipitated opioid withdrawal. Thus, reduced pituitary responses to peripheral CRH administration in acutely withdrawn rats may be related to CRH receptor downregulation and/or desensitization, induced by chronic activation of this receptor system.

Furthermore, we cannot exclude the possibility that hypothalamic CRH and/or AVP secretion in response to restraint stress is increased in acutely withdrawn rats. Indeed, previous studies have demonstrated enhanced activation of CRH neurones, in particular those coexpressing AVP, in the PVN of chronically stressed rats (25). There is also evidence that the expression of CRH and AVP in the PVN and/or their release into the hypothalamic-pituitary portal circulation may be increased in morphine-dependent rats undergoing withdrawal (57–59). Therefore, it is possible that hypothalamic

CRH and/or AVP function may be enhanced in acutely withdrawn rats, but that this effect is counteracted by reduced pituitary sensitivity to CRH, thereby resulting in an apparently normal ACTH response to restraint in these animals. Further studies are necessary to directly examine the neurocircuitry mediating the facilitated stress response observed in acutely withdrawn rats.

In contrast to the facilitated corticosterone responses to restraint in acutely withdrawn rats, rats tested 8 days after morphine withdrawal displayed dramatically attenuated ACTH responses to restraint stress. Moreover, restraint-induced increases in corticosterone were more sensitive to the negative-feedback effects of dexamethasone in these chronically withdrawn rats. Together, these data suggest that the reduced stress responses in chronically withdrawn rats involve enhanced sensitivity of negative-feedback systems to circulating glucocorticoids. Enhanced efficiency of negative-feedback processes in chronically withdrawn rats may be related to increased GR expression. Indeed, previous studies have shown that attenuated HPA responsivity to stress in handled rats is related to enhanced negative-feedback sensitivity and increased hippocampal GR expression (34). In the present study, we did not find evidence for changes in GR protein expression in the whole brain of chronically withdrawn rats. However, examination of GR expression within specific brain regions in these animals may reveal new information.

Although chronically withdrawn rats displayed reduced ACTH responses to restraint stress, the ACTH and corticosterone responses to peripheral CRH administration were normal in these animals. These data suggest that the reduced pituitary responses to restraint stress in chronically withdrawn rats probably do not involve changes in pituitary CRH receptor binding sites, or pituitary ACTH stores, but instead result from decreased hypothalamic function of CRH and/or AVP in response to restraint. This effect may be in part related to increased sensitivity of the negative-feedback systems to circulating glucocorticoids. Alternatively, chronic exposure to the stress of morphine dependence and the profound stress associated with withdrawal may have increased the threshold for activation of the stress axis, such that greater intensities of psychological and/or physiological stressors are necessary to activate the HPA axis in these animals. To our knowledge, the activity of CRH and/or AVP neurones during prolonged opioid withdrawal has not been investigated to date. It is possible that adaptive changes in the CRH and/or AVP systems mediate some of the hypo-responsivity observed in rats undergoing extended morphine withdrawal.

The studies presented here have focused on alterations in HPA activity that potentially mediate the facilitated and attenuated stress responsivity in rats undergoing acute and chronic morphine withdrawal, respectively. However, altered pituitary-adrenal responsivity to stress in these animals may be indirectly mediated through adaptive changes in other neurobiological systems. Indeed, previous studies have shown that chronic exposure to morphine affects noradrenergic innervation to the PVN (60–62). It remains to be determined if modifications in noradrenergic neurones potentially modulate CRH and/or AVP function in morphine-treated rats, thereby influencing pituitary-adrenal secretions under basal and stress conditions.

Interestingly, HPA function in acutely and chronically withdrawn rats appears to have common features with the HPA dysregulation observed in humans suffering from depression and post-traumatic stress disorder (PTSD), respectively. Dysregulation of HPA activity in depressed patients is manifested by cortisol hypersecretion, reduced sensitivity to the negative-feedback effects of dexamethasone and blunted ACTH responses to CRH administration (63). In contrast, PTSD is associated with reduced cortisol secretion and enhanced sensitivity to the negative-feedback effects of circulating glucocorticoids (64, 65). Furthermore, epidemiological and clinical studies show a high comorbidity between drug abuse, depression and PTSD (4, 5, 66, 67). However, it remains to be determined if drug abuse results in the neurobiological abnormalities underlying depression and PTSD (66, 67). The finding that chronic morphine treatment induces withdrawal time-dependent hyper- and hypo-responsivity of the HPA axis offers an intriguing animal model to study at least some aspects of drug abuse, depression and PTSD.

In conclusion, we have shown that the normal ACTH and exaggerated corticosterone responses to restraint in rats undergoing acute morphine withdrawal involve reduced sensitivity of negative-feedback systems to circulating glucocorticoids, reduced responsivity of the pituitary to CRH and enhanced adrenal sensitivity to ACTH. Furthermore, the reduced ACTH and normal corticosterone responses to restraint in rats undergoing chronic morphine withdrawal involve enhanced sensitivity of negative-feedback systems and reduced CRH and/or AVP function. These experiments shed light not only on changes in HPA activity associated with chronic opioid dependence, but they may also be relevant to studies of depression and PTSD.

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