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Chronic sustained stress increases levels of anterior pituitary prolactin mRNA☆

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Abstract

Our laboratory is investigating the effects of chronic stress on physiological, endocrine and behavioral measures, in order to elucidate the neuronal substrates for the pathophysiological consequences of stress in humans. In these studies, we have employed a rodent model of sustained stress in which rats are exposed to around-the-clock intermittent footshock that can be avoided or escaped by rats in the controllable stress group, but not by rats in the uncontrollable stress group. Each rat in the uncontrollable stress group is paired (yoked) to a rat in the controllable stress group such that the controllable stress group rat avoids or escapes shock for both rats. A third group of rats receives no shock (controls). We have previously reported that in male rats, plasma prolactin levels were elevated after 3 days of stress in both stress groups. In the present experiments we determined whether the increases in plasma prolactin were correlated with increases in anterior pituitary prolactin mRNA. In addition, we measured hormones and mRNA at three time points and we examined these responses in female as well as male rats. Adult male and female Sprague–Dawley rats were exposed to chronic stress for 1, 3 or 14 days. In unstressed control rats, levels of anterior pituitary prolactin mRNA were fivefold higher in female as compared to male rats. However, stress increased levels of anterior pituitary prolactin mRNA over baseline in both genders. After 1 day of stress, anterior pituitary prolactin mRNA levels increased in male and female rats belonging to both stress groups, with no significant difference seen between the uncontrollable vs. controllable stress groups. After 3 days of stress, anterior pituitary prolactin mRNA levels were even more elevated, and rats in the uncontrollable stress group had higher anterior pituitary prolactin mRNA levels than those in the controllable stress group. After 14 days of stress, there were no significant differences in control and stressed groups with respect to anterior pituitary prolactin mRNA. These data suggest that chronic sustained stress increases the synthesis of anterior pituitary prolactin mRNA during the first days of stress, and that levels return to prestress values sometime between 3 and 14 days of stress.

Keywords: Prolactin; Prolactin mRNA; Chronic stress; Corticosterone; ACTH

1. Introduction

In order to characterize the consequences of chronic stress on physiology and behavior, our laboratory has performed a number of studies using a rodent model of sustained stress [5,6,8,9,29–31,33,38]. In this paradigm, one group of control rats and two groups of stressed rats are generally studied in each experiment. One group of stressed rats can avoid or escape signaled intermittent around-the-clock (average frequency of one trial per 5 min) footshock by pulling a ceiling chain (controllable stress group). In the other group (uncontrollable stress or yoked stress), each rat is paired to a rat in the first group such that the rat with a ceiling chain terminates shock for both itself and the paired rat. We and others have reported that uncontrollable stress, in general, causes greater physiologic disruptions as assessed by various endpoints including plasma hormones, food intake and weight changes [29,30,57,58].

Although the paradigm is stressful, it is not debilitating. Rats avoid shock or escape during the shock sequence in

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>99% of the trials presented, continue to eat and drink, although food intake is decreased, and regain weight lost during the first days of stress as the stress period continues. The circadian rhythm of body temperature and sleep patterning are initially disrupted but return toward baseline as the stress continues. Both REM and non-REM sleep are decreased during the first 24 h of stress but only REM sleep in the controllable stress is still decreased on day 2. Acquisition is retarded and performance is initially impaired on working memory tasks but these return to baseline performances over time [8,9,29–31,38]. Estrous cycling is transiently disrupted during the first few days of stress but normal cycling resumes on the fourth or fifth day of the stress period [6,7]. Since for most of the measures we have examined there is a period of stress-induced disruption followed by a return to baseline over time, we have selected endpoints after 3 vs. 14 days of stress in most of our previous experiments to represent stress-induced alterations and adaptation, respectively.

Using this stress paradigm, we previously reported that although stress rapidly increased levels of plasma ACTH, after 24 h of stress plasma ACTH levels were equivalent to control values [33]. We have generally found that plasma corticosterone was elevated in animals stressed for 3 days in both the controllable and uncontrollable stress groups, with levels higher in the uncontrollable as compared to the controllable stress group [29]. Plasma corticosterone was not elevated after 14 days of stress. Results of chronic stress effects on plasma prolactin in our studies have been mixed. In published data from some studies using this chronic stress model we show stress-induced increases in plasma prolactin [29], but we have not found this in other studies [33]. A combined analysis of our collected data suggests that variability in results among studies is probably due to small sample sizes within individual studies and the inherent variability in plasma prolactin measures due to its pulsatile secretion.

Increased synthesis of anterior pituitary prolactin mRNA has been demonstrated following secretion of prolactin in response both to moderate hypoxaemia and to cortisol infusions in fetal sheep [44] and to nonstressful stimuli such as suckling or elevated estrogen [41,46]. The present studies were undertaken to determine whether chronic stress-increased levels of plasma prolactin were accompanied by increased levels of anterior pituitary prolactin mRNA.

2. Methods

2.1. Animals

Adult male and female Sprague–Dawley rats were purchased from Charles River and housed individually under controlled environmental and lighting (12-h light/dark cycle) conditions for at least 1 week, with food and water available ad libitum, before being used in experiments. In the first set of experiments using only male rats, body weights were 365±9.3 g (mean±S.E.M.). In the second set of experiments using both male and female rats, the females weighed 289±4.1 g and males weighed 454±11.9 g at the time they were placed in the operant boxes.

2.2. Experimental procedures

Nineteen experiments over a period of 3 years were performed. Twelve operant boxes were available for each experiment. Therefore, experiments were combined to yield a sufficient *n* for each study. In the first group of experiments, only male rats were used. Four rats each were assigned to unstressed control, controllable stress, and uncontrollable (yoked) groups. Following training (see below), the rats lived in the test chambers for 3 or 14 days. Then they were removed from the chambers and sacrificed. This experiment was replicated several times to yield a sufficient number of animals per group.

In a second series of studies, male and female rats were run in each experiment. That is, there were two unstressed control males, two unstressed control females, two controllable stress males, two controllable stress females, two uncontrollable stress males, and two uncontrollable stress females. Three stress durations of 1, 3 or 14 days were examined. At each duration, the paradigm was performed several times to yield a sufficient number of animals per treatment group.

2.3. Stress procedures

Rats were moved to the laboratory from the animal housing area and placed in standard operant cages located inside sound-attenuating chambers. Each cage was equipped with two levers, a triple cue light and sonalert (for the shock warning signals), a ceiling chain (see below), and a house light. (Cages, levers, chains, cue lights, chambers, etc. were purchased from Coulbourn Instruments.) House lights in the box were on from 0600 to 1800 h daily. The cage floor consisted of sixteen 0.6-cm-diameter stainless steel rods spaced 1.8 cm center-to-center through which footshock could be delivered. Food pellets (45 mg pellets, P.J. Noyes, Formula A) were delivered to a central food trough following a single press on either lever. Initially, peanut butter was placed on the lever to facilitate rats' learning to lever-press for food pellets. Water was freely available.

After all rats had learned to lever-press for food, the rats assigned to the controllable stress group were shaped to pull a ceiling chain to escape scrambled footshock delivered by the experimenter. Footshock was delivered from a programmable shocker (Coulbourn Instruments, model E13–10). A ceiling chain was suspended from the center of the cage and a 5-cm-diameter metal ring was attached to the end of the chain, which hung approximately 10 cm off the floor. During escape training, a strip of towel was attached to

the chain ring to make it more visible and to facilitate grabbing the chain. Rats in the uncontrollable stress (yoked) group were paired to a rat in the controllable stress group during training and thereafter such that the uncontrollable stress group rat received shocks whenever its paired partner did. After each controlling rat was trained, a procedure that generally required no more than 30 min of intermittent shock, shock delivery was subsequently controlled by a PDP11 computer programmed in SKED [53]. Shock presentation trials began with a 5-s illumination of a triple cue lamp, followed sequentially by a 5-s sonalert auditory warning tone, and then 5 s each of 0.16, 0.32, 0.65, 1.3, and finally 2.6 mA of footshock. Trials could be avoided or escaped at any point in the trial sequence by the controlling rat pulling the ceiling chain. The rats in the uncontrollable stress group had no control over shock delivery. Rats responding during the light or sound warning (first 10 s) did not receive any shock (avoided shock). Rats responding during the 25 s of shock did receive footshock as described above until the point in the sequence at which the ceiling chain was pulled (escaped from shock). Typically, rats' responses cluster at shock levels 2, 3, and 4 with only a few avoidance responses. Very few rats wait until the highest shock level to pull the ceiling chain and less than 1% of trials are not avoided or escaped. Rats respond about one level earlier during the dark as compared to the light hours. Since trial frequency was approximately, one trial per 5 min and since rats were sacrificed immediately after removal from the operant cages, the average length of time between the last shock and sacrifice was approximately 3 min (2.5 average time since last shock trial and 30 s from cage to sacrifice).

Shock trials were initially presented at average intertrial intervals of 1 min. Following 35 successful escapes, the average intertrial interval was increased to 5 min. A safeguard contingency stopped shock delivery if 20 consecutive escape failures from shock trials occurred; however, this condition was never met. Lever-press and chain pull data were collected and stored by the PDP11 for daily analysis.

2.4. Tissue and plasma collection

After 1, 3 or 14 days of stress, rats were decapitated immediately following removal from the operant cages during a 2-h period beginning 4 h following "lights on" in the morning to minimize circadian hormonal variation. This time period is within the normal trough in the diurnal rhythm when control levels of the stress hormones, ACTH, corticosterone and prolactin, are expected to be low [2,16,35,47]. In addition, morning sampling allowed us to avoid the increased plasma stress hormones in female rats due to the elevated estrogen levels during the afternoon proestrus surge [16,17,20,47]. Trunk blood was collected in a beaker containing heparin and aprotinin, a peptidase inhibitor, and then centrifuged at 4°C for 20 min at 3000 rpm. Aliquots of plasma were stored at -40°C until assayed for corticosterone, ACTH, prolactin, and estradiol. Anterior pituitaries were dissected out quickly and frozen on dry ice. Tissue samples were stored at -70° C until assayed for anterior pituitary prolactin mRNA.

2.5. Northern blot analysis

Total RNA from anterior pituitary was extracted using the modifications of a method described by Cathala et al. [19] and the Northern blots were generated as described earlier [22]. The RNA blots were incubated for 2 h at 42°C in prehybridization buffer [50% (v/v) formamide; $2 \times$ SSC ($1 \times$ SSC contains 0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0), 40 mM sodium phosphate buffer, pH 6.5; 200 μg/ml of tRNA (Baker's yeast) (Boehringer-Mannheim, Indianapolis, IN); 50 μg/ml of herring sperm DNA (Boehringer-Mannheim), $0.8 \times$ Denhardts solution (Sigma Chemicals, St. Louis, MO), 10 mM EDTA and 0.1% SDS]. Hybridization was carried out overnight at 42°C in prehybridization buffer containing 10% (w/v) dextran sulphate and 1×10^6 cpm/ml of ^{32}P-labeled 900-base-pair *Pst*I restriction fragment of rat prolactin cDNA [26] as described earlier [21]. After hybridization the Northern blots were washed twice with $2 \times$ SSC for 10 min at room temperature and twice with $0.1 \times$ SSC/0.1% SDS for 30 min at 42°C. The blots were then exposed for 24 h to Kodak XAR-2 X-ray films in cassettes with intensifying screen. The autoradiograms were quantitated by densitometric scanning (E-C Apparatus, St. Petersburg, FL). The relative amount of prolactin mRNA was calculated as the ratio of mRNA peak area obtained from the densitometric measurements of the Northern blots over the peak area of 28 S RNA obtained from the densitometric measurements of negative photographs of ethidium-bromide-stained gels.

2.6. Hormone assays

ACTH was measured using a radioimmunoassay kit (INCSTAR, Stillwater, MN). Human $ACTH_{(1-39)}$ was used as a standard. The antibody in the kit was generated against $ACTH_{(1-24)}$, which is identical in humans and rats. The assay was performed in 12×75 mm polypropylene tubes using an overnight incubation at 4°C. Assay sensitivity was approximately 10 pg/ml. The intraassay variation was 2.5% at 380 pg/ml and the interassay variation was $<5\%$. Materials for the prolactin assay were provided by the National Institutes of Health through the National Hormone and Pituitary program (NHPP). Intraassay variation was $<8\%$ and interassay variation $<12\%$. Corticosterone and estradiol were measured using an antibody-coated tube radioimmunoassay kits (COAT-A-COUNT, Diagnostic Products, Los Angeles, CA). The procedure described by the manufacturer was modified slightly to include an overnight incubation at 4°C. For corticosterone, assay sensitivity was 0.2 μg/dl and coeffi-

cients of variation were <5.7% for intraassay and <10.8% for interassay. In these assays corticosterone crossreactivity with progesterone was <0.9%. Assay sensitivity for estradiol was 5 pg/ml and the intraassay and interassay variations were 10%.

2.7. Data analysis

Data were analyzed using two-way analysis of variance to evaluate gender differences and any interaction effects, followed by one-way analysis of variance, where appropriate. Planned experimental group comparisons were made using a priori orthogonal contrasts [54] with coefficients based on our hypotheses that (1) plasma corticosterone and prolactin and anterior pituitary prolactin mRNA values would be greater in 1- and 3-day stressed groups than their control groups and (2) that the means of those measures in the yoked (uncontrollable stress) groups would be greater than the means for the controllable stress groups. Unplanned comparisons of means of stressed groups with the control were made using ANOVA and a posteriori Dunnett's *t* tests. Analyses were performed using SPSS 8.0 statistical software system for computers. Results were considered to be significant at $P<.05$.

3. Results

3.1. First series of experiments: effects of 3 or 14 days of chronic stress on plasma hormones and anterior pituitary prolactin mRNA in male rats

As shown in Table 1, three days of stress did not affect levels of plasma ACTH. Three days of stress increased levels of plasma corticosterone and prolactin (a priori contrast, $P<.02$ and $P<.01$, respectively, Table 1), but there were no significant differences between the two stress groups, controllable and uncontrollable. Anterior pituitary prolactin mRNA (Fig. 1) increased dramatically with stress (a priori contrast, $P<.001$) and the increase was greater in the uncontrollable stress group (a priori contrast, $P<.01$). After 14 days of stress, plasma prolactin was elevated in both stressed groups as compared to controls (Dunnett's *t* test, $P<.004$ for controllable stress vs. control and $P<.03$ for uncontrollable stress vs. control), but anterior pituitary prolactin mRNA was similar in control and stressed animals (Fig. 1).

Table 1
Effects of 3- vs. 14-day stress exposure on plasma hormones in male rats

	Plasma ACTH (pg/ml)	Plasma prolactin (ng/ml)	Plasma corticosterone (μg/dl)
3-Day stress			
Control (no stress)	48.9±4.5	1.4±0.3	0.8±0.3
Controllable stress	45.7±3.0	2.7±0.3[a]	3.2±0.7[b]
Uncontrollable stress	47.7±3.1	2.0±0.3[a]	2.4±1.1[b]
14-Day stress			
Control (no stress)	44.1±4.7	1.5±0.2	0.6±0.1
Controllable stress	56.2±5.3	4.5±0.7[c]	1.0±0.4
Uncontrollable stress	56.7±6.5	3.7±0.9[d]	1.2±0.4

Values represent the mean of 10–12 rats/group±S.E.M.

[a] Mean of stressed rats is significantly greater than control, a priori contrast, $P<.01$.

[b] Mean of stressed rats is significantly greater than control, a priori contrast, $P<.02$.

[c] Mean is significantly greater than control, Dunnett's *t* test, $P<.004$.

[d] Mean is significantly greater than control, Dunnett's *t* test, $P<.03$.

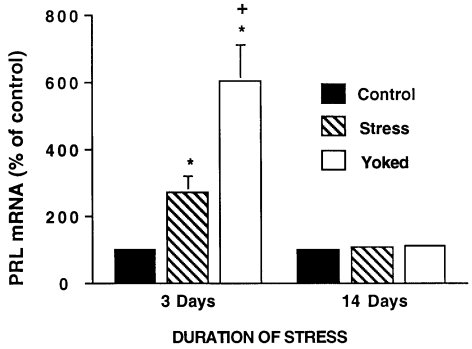

Fig. 1. Levels of anterior pituitary prolactin mRNA from first set of studies (male rats), expressed as percent of control (of same duration experiment). Values represent the mean of 10–12 rats per group. The absolute values for prolactin mRNA (expressed as ratio of PRL signal/28 S RNA) in controls were 0.28±0.01 and 0.44±0.09, respectively for 3 and 14 days duration of stress. Error bars are for S.E.M. * Significantly greater than control, a priori contrast, $P<.001$. + Significantly greater than controllable stress group, a priori contrast, $P<.01$.

3.2. Second series of experiments: effects of gender on anterior pituitary prolactin mRNA and plasma hormones

The data from control rats from the 1-, 3- and 14-day experiments are presented in Table 2 to show gender differences in the absence of stress. Morning (4–5 h after lights on) plasma ACTH and plasma prolactin concentrations were not significantly different in control male and female rats. Corticosterone levels were significantly higher in female controls (9.7±1.7 vs. 2.9±0.6 μg/ml, ANOVA,

Table 2
Gender effects in nonstressed rats

Measure	Male	Female
Plasma ACTH (pg/ml)	51.5±4.3	53.7±3.1
Plasma prolactin (ng/ml)	3.1±0.6	7.1±2.4
Plasma corticosterone (μg/dl)	2.9±0.6	9.7±1.7[a]
Prolactin mRNA (relative units)	0.63±0.09	2.16±0.17[b]

Values are means±S.E.M. Controls from 1-, 3-, and 14-day experiments were combined. $N=22$ per group. Relative units are as compared to Beta-Actin.

[a] Means of males and females are significantly different, ANOVA, $P=.001$.

[b] Means of males and females are significantly different, ANOVA, $P<.001$.

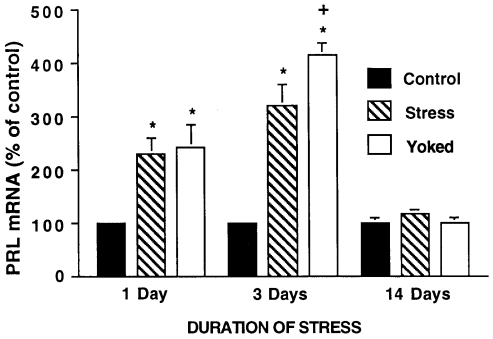

Fig. 2. Levels of anterior pituitary prolactin mRNA for male rats in the second study expressed as percent control (of same duration experiment). Values represent a mean of 6–10 rats/group. The absolute values for prolactin mRNA (expressed as ratio of PRL signal/28 S RNA) in controls were 0.54 ± 0.04, 0.28 ± 0.02 and 1.18 ± 0.14, respectively for 1, 3 and 14 days duration of stress. Error bars are for S.E.M. * Significantly greater than control, a priori contrast, $P < .001$. + Significantly greater than controllable stress group, a priori contrast, $P < .035$.

$P = .001$) as has been previously reported [20,32,39]. Control females had significantly greater amounts of anterior pituitary prolactin mRNA than male controls (2.16 ± 0.17 vs. 0.63 ± 0.09, ANOVA, $P < .001$). Two-way analysis of variance results indicate that anterior pituitary prolactin mRNA was significantly greater in females, in general, across all experimental conditions in all of these stress studies, 1-, 3- and 14-day, ANOVA, $P < .001$ for all three studies. When anterior pituitary prolactin mRNA levels are expressed in terms of amount of message relative to same gender within experiment controls, both gender (G) and gender by experimental treatment interaction (G × E) effects were significant in the 1- and 3-day stress experiments (two-way ANOVA, for 1-day stress study F_G, $P = .001$ and $F_{G \times E}$, $P = .048$; for 3-day stress study F_G, $P < .001$ and $F_{G \times E}$, $P < .01$. See Figs. 2 and 3).

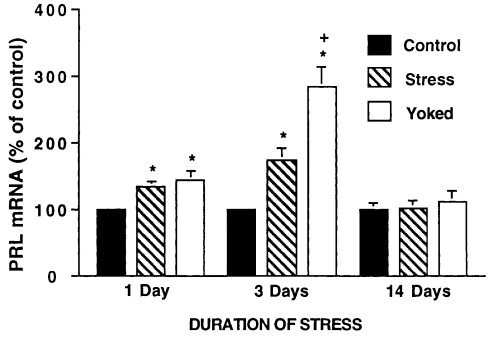

Fig. 3. Levels of anterior pituitary prolactin mRNA, for female rats in the second set of studies expressed as percent of control (of same duration experiment). Values represent a mean of 6–10 rats/group. The absolute values for prolactin mRNA (expressed as ratio of PRL signal/28 S RNA) in controls were 2.27 ± 0.14, 1.21 ± 0.06 and 2.91 ± 0.32, respectively for 1, 3 and 14 days duration of stress. Error bars are for S.E.M. * Significantly greater than control, a priori contrast, $P < .001$. + Significantly greater than controllable stress group, a priori contrast, $P < .01$. Absolute levels of anterior pituitary prolactin mRNA in these female rats were much higher than that in males (see text).

3.3. Second series of experiments: effects of 1, 3 or 14 days of chronic stress on plasma hormones and anterior pituitary prolactin mRNA in male and female rats

Male and female rats were exposed to 1, 3 or 14 days of no stress, controllable stress or uncontrollable stress prior to determination of the plasma hormones ACTH, prolactin and corticosterone and anterior pituitary levels of prolactin mRNA.

After 1 day of stress, no significant differences were seen between the two different stress groups (controllable vs. uncontrollable stress), but the values for stressed groups were greater than controls on most measures (Table 3, Figs. 2 and 3). Plasma ACTH levels were the exception, with no differences between stressed and unstressed animals of either gender. Plasma prolactin was significantly elevated in stressed males (a priori contrast, $P = .03$) but experimental group means were not different in females, possibly due to the larger variability of this measure in females. Plasma corticosterone was markedly increased in stressed rats of both genders (a priori contrast, $P < .001$ and $P = .03$ in males and females, respectively, Table 3). In the 1-day stress study the basal levels of prolactin mRNA were approximately fourfold higher in female control rats than in male controls (2.27 ± 0.14 vs. 0.54 ± 0.04). Anterior pituitary prolactin mRNA levels were increased similarly in both stress treatment groups as compared to controls. Anterior pituitary prolactin mRNA (expressed as percent control) was twice as high in 1-day stressed males as compared to controls (a priori contrast, $P < .001$, see Fig. 2) and increased by approximately 40% in stressed females (a

Table 3
Effects of 1-day stress exposure on plasma hormones

	Plasma ACTH (pg/ml)	Plasma prolactin (ng/ml)	Plasma corticosterone (μg/dl)
Male rats			
Control (no stress)	57.5 ± 5.5	2.1 ± 0.2[a]	3.4 ± 1.1[b]
Controllable stress	74.3 ± 7.0	5.1 ± 1.3	14.0 ± 3.2
Uncontrollable stress	56.6 ± 5.0	2.1 ± 0.2	10.7 ± 2.1
Female rats			
Control (no stress)	51.2 ± 1.4	5.5 ± 3.2	8.2 ± 2.5[c]
Controllable stress	65.7 ± 7.5	11.8 ± 6.0	24.7 ± 7.9
Uncontrollable stress	57.6 ± 6.6	5.2 ± 1.7	14.4 ± 7.3

Values represent the mean of 10 rats per group ± S.E.M.

[a] Mean of stressed rats is significantly greater than control, a priori contrast, $P = .03$.

[b] Mean of stressed rats is significantly greater than control, a priori contrast, $P < .001$.

[c] Mean of stressed rats is significantly greater than control, a priori contrast, $P = .03$.

priori contrast, $P=.001$, see Fig. 3). A statistically significant Gender × Experimental treatment interaction was indicated by two-way ANOVA, $P=.048$.

After 3 days of around-the-clock stress, anterior pituitary prolactin mRNA was greater in both male and female stressed rats vs. controls (a priori contrast, $P<.001$ for both male and female rats, see Figs. 2 and 3), while plasma hormone levels were not significantly different from controls for either gender (Table 4). Prolactin mRNA levels in anterior pituitary of both male and female rats differentially increased in the two stress groups. Results from a two-way ANOVA demonstrate a significant Gender × Experimental treatment interaction, $P=.006$. In male rats prolactin mRNA was approximately 320% and 413% of control values, respectively, in the controllable and uncontrollable stress treatment groups. This increase was greater in the uncontrollable stress group, a priori contrast, $P=.035$ (Fig. 2). Prolactin mRNA values in female rats were approximately174% and 283% of control values, respectively, in controllable stress or yoked groups (Fig. 3). This increase in anterior pituitary prolactin mRNA was significantly greater in the uncontrollable stress group (a priori contrast, $P<.01$). Thus, stress exposure for 1 or 3 days increased anterior pituitary prolactin mRNA in both male and female rats, but the increase relative to controls was greater in the male rats.

After 14 days of stress exposure, no significant differences among stress groups and controls were seen for either plasma hormones (Table 5) or prolactin mRNA as shown in Figs. 2 and 3. Prolactin mRNA levels in stressed male rats were approximately 116% and 98% of control values, and in stressed females 102% and 112% of control values, for the controllable and uncontrollable stress groups, respectively.

Plasma estradiol concentrations did not differ among 1-, 3- and 14-day stress studies; 18.07 ± 2.66, 18.55 ± 3.47, and 19.03 ± 3.47 pg/ml, respectively. Mean plasma estradiol concentrations did not differ among controllable stress, uncontrollable stress (yoked) and control groups for any of the stress periods: 17.46 ± 2.71, 18.95 ± 2.79, and 18.98 ± 3.67 pg/ml, respectively. Plasma hormone concentrations from each experiment are consistent with expectations for normally cycling S-D females with 4- to 5-day estrous cycles. Overall, plasma estradiol concentrations (samples taken 4–5 h after lights on) were bimodally distributed: mean of the higher concentration group was 43.65 ± 3.45 pg/ml, $n=13$. The mean of the lower concentration group was 12.28 ± 0.63 pg/ml, $n=53$. These values are consistent with those expected for populations of normally cycling female S-D rats on a 12-h light cycle [6,16,17]. The ratio of 4:1 for low vs. higher estradiol is not significantly different from expectations, $\chi^2=0.35$, $P>.5$. This suggests that the rats used in these studies were cycling normally. Plasma estradiol concentration was not correlated with plasma prolactin either in controls or in the overall population.

Table 4
Effects of 3-day stress exposure on plasma hormones

	Plasma ACTH (pg/ml)	Plasma prolactin (ng/ml)	Plasma corticosterone (μg/dl)
Male rats			
Control (no stress)	31.5 ± 3.1	5.2 ± 1.9	1.6 ± 0.5
Controllable stress	42.0 ± 8.8	3.1 ± 0.8	1.7 ± 0.4
Uncontrollable stress	41.0 ± 7.2	6.1 ± 2.3	9.2 ± 4.4
Female rats			
Control (no stress)	42.3 ± 6.4	7.7 ± 3.7	6.1 ± 1.9
Controllable stress	41.7 ± 5.9	4.2 ± 2.5	12.9 ± 5.6
Uncontrollable stress	38.7 ± 2.5	5.1 ± 2.9	5.2 ± 0.9

Values represent the mean of six rats per group ± S.E.M.

Table 5
Effects of 14-day stress exposure on plasma hormones

	Plasma ACTH (pg/ml)	Plasma prolactin (ng/ml)	Plasma corticosterone (μg/dl)
Male rats			
Control (no stress)	61.4 ± 8.2	2.5 ± 0.4	3.3 ± 0.8
Controllable stress	72.6 ± 6.9	9.5 ± 4.9	7.6 ± 2.7
Uncontrollable stress	68.9 ± 7.4	3.7 ± 1.5	10.6 ± 5.0
Female rats			
Control (no stress)	69.2 ± 5.1	9.3 ± 6.7	15.9 ± 3.7
Controllable stress	70.5 ± 11.6	3.8 ± 1.2	20.0 ± 11.7
Uncontrollable stress	71.5 ± 7.2	3.0 ± 0.6	7.0 ± 3.2

Values represent the mean of six rats per group ± S.E.M.

4. Discussion

In addition to the well-known pituitary adrenal hormonal response to stress, acute stress also elicits an increase in plasma prolactin levels [3,32,34–37,43,49,59]. In our laboratories, we have determined that short-duration immobilization, footshock, forced running, swimming, conditioned place aversion, or cold exposure elicits a robust increase in prolactin in rats [32,34–37]. The prolactin response to stress is proportional to the severity of the stressor over a moderate range and demonstrates habituation upon repeated exposure [3,15,43,59]. There are reported gender differences in the prolactin response to stress in rats. In females, prolactin is increased during proestrus, and stress at that time attenuates the elevation of plasma prolactin due to the proestrus surge [24,45]. In the present experiments, all animals were sacrificed in the morning prior to proestrus (which occurs in the late afternoon and evening using the light cycle employed here).

Prolactin is released by stimuli other than stress including suckling and estrogen administration. In mammary tissue, prolactin stimulates the transcription of proteins required for lactation. Although over 100 biological targets of prolactin have been reported [10,12,23,42,48], the significant downstream actions of stress-released prolactin are not known. One potential function of stress-released prolactin is its

ability to promote the immune response and thereby mitigate the immune suppressive effects of glucocorticoids, which are also increased by stress [1,11,42]. Prolactin has been shown to alter the transcription of many enzymes in different tissues and some of these effects may prove to play a role in either stress-induced pathophysiology or in stress resistance mechanisms [23,48].

In recent years, our laboratories have changed the focus of our research from acute stress to studies of chronic or sustained stress, as chronic stress is more likely to underlie various pathophysiological effects of stress in humans. Stress has been thought to be a contributing factor for a variety of human illnesses including cardiovascular, gastrointestinal and mental disorders [4,13,14,25,50,55].

In our studies, we have utilized the around-the-clock intermittent footshock paradigm detailed above. One aspect of this sustained stress model is that habituation is fairly rapid compared to repeated exposures to a stressor separated by many hours within the day and applied for several days. Thus, for example, ACTH levels in stressed rats are indistinguishable from control levels after 24 h of stress exposure in our chronic stress model. Both physiological and behavioral measures return to baseline over a period of several days even though the stress paradigm continues. Since rats avoid/escape more than 99% of the shock trials presented and rarely reach the top level of shock before terminating the trial, we hypothesize that the stress is moderate rather than severe. Both the moderate intensity and the continuous nature of the paradigm act to facilitate habituation.

Consistent with our previously published data, in the experiments we have presented here, plasma corticosterone was greater in stressed rats of both genders after 3 days of stress exposure, but not in rats stressed for 14 days in this chronic stress paradigm. In the first series of experiments (males only) reported herein, we saw increases in prolactin after 3 days of stress and differences among treatment groups at day 14. However, in the second set of experiments where both male and female rats were run simultaneously, we did not see significant elevations of plasma prolactin in either gender after 3 or 14 days of stress exposure. This apparent inconsistency is probably due to the lower number of animals/group in the second set of experiments. Our data with respect to chronic stress effects on plasma prolactin from earlier individual studies have not been consistent. In a recent study, we reported elevated plasma prolactin levels in both the controllable and the uncontrollable stress groups after 3 days of stress. In that study, levels in the 14-day uncontrollable stress group were greater compared to unstressed or controllable stress groups [29]. However, in other studies with less animals per experimental group, we have not seen prolactin increases at 3 days [33]. On the other hand, combining all of our data from numerous studies using only male Sprague–Dawley rats in this chronic stress model, we find that plasma prolactin is significantly greater in rats stressed for 3 days vs. controls. Means ± S.E.M. equal 1.77 ± 0.23, 2.79 ± 0.38, and 2.71 ± 0.39 ng/ml for controls ($n = 39$), controllable stress ($n = 33$) and uncontrollable stress ($n = 34$), respectively, Dunnett's t test, $P < .05$. Similarly, the summary of all data from our studies using this model for 14 days of stress with male rats alone show significantly greater plasma prolactin in stressed rats vs. controls. Means ± S.E.M. equal 1.83 ± 0.13, 3.61 ± 0.49, and 3.87 ± 0.73 ng/ml for controls ($n = 31$), controllable stress ($n = 28$) and uncontrollable stress ($n = 28$), respectively, Dunnett's t test, $P < .01$. Minor changes in the habituation rate from experiment to experiment possibly underlie the observed differences among experimental results in individual reports. In any case, the small elevation of plasma prolactin that we have reported in chronically stressed rats is markedly less than the 10- to 50-fold increases seen after acute stressors.

Others have reported that chronic repeated stress, twice daily sessions of footshock (3 mA of 1-s duration applied every 5 s for 30 min) repeated for 1, 3 or 7 days, did not significantly alter anterior pituitary prolactin mRNA [28]. On the other hand, it has been reported that nonstressful more continuous stimuli (e.g., suckling) increased prolactin release and increase levels of pituitary prolactin mRNA [41]. We report here that chronic sustained stress markedly increased anterior pituitary prolactin mRNA in both male and female rats, although baseline levels of prolactin mRNA were much higher in female rats as has been previously reported [56]. These effects were dependent on the time course of the experiment. Following 1 day of stress, anterior pituitary prolactin mRNA was elevated in both treatment groups (controllable and uncontrollable stress) in both genders, with no significant difference between the two stress-treatment groups. After 3 days of stress, levels of anterior pituitary mRNA were even more elevated than at 1 day and there were significantly higher levels in rats in the uncontrollable stress group. After 14 days of stress, anterior pituitary prolactin mRNA levels were equivalent to control levels.

Thus, the levels of anterior pituitary prolactin mRNA appear to be a sensitive marker for stress exposure, demonstrating a chronology of increased synthesis over the first 3 days of stress and then dropping to prestress levels sometime between 3 and 14 days. At the time of maximum increase, this biological marker also distinguishes between the stress treatment groups, suggesting as have other studies, that uncontrollable stress is more severe than controllable stress [29,57,58].

The increase in prolactin mRNA fits with other observations that report that increased physiological requirements for neurotransmitters or hormones increase mRNAs for either the needed substance itself or for key enzymes required to synthesize the compound. Stress has been shown to increase mRNA for proopiomelanocortin (POMC), the precursor for ACTH, for c-*fos*, for tyrosine hydroxylase (the key enzyme in catecholamine biosynthesis), for hypothalamic neurotensin, arginine vasopressin (AVP), enkephalin — and for CRH, a stress integrative hormone [27,28,40,52].

In the present studies, anterior pituitary prolactin mRNA levels were highest after 3 days of stress (as compared to 1 or 14 days), while prolactin levels were only slightly elevated (first set of experiments) or not different from control levels (second set of experiments) after 3 days of stress. Although stress-increased requirements for prolactin might drive prolactin mRNA production, the increased levels of plasma prolactin and prolactin mRNA would not necessarily be expected to share the same time line. In a recent report Castano et al. [18] demonstrate that there is a discordance among gene transcription, mRNA storage and hormone release in individual mammotropes. Furthermore, changes in pituitary prolactin mRNA and serum prolactin levels during the estrous cycle in rats are also not tightly coupled over time [51].

In summary, chronic sustained stress of 1 or 3 days duration elevates anterior pituitary prolactin mRNA, but mRNA levels are equivalent to control levels after 14 days of stress.

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