Nycthemeral differences in response to restraint stress in CD-1 and C57BL/6 mice

Andrew K. Hotchkiss*, Leah M. Pyter, Gretchen N. Neigh, Randy J. Nelson

Department of Psychology, The Ohio State University, 48a Townshend Hall, 1885 Neil Avenue Mall, Columbus, OH 43210, USA
Department of Neuroscience, The Ohio State University, Columbus, OH 43210, USA

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Abstract

Restraint represents psychological and physical stress. Methods used to model restraint stress in mice vary in duration, time of day during which restraint is applied, and the strain of mouse tested. The goals of this study were: (1) to identify the optimal daily time periods during which the stress response is maximized, and (2) to describe mouse strain differences, if any, in response to restraint. Groups of outbred CD-1 and inbred C57BL/6 mice were restrained for 3 h during three time points of the daily light–dark cycle: (1) the late light phase, (2) the transition between the light phase and the dark phase, and (3) the mid-dark phase. Additional mice served as control groups for food deprivation or were unhandled except for blood sampling. Mice of both strains lost significant body mass after 3 days of restraint. Unrestrained food-deprived mice lost body mass, particularly if food-deprived during transition periods. Corticosterone was elevated in restrained mice compared with control mice. Neither basal nor postrestraint corticosterone differed between strains. Corticosterone was elevated by food deprivation during transitional periods in CD-1 mice and during both transition and dark phases in C57 mice. Corticosterone response in restrained CD-1 mice was increased during the dark phase. These results suggest that the physiological response to restraint is similar in both strains. However, corticosterone responses to both restraint and food deprivation were highest during the transitional and dark phases.

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1. Introduction

The stress response in mice involves the perception and central processing of a stressor, followed by an endocrine cascade including the release of corticotropin releasing hormone (CRH) from the hypothalamus, subsequent release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, and finally, the secretion of corticosterone from the adrenal cortex [1]. This hormonal sequence constitutes part of the hypothalamic–pituitary–adrenal (HPA) axis. Hormones, such as the glucocorticoid, corticosterone, affect several parameters of physiological homeostasis during stress including the allocation of energy reserves, as well as the endocrine and immune systems. In addition to mediating the stress response, plasma concentrations of HPA hormones display a strong circadian rhythm. ACTH secretion peaks during the light phase of the daily photoperiod when nocturnal rodents are inactive. The subsequent peak of glucocorticoids occurs immediately prior to the active phase [1]. Functionally, increased glucocorticoid concentrations mobilize energy reserves to prepare the individual for activity [2].

Several experimental models have been used to study the effects of HPA activation on various physiological and behavioral endpoints. These models are designed specifically to mirror psychological, metabolic, and physical stressors. However, because it is difficult to represent accurately the human perception of stress using animal models, there is no consensus regarding which approach is most appropriate for inducing each type of stress. Models of psychological stressors (open-field, acoustic startle) involve subjecting the animal to the respective treatment without physical discomfort, whereas physical stressors (footshock, forced swim) certainly comprise psychological stressors [3]. Variation in stress models, experimental methods, and...
animal strain significantly affects endpoints being compared [3–9]. Additionally, the basic definition of stress is an “ethereal” concept [10]. For this study, we used an operational definition of stress as any homeostatic perturbation necessitating an increase in glucocorticoids; a successful stressor should elevate the primary glucocorticoid in mice, corticosterone.

One of the most commonly used stress paradigms is restraint. In this procedure, the rodent is placed in a well-ventilated container and immobilized without apparent physical harm. Methods for restraint vary both in duration (15 min to 16 h) and time of day restraint is applied [11–14]. Additionally, previous studies in mice have shown significant strain differences (BALB/cByJ and C57Bl/6ByJ mice) in responsiveness to mild stressors [15–17]. These strain differences have included differing ACTH, corticosterone [15], and amine concentrations [16,17] in response to stressors such as footshock and cold swim. Studies in nocturnal rodents, including several strains of mice, report increased corticosterone concentrations after restraint and exposure to constant bright light [18–20]. Bright light is thought to be a necessary component of the stress response in nocturnal animals. However, restraint may be more effective as a stressor if these animals are restrained during their active period: the dark phase. Alternatively, the circadian rhythm of corticosterone peaks around the transition from light to dark, suggesting that the HPA axis may be more responsive to stress during the dark phase. Although corticosterone responses to restraint during the dark phase of the daily light–dark cycle have been examined [21–23], the question of an optimal restraint time resulting in maximal corticosterone elevation has not been addressed and no standard procedure exists. In this study, two strains of mice were restrained at three different time points during the light–dark cycle: (1) the late light phase, (2) the transition between the light and dark phase, and (3) the mid-dark phase. Corticosterone values obtained during these three time periods allow for identification of effective periods of psychological stress and elucidate possible strain differences in the stress response.

2. Methods

Two parallel experiments were conducted simultaneously with either adult male (3–4 months) CD-1 or C57BL/6 (C57) mice. Both commonly used strains were purchased from Charles River (Wilmington, DE). Mice were singly housed, provisioned with ad libitum food and water, and maintained on a 14:10-h light–dark cycle (lights off at 15:00 EST). At the initiation of the experiment, mice were weight-ranked and assigned randomly to treatment groups to control for weight differences among groups. Body mass was also recorded at the conclusion of the study. Percent change in body mass was calculated as: (final body mass – initial body mass)/initial body mass × 100. One group of each strain (R, n = 7–8) underwent 3 h of restraint stress at one of three different times for three consecutive days. During the light phase and the illuminated portion of the transition period, a 60-W white light was placed 1.0–1.3 m above the restrained animals in order to supplement ambient light. The times of restraint were mid-light phase (08:00–11:00), during the transition from light to dark (13:30–16:30), and mid-dark phase (19:00–22:00). Each restrained group was matched with a nonrestrained, food-deprived control group that remained in their home cages (FC, n = 7–8). Food deprivation occurred during the same 3 h that the restraint was applied. An additional control group consisted of mice that were left unhandled in their home cages with access to food during the entire experimental period (UC, n = 5). Mice were restrained individually in well-ventilated restraint tubes. To accommodate the different body sizes of CD-1 and C57 mice, two different sizes of tubes were used. Dimensions for the restraint tubes used for the larger CD-1 mice (mean body mass = 36.2 g) were 10.5 cm long with an internal diameter of 3.7 cm. Restraint tube dimensions for the smaller C57 mice (mean body mass = 26.6 g) were 9.7 cm long with an internal diameter of 2.8 cm. Restrainted animals were isolated from control animals during the restraint period.

Blood samples were collected from all mice on Days 1 and 3. Immediately (<2 min) following the restraint and control procedures on Day 1 and Day 3, animals were lightly anesthetized with isoflurane and a 50-μl retro-orbital blood sample was taken and stored on ice until further processing. Samples were centrifuged at 4 °C at 900 × g for 30 min. Plasma was collected and stored at −70 °C until assessed for corticosterone concentration. Corticosterone was measured in duplicate using a mouse 125I radioimmunoassay kit (ICN Biomedical) as directed by the manufacturer. The intra-assay coefficient of variation was <10%. The detection limit of the assay was 5 ng/ml.

2.1. Statistics

Data in the experiment were analyzed using SAS Statistical Software Version 8 (SAS Institute, Cary, NC, 2001). The mixed statistical model consisted of stress treatment as the independent variable, day as the repeated measure, and individual animal as a random variable. Dependent variables included body weight and corticosterone concentration. All interactions between stress treatment, time of day, and strain were assessed using ANOVAs with unequal variances. Error structures that best fit each experiment were determined using Bayesian Information Criteria before analysis. Following significant interactions, pairwise comparisons were made using the Fisher’s Protected LSD Means Difference test under the a priori hypothesis that that restraint would elevate corticosterone and decrease body mass. Individual t tests were also run between strains to determine strain differences. Mean differences were considered significant when P ≤ .05.
3. Results

3.1. Body mass

3.1.1. CD-1

Mice restrained at each of the three time points lost body mass compared with the unhandled control mice \( F(8,52) = 8.40; P < .001; \text{ Fig. 1a}. \) Mice food-deprived during the transition period also lost body mass \((P < .001)\) comparable to the decrease measured in the animals restrained during the light and dark phase. Body mass decreased less dramatically in food-restricted mice during the light or dark phases \((P < .05)\). Body mass of unhandled control mice did not change or slightly increased at all time periods.

Fig. 1. Body mass changes in response to 3 h of restraint in both CD-1 and C57 male mice \((n = 7\text{–}8\) per group\). UC = unhandled control, FC = food-deprived, R = restrained. The same experimental design was used for CD-1 and C57 mouse strains. (a) All restrained CD-1 mice lost body mass compared to unrestrained mice. Food deprivation also decreased body mass. (b) Restrainted C57 mice also lost significant body mass at each time point. During the transition phase, food-deprived animals lost significant body mass compared to controls. Dissimilar letters indicate statistical significance \((P < .05)\).
3.1.2. C57

Mice restrained at each of the three time points lost more body mass than both the unhandled control and food-deprived control mice \( F(8,52) = 12.42; P < .05 \) in each case, Fig. 1b). During the transition phase, food-deprived mice lost body mass compared with unhandled control animals \( P < .001 \).

3.1.3. Strain comparison

During the transition period, restrained C57 mice lost more body mass relative to Day 1 values than the restrained CD-1 mice \( t = 2.85, P < .01 \). Similarly, during the light phase, food-deprived CD-1 mice lost more body mass than C57 food-deprived mice \( t = 1.98, P < .05 \). In contrast,
unhandled C57 mice gained mass during the transitional period ($t = 3.69, P < .01$), whereas body mass of CD-1 mice did not change.

3.2. Plasma corticosterone concentrations

3.2.1. CD-1

CD-1 mice restrained during each of the three phases significantly increased corticosterone concentrations compared with unhandled control and food-deprived control mice ($F(8,50) = 6.10; P < .05$, Fig. 2a). Corticosterone elevation was highest in the group restrained during the dark phase ($P < .05$). Animals restrained during the dark phases significantly decreased the corticosterone response from Day 1 to Day 3 ($P < .01$). Food-deprived CD-1 mice showed an increase in corticosterone ($P < .05$) during the transition phase only. Unhandled control CD-1 mice exhibited basal corticosterone levels throughout the day, with a nonsignificant decreasing trend ($P > .05$). During the light phase, both the unhandled control and food-deprived control mice displayed nonsignificant increases in corticosterone between Days 1 and 3.

3.2.2. C57

C57 mice restrained during each time point increased plasma corticosterone concentrations compared with unhandled control mice ($F(8,50) = 2.98; P < .01$ in each case, Fig. 2b). Restraint during the transition period increased corticosterone concentrations significantly more than restraint during the light phase ($P < .05$). However, the mice restrained during the transition and dark periods also decreased corticosterone concentrations on Day 3 compared with Day 1 ($P < .01$), whereas the concentrations of the restraint mice during the light phase remained constant. Food-deprived C57 mice increased corticosterone concentrations during the transition and dark phases as compared to unhandled controls ($P < .001$). During the transition phase, the corticosterone increase in food-deprived mice was large enough to match that of the restrained animals. However, during the dark phase, these mice had smaller increases in corticosterone as compared to the restraint groups. Food-deprived mice from the transition and dark phases also displayed significant decreases in corticosterone response on Day 3, similar to the significant trend of habituation in the restraint mice ($P < .001$). Unhandled control C57 mice exhibited a trend of decreasing corticosterone concentrations throughout the day. However, no habituation of corticosterone response was seen in these unhandled control mice ($P > .05$).

3.2.3. Strain comparison

Corticosterone concentrations following treatments on Day 1 revealed strain differences in the food-deprived control mice during the transition and dark phases. Food-deprived C57 mice displayed elevated corticosterone compared with C57 mice that were not food deprived during the transition period ($P < .01$) and during the dark phase ($P < .01$). In comparison, food-deprived CD-1 mice showed elevated corticosterone only during the transition period ($P < .05$). In terms of direct corticosterone concentration comparisons, food-deprived CD-1 and C57 mice were different during both the transition ($t = 3.49, P < .001$) and dark phase ($t = 4.18, P < .001$). Day 3 corticosterone concentrations were elevated in food-deprived versus unhandled control C57 mice during the transition ($P < .05$) and dark ($P < .05$) phases but food deprivation did not cause elevated corticosterone in CD-1 mice ($P > .05$). Restraint caused a similar increase in corticosterone in both strains across all time periods ($P < .05$).

4. Discussion

In this study, 3 h of restraint elevated corticosterone and decreased body mass across strains and light periods. Unrestrained, food-deprived mice also lost body mass, particularly during transition periods. Corticosterone was elevated by food deprivation during transition periods in CD-1 mice and during both transition and dark phases in C57 mice. Finally, corticosterone response in restrained CD-1 mice was increased during the dark phase and tended to be higher during the transition and dark phases in C57 mice.

The decreased body mass in restrained animals of this study is consistent with that observed in other restraint studies [24–27]. Loss in body mass is suspected to be caused by food deprivation, possible hypophagia (caused by an increase in corticosterone), and increased fecal elimination during restraint [2,28,29]. Although the experimental impact of reduced body mass depends on the criteria and endpoints of specific studies, such body mass reductions may have substantial and dramatic effects. Nutritional deprivation resulting in reduced body mass can cause immunosuppression [30]. For example, 48 h of starvation resulted in a 69% reduction in T-cell priming as measured by delayed-type hypersensitivity [30]. Therefore, the duration of restraint and subsequent decrease in body mass can be important for studies of immune function. Restraint quickly elevates corticosterone in mice and significant effects may be observed by 20 min [31], although, whether restraint of this short duration would also reduce body mass is unknown. It is possible that a shorter restraint period may eliminate the effects of food deprivation on the stress response. However, most studies impose restraint for considerably longer periods of time, making body mass reduction more relevant [12,32]. Reduced body mass in food-deprived control animals likely results from compressed feeding time during their active (dark) period [2,33]. Thus, mice that are food deprived during the dark phase display a reduction in body mass [14]. If reductions in body mass are indicative of feeding times, then C57 mice appear to confine their eating bouts...
to the transition periods, whereas CD-1 mice eat throughout the day, although circadian food intake was not specifically measured.

Corticosterone concentrations observed in this study were similar to those in other published reports [12,14]. We did not observe a strong circadian rhythm of corticosterone in the unhandled control mice. Based on previous studies, corticosterone is expected to peak around the transition period between lights on and lights off [34,35]. However, our three selected time points are likely insufficient to detect a clear circadian rhythm. Results of previous studies indicate that animals can entrain to nonphotic cues such as routine cage changing [36]. Because our mice were housed in a large colony room, the animals may have become partially entrained to daily human activity, possibly shifting the corticosterone peak earlier in the light phase, discordant with our sampling times.

Food deprivation increased corticosterone in C57 mice during transitional and dark periods. The magnitude of the increase for the CD-1 mice during the transition period was less than that of C57 mice, implying that food deprivation is less taxing in CD-1 mice, perhaps due to a larger initial body size. Other studies have documented metabolic as well as psychogenic effects of restraint due to elevated corticosterone [37,38]. Interactions between these inputs could explain some of the effects of food deprivation in the restraint paradigm used in this study. Alternatively, C57 mice may limit the majority of their food intake to the transition and dark phases; food deprivation during these periods may be more traumatic in C57 mice than in strains that maintain a more constant food intake over the day. Therefore, mice that are food deprived during periods of the day when normally feeding may display elevated corticosterone due to perceived stressful conditions. C57, but not CD-1, mice habituated to food deprivation by Day 3. These results are consistent with other studies showing that 4–5 days of repeated exposures to stressors can habituate induction of early response genes [39,40]. Differences between mouse strains previously have been reported [41]; however, the consequences of these subtle strain differences await additional investigation.

In both mouse strains, a small, yet significant, time of day effect was detected in the corticosterone response to restraint. C57 mice displayed the largest corticosterone response to restraint during the transitional and dark phases; CD-1 mice had the largest response during the dark phase. Restraint during the light phase also elevated corticosterone in both mouse strains. It is unclear whether a slight difference in responsiveness to restraint during different light periods has biological significance. For unspecified reasons, mice restrained during the light phase did not habituate to restraint as seen in mice during the transition and dark phases. Similar circadian differences in habituation have been observed in rats, and have been attributed to the ability of rodents to habituate to mild stressors during their active phase [42].

Several critical issues surrounding the experimental use of restraint were addressed in this report. First, subtle strain differences were observed in body mass and corticosterone response to restraint stress in CD-1 and C57 mice. The implications of these differences remain to be determined and depend on the endpoints and overall objectives of individual studies. Second, reductions in body mass due to acute restraint were significant and could influence the results of studies using animals with compromised health or low initial body mass. Thus, the effect of restraint on body mass should be incorporated into both study design and interpretation of results. Third, although unhandled control animals maintained relatively low corticosterone concentrations throughout the experiment, food-deprived animals displayed elevated corticosterone. Use of food-deprived animals as controls for restraint-stressed mice may mask effects due to increases in glucocorticoids and the associated decrease in body mass. However, if partitioning of stress is of interest, then inclusion of a food-restricted group, as well as unhandled control animals, would be required. Finally, we observed a small, yet significant, time of day effect on the responsiveness of the HPA axis to restraint. This effect, however, was not dramatic and overall restraint appeared to be similarly effective at various time points in a 24-h period for both strains of mice.

Although several important issues were addressed in this study, several more remain. For example, do CD-1 and C57 mice display circadian variations in corticosterone concentrations? Although we did not see evidence of circadian variation in corticosterone, a more detailed study is required to adequately address this question. Another issue is whether the habituation of corticosterone response in C57 mice would translate into habituation of other endpoints affected by glucocorticoids. In any case, the results presented in this report suggest careful consideration of both time of day and mouse strain is necessary for studies that use restraint as a stress paradigm.

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References


