

Restraint Stress Delays Reentrainment in Male and Female Diurnal and Nocturnal Rodents

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Abstract A temporary loss of normal circadian entrainment, such as that associated with shift work and transmeridian travel, can result in an array of detrimental symptoms, making rapid reentrainment of rhythmicity essential. While there is a wealth of literature examining the effects of stress on the entrained circadian system, less is known about the influence of stress on circadian function following a phase shift of the light:dark (LD) cycle. The authors find that recovery of locomotor activity synchronization is altered by restraint stress in the diurnal rodent *Octodon degus* (degu) and the nocturnal rat. In the first experiment, degus were subjected to a 6-h phase advance of the LD cycle. Sixty minutes after the new lights-on, animals underwent 60 min of restraint stress. The number of days it took each animal to reentrain its activity rhythms to the new LD cycle was recorded and compared to the number of days it took the animal to reentrain under control conditions. When subjected to restraint stress, degus took 30% longer to reentrain their activity rhythms ($p < 0.01$). In a second experiment, rats underwent a similar experimental paradigm. As with the degus, stress significantly delayed the reentrainment of rats' activity rhythms ($p < 0.01$). There was no interaction between sex and stress on the rate of reentrainment for either rats or degus. Furthermore, there was no effect of stress on the free-running activity rhythm τ of degus, suggesting that the effect of stress on reentrainment rate is not secondary to alterations of period length. Together, these data point to a detrimental effect of stress on recovery of entrainment of circadian rhythms, which is independent of activity niche and sex.

Key words circadian, stress, *Octodon degus*, activity, rat, jetlag, entrainment, phase shift

Circadian rhythms, using light as the primary source of temporal information, tightly regulate physiological and behavioral functioning. A rapid change in the light:dark (LD) cycle alters the phase relationship between the organism and the outside world, during which time the internal synchrony of the individual is disordered. In humans, this phenomenon is commonly referred to as jetlag and has been linked to physical, emotional, and psychiatric problems such as ulcers, depression, and emotional distress (Katz et al., 2001; Katz et al., 2002; Winget et al., 1984).

The time it takes for circadian rhythms to reentrain is dependent, at least in part, on the size of the phase shift. There is also individual variation in recovery time following a photic phase shift and, in some species, a difference in reentrainment rate between sexes (Goel and Lee, 1995; Labyak and Lee, 1997). It is unclear what accounts for the interspecies and intraspecies variation in time to reentrain. One possibility is that individual variation in the stress response is mediating reentrainment. Variability in the stress response has been well documented in both humans and ani-

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mals (Berger et al., 1987; Piazza et al., 1991; Singh et al., 1999), and this disparity appears to affect circadian function (Bouyer et al., 1998; Weibel et al., 2002). For example, there is a positive correlation between corticosterone reactivity to restraint stress under entrained conditions and the time it takes female rats to subsequently reentrain locomotor activity after an 8-h phase advance of the LD cycle (Weibel et al., 2002). The direct effects of stressors and/or glucocorticoid manipulation presented following a photic phase shift, however, have not been documented.

Corticosterone and cortisol (CORT) reactivity is primarily controlled by the hypothalamic-pituitary-adrenal (HPA) axis, which allows organisms to adapt to environmental influences. Under normal conditions, an applied stressor results in the release of glucocorticoid (i.e., corticosterone or cortisol) at the adrenal cortex. In most animals, a normal, daily rise in CORT is seen just prior to the start of the active period following a decline during the rest period. For diurnal animals, such as humans (and *Octodon degus*), there is a CORT rise during the early hours of the day and very low levels at night (Bailey and Heitkemper, 2001; Mohawk et al., 2005). Phase shifts and the associated symptoms, however, disturb the functioning of the HPA axis (Caufriez et al., 2002; Cho et al., 2000; Desir et al., 1981; Weibel and Brandenberger, 1998).

After a rapid shift of the LD cycle, humans exhibit a splitting of the cortisol rhythm (with 2 acrophases and/or nadirs seen in 1 circadian cycle) as the subject adapts to the phase shift (Desir et al., 1981). Since the HPA axis is not functioning normally and not appropriately entrained to the LD cycle during recovery from a phase shift, it is likely that the CORT rhythm is not synchronized with other physiological systems. This may lead to some of the symptoms associated with the recovery period after a photic phase shift.

In addition to general disruption of HPA functioning, rapid phase shifts may induce an acute, or even chronic, stress response. A study conducted with human subjects found a correlation between circadian desynchrony and elevated levels of CORT (Cho et al., 2000). Cho and colleagues took saliva samples from airline workers, both cabin crew on flight days and ground crew. Cabin crew who had undergone a large time change had CORT levels elevated above those of ground crew, providing evidence for HPA activation in response to a photic phase shift. Glucocorticoid levels have also been shown to increase in rats (Sei et al., 2003) and degus (Mohawk et al., 2005) following a phase shift of the LD cycle.

It is worth noting that rarely does a phase shift occur in the absence of other environmental changes. For the human transmeridian traveler, a variety of events may take place that could cause or exasperate a stress response, and these added stressors could also affect recovery of normal circadian entrainment. It is therefore useful to look at the effect of stressors presented following a shift of the LD cycle on reentrainment rate.

Stress does not appear to have a robust effect on the central circadian pacemaker (located in the mammalian suprachiasmatic nucleus) of rats or hamsters, as measured by free-running rhythms (Meerlo et al., 1997; Moberg and Clark, 1976; Van Reeth et al., 1991). Stress can, however, alter a number of circadian properties (Meerlo et al., 2002). Glucocorticoids have been found to delay feeding-induced resetting of circadian rhythms in the kidney and liver of mice (Le Minh et al., 2001). Stress also has some masking effects on circadian variables. Decreases in the amplitude of body temperature rhythms in rats follow both social stress (Meerlo et al., 1997) and foot shock (Kant et al., 1991). Surgical stress increases rats' heart rates and body temperature amplitudes (Harper et al., 1996). Stress has also been demonstrated to alter the circadian rhythms of growth hormone and thyroid-stimulating hormone (Marti et al., 1993) as well as food intake (Bhatnagar and Dallman, 1999).

Given the effects of stress and CORT on entrained circadian properties and feeding-induced resetting, it is reasonable to expect that exogenous stressors would influence reentrainment following a shift of the LD cycle. To test this hypothesis, animals in the current study were exposed to restraint stress following a photic phase advance. In addition to an effect of stress on reentrainment rate, it was expected that sex and restraint stress would interact to influence recovery of normal circadian entrainment. This seemed likely because the HPA axis response to stress is sexually dimorphic in a number of species (Figueiredo et al., 2002; Viau, 2002; Yoshimura et al., 2003; Young et al., 2001).

An important step in testing the proposed hypotheses was to establish a model of the human jetlag experience. Experiment 1 uses *O. degus*, a diurnal species, while experiment 2 employs the nocturnal rat. The use of both a diurnal and nocturnal species allows the results to be generalized across circadian niches. This is important given that there are some differences in the functioning of the suprachiasmatic nuclei and the relationship of CORT rhythms to the circadian photic

phase response curve between diurnal and nocturnal animals (see Smale et al., 2003, for review). Degus are hystricomorph rodents native to Chile. This rodent is a good diurnal model for examining the effect of the stress axis on circadian behaviors, as it is active above ground during the day (Fulk, 1976; Kenagy et al., 1999; Kenagy et al., 2004), is highly social (Fulk, 1976), and has cortisol as its primary glucocorticoid (Kenagy et al., 1999), which peaks soon after lights-on (Mohawk et al., 2005), consistent with a diurnal activity pattern. The degu has a reliable rate of activity reentrainment (following a shift of the LD cycle) of 1 to 3 days for each hour of phase shift (Goel and Lee, 1995; Labyak and Lee, 1997).

In the present study, the effect of restraint stress on the reentrainment rate of the diurnal degu was compared to that of the nocturnal rat. Rat locomotor activity rhythms reentrain after an average of 6 (Takamura et al., 1991; Yamazaki et al., 2000) to 14 (Nagano et al., 2003) days following a 6-h phase advance of the LD cycle, a rate similar to that of degus. The primary glucocorticoid in the rat is corticosterone, the rhythm of which peaks near the time of lights-off (D'Agostino et al., 1982; Ixart et al., 1977), consistent with a nocturnal circadian activity pattern.

In addition to examining the effect of restraint on the reentrainment rate of *O. degus*, the effect of restraint, as well as the combination of restraint and a light pulse, on free-running period (τ) was also determined for degus. If restraint stress alters τ , then it is possible that the effects of stress on the reentrainment rate result from a change in period length. Unlike the rat, for which no effect of mild restraint stress on τ has been found (Barrington et al., 1993), the effect of stress on basic circadian parameters has not been previously examined in the degu. It was hypothesized that any effect of restraint on the reentrainment rate was due to actions directly on the recovery of circadian rhythms and not secondary to changes in τ .

MATERIALS AND METHODS

Observation of Cortisol Response to Restraint Stress in *Octodon degus*

Glucocorticoid response to restraint stress in *O. degus* has not previously been documented. A preliminary study was therefore carried out to determine if a rise in cortisol concentration is seen following restraint stress.

Six adult male degus, between 2 and 3 years of age, were obtained from a colony at the University of Michigan and entrained to a 12:12 LD cycle. Blood samples were taken between ZT 2.5 and ZT 3. This is a time when CORT levels would normally be near their circadian peak in the degu (Mohawk et al., 2005). Basal CORT blood samples were obtained within 3 min of removal from the home cage. Animals were allowed to recover for at least 4 days prior to undergoing restraint stress. All degus were then put into Plexiglas restraint devices (length = 20.3 cm, diameter = 6.4 cm), and blood samples were collected following 30 min of restraint.

In order to collect blood, all animals were anesthetized with 2% isoflurane (Aerrane, Baxter Pharmaceutical, Deerfield, IL), and 0.5 cc of blood was collected via intracardiac puncture. Blood was clotted on ice for 2 h, centrifuged, and then stored at -20°C before being assayed for cortisol.

Plasma CORT concentration was measured using a prepared radioimmunoassay kit (GammaCoat Cortisol ^{125}I , DiaSorin, Stillwater, MN). The plasma was diluted 1:1 (5 μL plasma, 5 μL serum) in human serum blank (AB 1-40, DiaSorin). The minimum detection threshold was 0.21 $\mu\text{g}/\text{dL}$, and the inter- and intra-assay coefficients of variation were 9.2% and 7.0%, respectively.

Thirty minutes of restraint stress resulted in CORT levels 2.4 times higher than those observed under basal conditions, $t(5) = -4.17, p < 0.05$. The mean CORT concentrations were 54.15 ± 12.52 under basal conditions and 130.96 ± 9.40 following 30 min of restraint stress. The magnitude of CORT increase under restraint conditions was impressive, particularly since the basal levels were taken at a time when cortisol levels had reached their circadian peak (Mohawk et al., 2005).

Experiment 1: Effect of Acute Stress on Reentrainment Rate in *Octodon degus*

Fifteen adult degus (7 intact males, 8 intact females), between 2 and 4 years of age, were obtained from a colony at the University of Michigan. Degus were housed in 26.7×20.3 -cm Nalgene cages equipped with infrared (IR) devices (Slimline PIR, SmartHome, Irvine, CA) to monitor activity. Activity data were collected in 10-min bins with Vitalview (Minimitter, Bend, OR) software. Prior to entering the experiment, all animals were maintained in LD 12:12, with lights on at 0600 h and off at 1800 h.

The light cycle was then advanced 6 h, with the lights coming on 6 h earlier than they had the previous day (new lights on from 2400 h to 1200 h). At 0100 h on the first day of the shift (60 min after the lights came on at the new time), 7 animals (stress group) were put into cylindrical, Plexiglas restraint devices (length = 20.3 cm, diameter = 6.4 cm) for 60 min. Animals were randomly assigned to an initial treatment group with an equal number of males and females in each condition. The other 8 animals remained unhandled (control group). After the first day of the shift, animals were left undisturbed except for weekly cage changes and daily monitoring of their food and water supply. After all animals' locomotor activity reentrained, the light cycle was again advanced 6 h, with the lights coming on at 1800 h (new lights on from 1800 h to 0600 h). The experimental procedures were then repeated as above, with the former "control" group subjected to restraint and the former "stress" group being left unhandled, resulting in a crossover experimental design.

Analysis of activity data. Phase angles of entrainment (Ψ), based on activity onset, were obtained for each animal prior to each photic phase advance. Activity onset was defined as 3 continual bins of activity, subsequent to 3 h of inactivity, at a level equal to at least 25% of the animal's average daily activity. The time of activity onset was averaged over 3 days, and the phase angle of the mean activity onset compared to the time of lights-on was recorded.

Following the phase shift, activity rhythms were allowed to reentrain with the new LD cycle. An animal was considered reentrained after displaying 3 days of a consistent Ψ , ± 30 min from the original phase angle. The number of days between the start of the phase shift and the first day of reentrainment was recorded for all animals for both shifts. A minimum of 3 weeks occurred between phase shifts.

In the event of an interaction between sex and treatment condition (restraint stress vs. control), estrous cycle stage was determined based on degu activity data (Labyak and Lee, 1995). Activity amplitude and phase are significantly altered on the day of estrus, allowing one to readily determine phase of the cycle at the onset of the phase shift and restraint.

Experiment 2: Effect of Acute Stress on Reentrainment Rate in Rats

The aim of experiment 2 was to determine whether an effect of restraint stress on reentrainment rate could be generalized across species/circadian niches. Experiment 2 was similar to experiment 1 with a few important differences. First, experiment 1 used a crossover design, whereas experiment 2 did not. This change in experimental design results in a confounding variable when comparing the 2 species used (degus in experiment 1 and rats in experiment 2) and introduces the possibility of an order effect but also eliminates the possibility that a subtle, long-lasting effect of restraint stress presented in an initial phase shift is responsible for the difference in reentrainment time observed in later phase shifts. While no residual effects of restraint stress were found in subsequent phase shifts (see experiment 1 results), the design of experiment 2 further verified that any difference in reentrainment rate between control and restraint stressed conditions could not be due to a long-lasting effect of restraint on reentrainment (i.e., restraint at the beginning of 1 phase shift did not result in acceleration of reentrainment in subsequent phase shifts, such as might be expected if restraint allows animals to habituate to the phase shift experience). A second control shift included at the end of experiment 2 would have been ideal, but this was not included in the experimental paradigm. However, we have no reason to expect that a second control shift would have shown us anything of significance, based on the data from the degus. Another difference between the 2 experiments was the mode of data collection. Degus's activity data were collected using IR devices so that any effect of restraint on the reentrainment rate would not be confounded by the ability of unrestrained animals to begin wheel-running during the first few hours following the phase shift. Because there was no effect of treatment condition (phase shift with or without concurrent restraint stress as compared to entrained animals) on activity level found in the degus, rat data were collected using running wheels, which result in clearer actograms. While the change in mode of activity collection may result in confounding effects when comparing the data from rats in experiment 2 to degus in experiment 1, it also improves the clarity of the activity records.

Eighteen adult Sprague-Dawley rats (9 males, 9 females), approximately 60 days of age, were obtained from Charles River Laboratories (Wilmington, MA).

Rats were housed in 26.7 × 20.3-cm Nalgene cages equipped with running wheels to monitor activity. Activity data were collected in 10-min bins with Vitalview (Minimitter, Bend, OR) software.

Removal of ovarian hormones from the female rats was necessary to control for cycle effects because, unlike the degu, there is not a known reliable behavioral assay for cycle stage available in this species. To control for estrous cycle effects on the stress response (Figueiredo et al., 2002; Viau and Meaney, 1991), adult female rats were bilaterally ovariectomized under 5% isoflurane (Aerrane, Henry-Schein/Sullivan Schein, South Natick, MA) gas anesthesia. They were then implanted subcutaneously with two 10-mm Silastic capsules containing estradiol (0.062 ID, 0.125 OD) and one 10-mm Silastic capsule containing progesterone (0.132 ID, 0.183 OD), mimicking the endocrine levels seen in the diestrous phase of the estrous cycle (Bowman et al., 2002; Viau and Meaney, 1991). Male rats underwent a sham procedure. Upon completion of the experiment, vaginal lavage and subsequent cytology confirmed abolishment of estrous cyclicity in all females.

Prior to entering the experiment, all animals were maintained in LD 12:12, with lights on at 0600 h and off at 1800 h. Animals were allowed to recover from the surgical procedure for at least 14 days prior to the start of the experiment. The light cycle was then advanced 6 h, with the lights coming on 6 h earlier than they had the previous day. After the first day of the phase shift, animals were left undisturbed except for weekly cage changes and daily monitoring of their food and water supply ("control" shift). Once all animals were reentrained, the light cycle was again advanced 6 h, with the lights coming on at 1800 h (new lights on from 1800 h to 0600 h). At 1900 h on the first day of the shift (60 min after the lights came on at the new time), animals were put into cylindrical, Plexiglas restraint devices (length = 20.3 cm, diameter = 6.4 cm) for 60 min. This constituted the "stress" shift.

Analysis of activity data. Activity data were scored as in experiment 1, with the exception that the phase angle of the activity onset was recorded relative to lights-off.

Experiment 3: Effect of Acute Stress on Free-Running Period of *Octodon degus*

Sixteen adult degus (female), 2 to 4 years of age, were housed in 26.7 × 20.3-cm Nalgene cages. All

cages were equipped with IR devices (Slimline PIR, SmartHome, Irvine, CA) to monitor activity. Activity data were collected in 10-min bins with Vitalview (Minimitter, Bend, OR) software. Prior to entering the experiment, all animals were entrained to a 12:12 LD cycle, with lights on at 0600 h and off at 1800 h.

Animals were assigned to one of three experimental conditions: 1) control, 2) restraint, or 3) light + restraint. The light pulse was intended to simulate onset of light during a 6-h advance of the LD cycle. In this way, experiment 3 approximated the experimental conditions that degus were exposed to in experiment 1. On the day of the experimental treatment, the lights went out for all groups at the normally scheduled time (1800 h). The control group ($n = 6$) received no manipulation. The restraint group ($n = 5$) was subjected to 1 h of restraint stress beginning at ZT 19 (0100 h). The light + restraint group ($n = 5$) received a 2-h light pulse beginning at ZT 18 (2400 h) and 1 h of restraint stress beginning at ZT 19 (0100 h). Restraint stress consisted of 60 min in a Plexiglas restraint device (length = 20.3 cm, diameter = 6.4 cm). At ZT 20 (0200 h), all animals were released into constant darkness (see Fig. 1 for experimental design). This allowed for the subsequent measure of free-running rhythms as a consequence of treatment condition.

Analysis of activity data. Period (τ) was calculated from daily activity onsets (as described for experiment 1) using ActiView software (Minimitter) from a total of 14 days of data collected in constant darkness. Data from days 3 through 16 of constant darkness were used in the calculations. Phase angles of activity onset were recorded prior to the experiment and on days 1 and 2 following release into constant darkness (see experiment 1 for description of activity onset determination). Any change in ψ following experimental treatment was then calculated and recorded.

STATISTICAL ANALYSES

For experiments 1 and 2, the number of days it took animals to reentrain under each condition was compared using a 2 (sex) × 2 (conditions: stress vs. control) repeated-measures analysis of variance (ANOVA) testing the main effect of restraint stress, the main effect of sex, and the interaction between sex and restraint stress on reentrainment rate. All rates of reentrainment were determined by 2 analysts blind to the experimental condition of the animals. The aver-

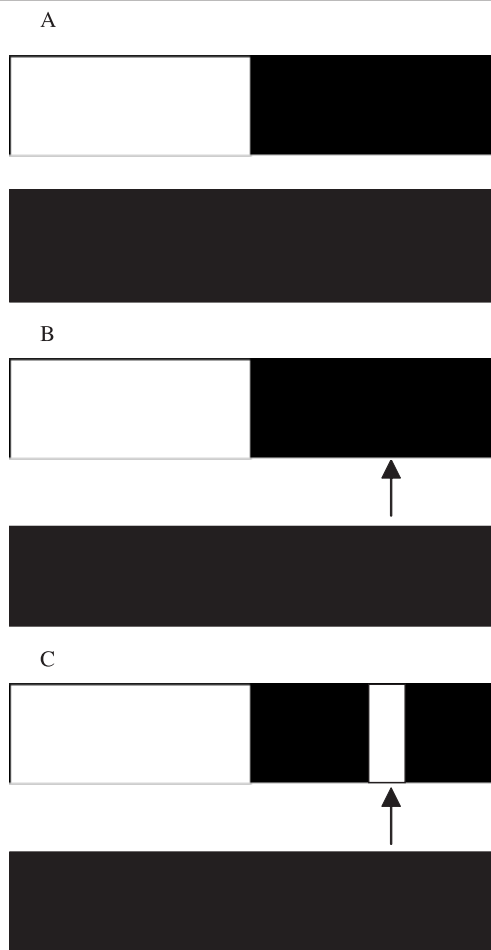


Figure 1. Experimental design for experiment 3. The female degus were kept in LD 12:12 (as indicated by the upper LD bars). The control group ($n = 6$) was released into DD following lights-off on the experimental day, receiving no other manipulation (A). The restraint group ($n = 5$) underwent 60 min of restraint stress (beginning at ZT 19), as indicated by the arrow, and was then released into DD (B). The light + restraint group ($n = 5$) was subjected to a 2-h light pulse, beginning at ZT 18 (indicated by the white box within the dark portion of the photoperiod). These animals also experienced 60 min of restraint stress (again, beginning at ZT 19, as indicated by the arrow) before being released into DD (C).

age of the mean daily activity level (defined as number of times the animal crossed the IR beam per 10-min bin for degus; number of wheel revolutions per 10-min bin for rats) was recorded for each animal for the 3 days immediately preceding the first phase shift (representing the animal's activity level during normal, entrained conditions) and across the first 3 days immediately following each LD phase shift. Average activity level was compared within animals across conditions (entrained, control shifting, and stress shifting) using a repeated-measures ANOVA. For experiment 1, the effect of order of treatment on

reentrainment rate was also tested within each treatment.

For experiment 3, τ was compared across treatment conditions (control, restraint, light + restraint) using ANOVA with Bonferroni post hoc comparisons. Change in ψ was also compared across treatment conditions using ANOVA with Bonferroni post hoc pairwise comparisons. Both the mean change in ψ (the average change with delays represented by a negative number and advances by a positive number) and the absolute change in ψ (absolute value of the change in minutes, regardless of whether the change was an advance or delay) were recorded. Absolute change in ψ was recorded to determine whether restraint stress results in a nondirectional shift in ψ (i.e., does restraint change the phase angle of reentrainment in a nonspecific way?).

RESULTS

Experiment 1: Degu Reentrainment with and without Restraint

There was a main effect of restraint stress on reentrainment rate, $F(1, 13) = 13.342, p < 0.01$ (Table 1 and Fig. 2), with restraint significantly delaying reentrainment for degus. The crossover design resulted in no residual order effect of phase shift on mean time to reentrain (stress condition by phase shift order $t(13) = 0.166, p > 0.05$; control condition by phase shift order $t(13) = -0.199, p > 0.05$). There was a main effect of sex on reentrainment rate, $F(1, 13) = 7.013, p < 0.05$, with females reentraining faster than males. There was no interaction between restraint stress and sex, $F(1, 13) = 0.026, p > 0.05$. Mean reentrainment time for males was 15.7 ± 0.9 days in the stress condition and 12.6 ± 1.1 days in the control condition. Mean reentrainment time for females was 13.0 ± 0.8 in the stress condition and 10.1 ± 0.8 days in the control condition. Since there was no interaction between stress and sex, female degu estrous cyclicity data are not presented here. Average activity level did not differ significantly between entrained and phase-shifting conditions, $F(2, 28) = 1.051, p > 0.05$.

Experiment 2: Rat Reentrainment with and without Restraint

There was a main effect of restraint stress on reentrainment rate, $F(1, 16) = 11.678, p < 0.01$ (Table 1

Table 1. Experiments 1 and 2: Mean \pm SEM Days to Reentrain following a 6-h Phase Advance in the LD Cycle under Control and Restraint Stressed Conditions

	Days to Reentrain		n
	Control	Restraint Stress	
Degu	11.3 \pm 0.7	14.3 \pm 0.7*	15
Rat	9.9 \pm 0.6	12.1 \pm 0.7*	18

NOTE: Restraint stress resulted in a significant delay in reentrainment. Days to reentrain for degus are based on general activity data; days to reentrain for rats are based on wheel-running data. SEM = standard error of the mean.

* $p < 0.05$ compared to control condition; paired t -test.

and Fig. 3), with restraint significantly delaying reentrainment for rats. There was no main effect of sex on reentrainment rate, $F(1, 16) = 2.657$, $p > 0.05$. There was no interaction between restraint stress and sex, $F(1, 16) = 0.376$, $p > 0.05$. Mean reentrainment time for males was 13.1 ± 0.9 days in the stress condition and 10.6 ± 0.7 days in the control condition. Mean reentrainment time for females was 11.0 ± 1.0 days in the stress condition and 9.2 ± 0.9 days in the control condition. Average activity level (wheel revolutions/15-min bin) did not differ significantly between entrained and phase-shifting conditions, $F(2, 34) = 3.24$, $p = 0.051$. Given the trend toward significance in activity level revealed by the repeated-measures ANOVA, post hoc comparisons were made using the Tukey honestly significant difference test. No pair of conditions (entrained, phase shifting with stress, phase-shifting control) significantly differed in activity level ($p > 0.10$ for all pairs).

Experiment 3: Free-Run in Degus following Restraint or Light + Restraint

There was no effect of restraint stress, with or without a concurrent light pulse, on free-running period, $F(2, 13) = 0.367$, $p > 0.05$ (Table 2). There was an effect of treatment condition on the mean and absolute change in ψ , $F(2, 9) = 7.842$, $p < 0.05$ (Table 2), with degus in the light + restraint condition exhibiting a greater change in phase angle than degus in either of the other groups ($p < 0.05$ compared to control and restraint-only conditions). All but one of the light + restraint animals exhibited a phase advance. The phase-delaying light + restraint animal's data were therefore excluded from the reported mean change in ψ (Table 2) but included in the absolute change data (Table 2) and statistical analyses. Degus in the control and restraint groups did not exhibit a significant change in ψ following

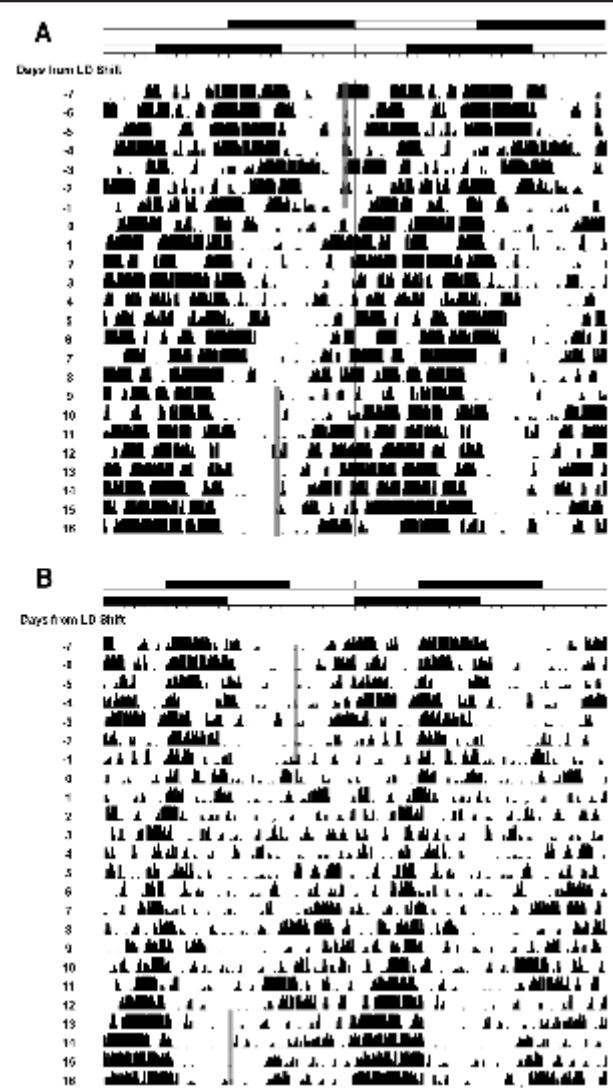


Figure 2. Experiment 1: actograms from a representative degu in the control (A) and stressed conditions (B). The upper LD bar denotes the original light cycle; the lower LD bar denotes the light cycle following the 6-h phase advance. Stress consisted of 60 min in a restraint tube beginning 1 h after the onset of the new LD cycle. Days from the beginning of the LD shift are plotted along the y -axis, with day 0 being the day of the LD shift and, in the case of (B), stress. The gray bars denote phase angle of entrainment prior to and following recovery from the phase shift.

experimental treatment. While the standard errors of the mean (SEMs) for the changes in phase angle vary between experimental conditions, they do not differ significantly.

DISCUSSION

Sixty minutes of restraint stress, 1 h after the onset of a phase shift, delayed the rate of reentrainment for

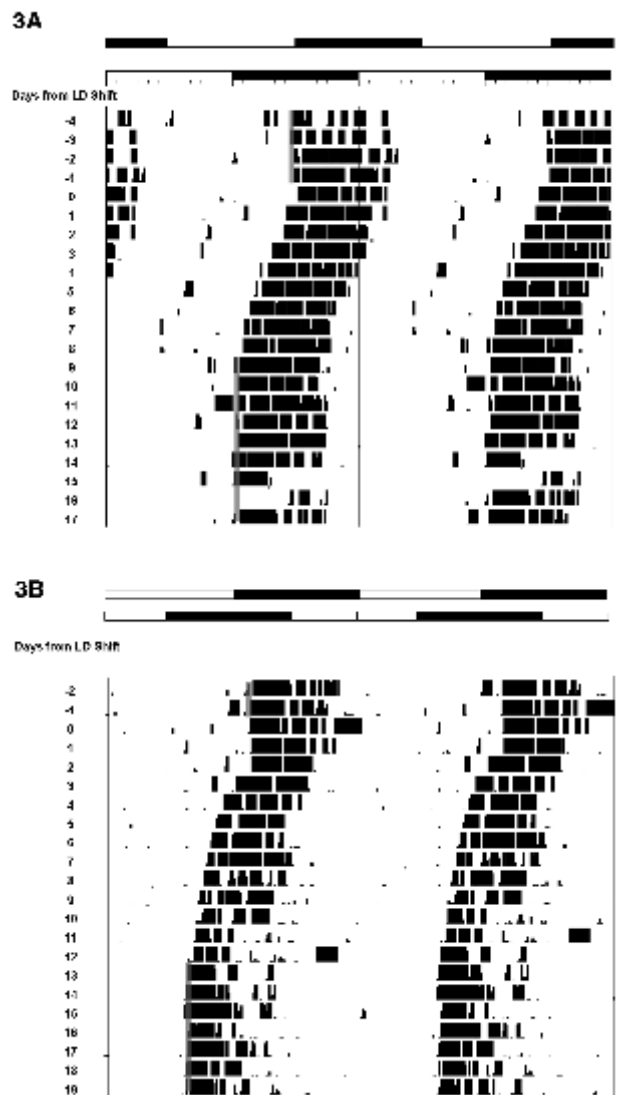


Figure 3. Experiment 2: actograms from a representative rat in the control (A) and stressed conditions (B). The upper LD bar denotes the original light cycle; the lower LD bar denotes the light cycle following the 6-h phase advance. Stress consisted of 60 min in a restraint tube beginning 1 h after the onset of the new LD cycle. Days from the beginning of the LD shift are plotted along the *y*-axis, with day 0 being the day of the LD shift and, in the case of (B), stress. The gray bars denote phase angle of entrainment prior to and following recovery from the phase shift.

both degus and rats. On average, animals took 20% to 30% longer to recover from a 6-h phase advance when they were subjected to 1 h of restraint on the first day of the shift than when they were left undisturbed. There was no interaction between sex and the effect of restraint stress on the reentrainment rate for either degus or rats. Restraint, with or without a concomitant light pulse, did not alter degus's free-running period, consistent with what has been previously

Table 2. Experiment 3: Mean Period and Change in Phase Angle of Activity Onset

	τ		Change in ψ		Absolute Change in ψ	
	Mean (h)	SE	Mean (min)	SE	Mean (min)	SE
Control	23.69	0.03	-5	5.00	5	5.00
Restraint	23.74	0.01	+15	29.24	39	23.04
Light + restraint	23.77	0.06	+125*	18.03	116*	15.46

NOTE: ψ activity onset shifted significantly more for animals exposed to light + restraint than to controls or those treated with restraint alone. $n = 6$ in the control condition and 5 in both the restraint and light + restraint conditions.

* $p < 0.05$ compared to all other conditions; analysis of variance (ANOVA).

reported for the rat (Barrington et al., 1993). It can therefore be said that the effect of restraint on the reentrainment rate is not secondary to acute effects of stress on the clock's endogenous period length. This is the first report of an acute bout of restraint stress affecting the reentrainment rate in any mammalian species, and we find the effect to be identical in the 2 species tested.

It was somewhat surprising that no interaction between sex and stress was observed, given the usual sexually dimorphic nature of the stress response. It should be noted that female rats in experiment 2 were ovariectomized and hormone replaced, while female degus (experiment 1) were not. Since the female rats were replaced with diestrus levels of estrogen and progesterone, they may not have achieved the maximum possible adrenocorticotropic hormone (ACTH) and CORT response to stress, which would be expected in the proestrous phase of the estrous cycle (Viau and Meaney, 1991). The degus remained intact during experiment 1, and yet there also was no sex difference in the effect of acute restraint stress on the rate of reentrainment. Since CORT levels were not measured, it is still possible that females mounted a larger response than males without causing a longer delay in recovery.

Degus in experiment 3 exhibited only changes in phase angle as a result of the light + restraint condition. Since restraint stress alone did not affect ψ , it can be concluded that the light pulse was responsible for the observed changes in phase angle. The light pulse (2 h in length) occurred at ZT 19 on an entrained, LD background. The size and magnitude of the phase shifts were consistent with what has previously been reported in this species (Kas and Edgar, 2000; Lee and Labyak, 1997), although prior research has examined

the phase response to shorter light pulses in animals that were housed in constant darkness prior to the occurrence of the light pulse. Also of note, there was a variable direction of phase change for the animals in the restraint-only condition as compared to the light + restraint condition, suggesting that restraint stress is not acting as a reliable zeitgeber.

Experiment 3 replicated in the degu what has previously been reported for the rat (Barrington et al., 1993): free-running rhythm is not significantly affected by restraint stress. Since experiment 3 was meant to simulate the conditions to which degus were exposed in experiment 1 (i.e., a 6-h phase advance with 60 min of restraint stress occurring 1 h after the new lights-on), our results can only be generalized to stressors presented at a specific zeitgeber time (ZT 19). The possibility that stress occurring at a different time of day could result in alterations of τ cannot be ruled out. However, since experiment 3 did present the stressor at the same time of day as it was presented in experiment 1 (and experiment 2), it can be concluded that the delay in reentrainment rate as a result of the stressor in experiment 1 cannot be secondary to alterations in τ .

The method by which restraint stress affects the reentrainment rate remains unclear, but the current literature points to a role of HPA function in influencing reentrainment rates (Mohawk et al., 2005; Sage et al., 2004; Weibel et al., 2002). There are at least 3 likely mechanisms by which HPA activation could modulate reentrainment: 1) via corticotropin-releasing hormone (CRH) effects on central nervous system activity, 2) through CORT action in the periphery, and 3) through CORT action on the SCN (either directly or via other central nervous system structures). Arginine vasopressin (AVP) or CRH could increase following stress, which in turn could have effects at either the SCN or other areas downstream of SCN function. There is an increase in CRH immunoreactivity in the rat paraventricular nucleus following brief exposure to light (Daikoku et al., 1992), making this structure a possible site of integration for information about light and stress. Release of AVP within the SCN in response to a stressor has been demonstrated (Engelmann et al., 1998), and since AVP can alter SCN neuronal firing (Ingram et al., 1996; Mihai et al., 1994), this may be another possible route by which stress can alter circadian reentrainment.

Another explanation for the effects of restraint on reentrainment rate is that a rise in CORT (due to pharmacological manipulation, the phase shift itself, or an applied physical or psychosocial stressor) causes a

decoupling of peripheral oscillators from the central pacemaker. An elevation in glucocorticoid levels could force any rhythms sensitive to the circadian production of these hormones (e.g., liver, immune system) to become out of phase with the SCN and each other. This internal desynchrony may lead to altered rates of reentrainment for peripheral oscillators and contribute to delayed recovery of entrained circadian activity.

Glucocorticoids could also have central SCN effects on circadian rhythmicity. Indeed, CORT activation appears to have effects on the SCN *in vivo*. Fos immunoreactivity increases in the SCN, particularly the light-responsive ventral portion of the SCN, in response to administration of the glucocorticoid agonist dexamethasone (Briski et al., 1997), suggesting that the central pacemaker is sensitive to CORT. The paraventricular nucleus of the thalamus (PVTh) has also been implicated in the integration of circadian and stress functions. The PVTh has reciprocal connections with the SCN (Moga et al., 1995; Watts and Swanson, 1987; Watts et al., 1987), contains glucocorticoid receptors (Morimoto et al., 1996), modulates phase shifting of drinking behavior (Salazar-Juarez et al., 2002), and regulates aspects of circadian rhythmicity with regards to the stress experienced by an animal (Bhatnagar and Dallman, 1999). Further research will be necessary to determine the mechanism underlying the attenuating effect of stress on reentrainment.

The finding that restraint stress can influence reentrainment rate provides a new perspective with which to view the current literature. In Cho and colleagues' studies of airline workers (Cho, 2001; Cho et al., 2000), the elevated cortisol levels (of the cabin crew) may be due solely to the transmeridian travel itself or may be confounded by the work conditions (i.e., cramped airplane, job stress). Whatever the cause of the elevated cortisol levels, the increase in CORT could be leading to extended loss of entrainment, following transmeridian travel, as suggested by our data. This, in turn, could perpetuate the chronic elevation of CORT and result in long-term changes in physiological and psychological function. Our data also provide further support for the findings of Weibel and colleagues (2002), which suggest that individual HPA responsiveness is correlated with individual reentrainment rate.

In summary, the experiments presented here demonstrate that restraint stress is capable of delaying reentrainment, following a 6-h phase advance, in both

the diurnal degu and nocturnal rat. They further demonstrate that this effect is not dependent on a change in free-running period. In the future, it would be worthwhile to examine the effects of additional stressors (e.g., immune challenge, cold stress), manipulation of the time of stressor presentation relative to the LD cycle, and chronic/repeated stress on the reentrainment process. The effect of stress on reentrainment rate could be of profound importance with regards to the health of shift workers, transmeridian travelers, and the interaction between depression and circadian dysfunction, particularly if the effect proves to generalize across a wide variety of stressors.

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