ORIGINAL ARTICLE

Diurnal behavioral and endocrine effects of chronic shaker stress in mice

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Abstract

Experiments were performed in C57BL/6J male mice to determine 1) light/dark effects of acute and chronic shaker stress on open field behavioral patterns and 2) light/dark effects of chronic stress on plasma corticosterone and oxytocin. Shaker stress was applied acutely (15 min) or chronically (3 or 7 days). Mice were tested in the open field in the light or dark phase of the circadian cycle. For the endocrine study, mice were exposed to 3 days of intermittent shaker stress and sacrificed after the last stress event (09:00 or 19:00 h). Acute or chronic shaker stress had no significant effects on intensity of motor activity and rearing of mice tested under either light condition. Mice tested in the dark phase had higher motor activity and exhibited lower anxiety-like behavior as expressed by central zone activities and had higher emotionality as expressed by increased defecation. Chronic stress increased corticosterone with a greater absolute increase in the dark period. However, the percentage stress-induced increase was not different between the day and night periods. The oxytocin response to stress was observed only during the light phase with no change seen at dark phase. These results show that there is a marked difference in the light/dark pituitary stress response with no alteration in stress induced behavioral changes. They also suggest that there are circadian interactions in the endocrine stress axis that are without consequences for open field behavior.

INTRODUCTION

Chronic stress is considered to be an important risk factor for the development of various physical and mental illnesses (Jacobs and Bovasso, 2000; Rudolph *et al*., 2000; Sandberg *et al*., 2000; Korte, 2001; Schneiderman *et al*., 2005). The investigation of stress-related diseases as well as treatment strategies requires the development of appropriate animal models. Our investigations have employed shaker stress, a psychological stress, in which animals are exposed to brief periods of unexpected shaking. Shaker stress elevates blood pressure and heart rate, increases plasma catecholamines, corticosterone and oxytocin and activates the central secretion of amines and peptides (Hashiguchi

et al., 1997; Nishioka *et al*., 1998, Bernatova *et al*., 2002a; Bernatova *et al*., 2004). The advantage of shaker stress is that it is a mild, pain-free stimulus that elicits reproducible cardiovascular and endocrine changes.

Acute (single) and chronic (repeated, long-term) stress causes behavioral as well as cardiovascular and endocrine changes (Gavrilovic and Dronjak, 2005; Umegaki *et al*., 2006). Behavioral changes are usually observed immediately after stress, but in many cases they persist after discontinuation of the stressful stimuli (Meerlo *et al*., 1996; Hashiguchi *et al*., 1997; Takahashi *et al*., 2005). Behavioral alterations can be manifested in activity level, emotionality and anxiety, as well as in learning and memory processes (Ramos and Mormede, 1998; Hata *et al*., 2001; Keeney *et al*., 2001; Shors, 2001; King *et al*., 2005).

The light/dark circadian cycle influences physiological function since it controls the rhythm of all activities. The rhythms originate in the central nervous system (CNS) pacemakers, acting to modulate behavior, temperature, endocrine secretion and other physiological functions. The circadian variations in adrenal hormonal secretion have long been recognized as a standard example of a daily biological rhythm, e.g. a rise in circulating corticosterone during the active period (Simon and George, 1975; Kelliher *et al*., 2000). Likewise, diurnal variations are seen in neurohypophyseal hormone secretion, vasopressin and oxytocin (Forsling and Williams, 2002). These endocrine rhythms are thought to be regulated by changes in melatonin, the indoleamine secreted from the pineal gland. For the cardiovascular system, there is also a circadian pattern with highest blood pressure and heart rate levels seen during the night (active) period in nocturnal rodents and during the day (active) period in humans (Contreras *et al*., 2000; Li *et al*., 2000; Roemer and Griefahn, 2002). Circadian variations are also seen in the CNS, related to neurotransmitters, neurotransmitter receptors and receptor-associated secondary messenger systems (Aldegunde and Arnaez, 1984; Lemmer *et al*., 1985; Kafka *et al*., 1986; Wirz-Justice, 1987). There is experimental evidence to show interactions between stress responsiveness (endocrine, cardiovascular and behavioral) and the circadian cycle. For example, the effect of restraint stress on paradoxical sleep as well as hypothermia was dependent on the phase of the circadian rhythm (Koehl *et al*., 2002; Peloso *et al*., 2002). Social stress-induced disruptions of body temperature, heart rate and locomotor activity were also found to interfere with light/dark rhythms (Meerlo *et al*., 2002; Mazzoccoli *et al*., 2004).

In view of the fact that CNS pacemakers regulate bodily rhythms and that diurnal changes are important in health and disease, information on the nature of the stress cascade is clearly needed. Therefore, the present experimental study investigated the effects of shaker stress on selected behavioral and endocrine variables in relation to light/dark rhythm.

MATERIALS AND METHODS

Animals

Male C57Bl/6J mice (Harlan, Chicago, IL; average body weight 28.3±2.1 g, age 2 month) were housed singly in plastic cages with wooden shavings in a temperature controlled room (22–23 °C) with 12:12h light:dark cycle (lights off 17:00 h). A standard pellet diet and tap water were provided *ad libitum*. After 10 days of acclimatization the mice were divided into experimental groups for the behavioral studies (controls, acute stress and chronic stress under light and dark conditions; n=9–10 mice/group). The endocrine experiment used separate groups of animals, controls and stressed (n=5–6 mice/group) under light and dark conditions. Animals were handled for 3 days before initiation of the experiments in order to minimize handling-related stress.

Shaker stress

Shaker stress was administered using an electric shaker (Model 5901, Eberbach Inc, Ann Arbor, MI) with a linear horizontal stroke of 2.86 cm and a speed of 150 cycles/min. Animals were kept in their home cages which were inserted into a cage rack with an automated watering system. Mice in the chronic stress group were exposed to intermittent shaker stress for 90 min/day for 3 or 7 days. This total time was distributed into 45, 2-min shaking periods separated by randomized still periods with a mean duration of 30 min (13–44 min). Acutely stressed mice were exposed to a single 15 min period of continuous shaking. Control mice were housed under the same conditions without stress exposure.

Open field test

The behavioral evaluation was conducted by means of an automated open field system using infrared photobeams (Motor Monitor, Version 3.11, 2000, Hamilton Kinder, Poway, CA). The open field sized 40.6×40.6 cm was divided into central (20.3×20.3 cm), intermediate and peripheral (both 5.1 cm wide) zones. For the test, the mouse was placed in the center of the open field arena and the following variables of motor activity were recorded: locomotor activity, fine movements (grooming), rearing, distance traveled, total time, rest time, number of entries and head pokes in individual zones.

At the beginning of experiment a baseline level of locomotor activity was measured in all subjects under both light conditions and assigned to groups based on whole body movements. Three days later, they began either 7 days of intermittent shaking or a single 15 min period of shaker stress. Mice for the light phase experiment were put on the shaker in the morning at 09:00 h and for the dark experiment in the evening at 18:00 h. The behavioral test was conducted immediately after the end of the stress (Day 1) and 7, 14 and 21 days thereafter to evaluate possible delayed or long-term effects. The mice were exposed to the open field in 15-min sessions once daily in the same time schedule. The mice were tested

in the morning between 09:00–13:00 h (light phase) and in the evening beginning 1 hr after lights went off, between 18:00–22:00 h (dark phase). In the dark phase, the testing room was illuminated with red light lamps. After the session, the number of fecal pellets (defecation rate) was noted for assessment of emotional reactivity and the open field arena was cleaned with a 70% alcohol solution.

Endocrine Experiments

Separate groups of mice were used for this experiment. Animals were sacrificed 5 or 30 min after the last stress event (3 day intermittent stress) at 09:00–10:00 h and 19:00–20:00 h (light and dark phase, respectively). The collection times were chosen on the basis of the results of previous study on light/dark rhythms in cardiovascular stress responses (Bernatova *et al.*, 2002a). The 5-min post stress time was used for oxytocin and 30 min post stress for corticosterone because of the time course of pituitary and adrenal secretion. After decapitation the trunk blood was collected for endocrine assays. Plasma corticosterone was determined using the ImmuChem™ double antibody RIA kit (ICN Biochemicals Inc., Costa Mesa, CA). The assay requires 2 µl of plasma. Plasma oxytocin was measured using a radioimmunoassay (RIA). Plasma samples (50–100 µl) were thawed on ice, plasma proteins were precipitated with acetone and the supernatant was extracted with petroleum ether. After lyophylization, the extracts were resuspended in assay buffer. ^{125}I oxytocin was purchased from Dupont Inc (Boston, MA). A non-equilibrium assay was used with an incubation volume of 500 µl and an incubation time of 3–4 days at 4 °C. The oxytocin antiserum, developed by Morris and colleagues is specific for the amidated peptide with no cross-reactivity with vasopressin or other related peptides. Results were calculated using logit transformation of the data.

Statistical Analysis

Data were analyzed by repeated measures ANOVA with two factors (light conditions – light or dark phase, treatment – non-stressed or acutely and chronically stressed mice) followed by Tukey's honest significant difference (HSD) test for unequal N (Spjotvoll/Stoline post hoc test). The endocrine study used 2-way ANOVA followed by Newman-Keul's post-hoc test. The software used was STATISTICA, `99 Edition (StatSoft, Inc., Tulsa, OK). The results are presented as mean ± SEM ($p<0.05$ was considered to be significant).

RESULTS

General health and body weight

There was a significant main effect of time [$F(11,486)=3.19$; $p<0.002$] and treatment [$F(2,486)=18.28$; $p<0.001$] on body weight. Post-hoc comparison of treatment revealed a significant decrease in body weight during and shortly after chronic stress under both light conditions ($p<0.01$). However, on individual days, this decrease failed to be statistically significant compared to controls or acutely stressed mice. After discontinuation of chronic stress, the body weight gradually approached that of controls. Figure 1 shows the body weight of animals from the light phase experimental group. Results were almost identical in the dark phase group (data not shown).

Open field test

Locomotor activity: Repeated exposure to the open field resulted in habituation of locomotor activity both in stressed and non-stressed animals under both light conditions [$F(3,138)=3.82$; $p=0.0114$]. Despite similar baseline values of locomotor activity both in the light and dark phase, repeated testing revealed a significant main effect of light conditions. Mice tested in the dark phase had a significantly higher activity level compared to those in the light phase; total activity counts in the light phase: 1 999.90±44.52 and in the dark phase: 2 160.96±31.88 [$F(1,46)=5.03$; $p=0.0298$]. There was no significant main effect of treatment, light conditions or interaction of the factors (data not shown).

Rearing: Similar to locomotor activity, repeated exposure to the open field resulted in rapid habituation of rearing in all experimental groups under both light conditions [$F(3,138)=7.07$; $p=0.0002$]. There was no significant main effect of treatment, light conditions or interaction of the factors (data not shown).

Zone analysis: Repeated measures ANOVA demonstrated gradual decrease (habituation) in all behavioral activities performed in the center of the open field during repeated testing of the mice. A significant main effect of time was found for the distance traveled [$F(3,138)=4.75$; $p=0.0035$], rest time [$F(3,138)=50.92$; $p<0.0001$] and number of entries [$F(3,138)=4.47$; $p=0.0049$] (data not shown). Mice tested in the dark phase exhibited higher activity in individual variables compared to those for mice tested in the light phase. However, except to the number of pokes, the differences failed to be statistically

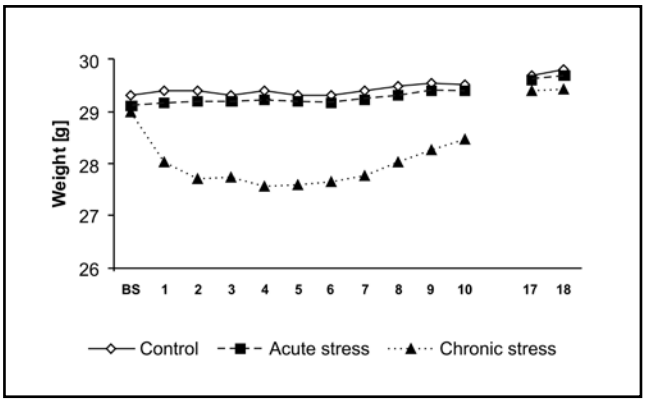

Figure 1. Effect of acute and chronic shaker stress on body weight of mice in the light phase experiment. BS – basal values, 1–7 - seven days of shaker stress, 8–18 - days after discontinuation of stress. Significant overall effect of chronic stress ($p<0.01$).

Table 1. Main effect of light conditions on the behavior of mice in the central zone of the open field.

Variables	Light phase	Dark phase	F(1,46)	p-value
Distance traveled (cm)	335.42±20.49	412.24±25.78	2.26	0.1389
Total time (s)	25.52±1.90	34.54±3.18	2.82	0.0994
Rest time (s)	1.17±0.27	3.53±0.72	3.30	0.0758
Number of entries	16.27±0.96	20.05±1.18	2.31	0.1354
Number of pokes	11.51±0.63	15.90±0.90*	4.98	0.0305

*p<0.05 - significant difference between light and dark phase

Table 2. Main effect of light conditions on the behavior of mice in the peripheral zone of the open field.

Variables	Light phase	Dark phase	F(1,46)	p-values
Distance traveled (cm)	3040.03±58.54	3124.47±64.61	0.25	0.6182
Total time (s)	702.95±8.74	663.82±12.16	2.32	0.1345
Rest time (s)	180.72±6.83	142.56±5.63**	8.84	0.0047
Number of entries	65.00±2.45	79.15±2.30*	6.65	0.0137
Number of pokes	31.82±1.49	37.25±1.84	1.97	0.1668

*p<0.05; **p<0.01 - significant difference between light and dark phase

significant (Table 1). Habituation in individual variables recorded in the peripheral zone was also found. A significant main effect of time was found for the rest time in the periphery [F(3,138)=3.46; p=0.0181] (data not shown). There was a significant main effect of light conditions for the rest time and number of entries in the periphery. Mice tested in the dark phase rested in the periphery less and entered into this zone more frequently than those in the light phase (Table 2). There was no significant main effect of treatment or interactions between the factors in both central and peripheral zones of the open field.

Emotional reactivity: Repeated measures ANOVA showed a significant main effect of time on emotional reactivity in mice. In contrast to habituation of locomotor and rearing activity, there was an increase in the number of fecal boli during repeated exposure of mice to the open field [F(3,138)=13.87; p<0.0001]. There was also a significant main effect of light conditions, with mice tested in the dark phase having an increased mean number of boli compared to those tested in the light phase [F(1,46)=11.14; p=0.0017]. The mean values of number of boli tended to be lower on day 1 of testing in acutely stressed mice in the light phase and both in acutely and chronically stressed mice in the dark phase. However, the differences failed to be statistically significant (Figure 2).

Endocrine analysis

Shaker stress of 3 days produced activation of both the adrenal and pituitary axes in mice (Figure 3). ANOVA demonstrated a significant main effect of light conditions [F(1,28)=25.14; p<0.0001], treatment

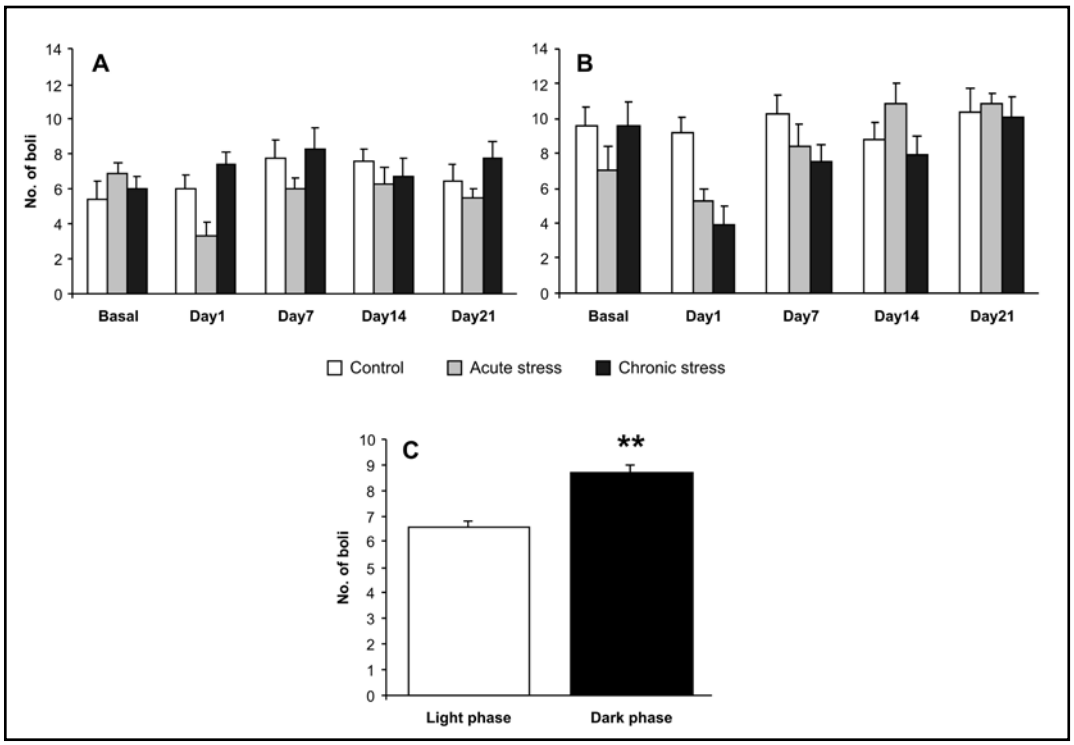

Figure 2. Effect of acute and chronic shaker stress on emotional reactivity of mice in the light (**A**) and in the dark phase (**B**) and the sum for the light and dark phase (**C**). Significant main effect of time (p<0.01) and light conditions (p<0.01) **p<0.01 - significant difference compared to the light phase.

[F(1,28)=131.01; p<0.0001] and interaction of both factors [F(1,28)=18.44; p<0.0002] on plasma corticosterone. Post hoc comparison showed that baseline corticosterone was non-significantly increased in the dark phase as compared to the light phase (light phase: 10.5±1.62 vs. dark phase: 22.0±2.12). There was a significant increase of corticosterone in chronically stressed mice compared to controls under both light conditions (p<0.01). The effect of chronic stress was more pronounced in the night period (increase in the dark compared to the light phase, p<0.01). However, corticosterone stress responsiveness expressed as a percentage change showed balanced increase in both phases of circadian rhythm (more then 10 times increase compared to controls).

For the pituitary oxytocin system, there was an increase during the light phase, but not during the dark period. ANOVA showed main effect of light conditions [F(1,23)=16.13; p<0.0005], treatment [F(1,23)=5.73; p<0.02] and interaction [F(1,23)=4.53; p<0.05]. Post hoc tests showed no significant differences in the baseline oxytocin between the light and dark periods. The oxytocin response to stress was observed only during the day (p<0.01) with no change seen at the dark phase. In contrast to corticosterone, the oxytocin stress response, expressed as percentage change, was significantly increased only during the light phase (approximately 60 % increase compared to control).

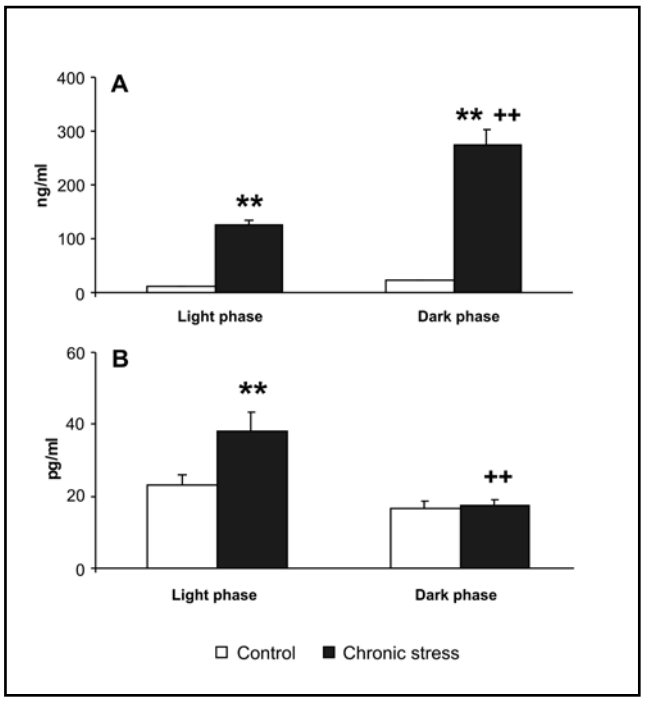

Figure 3. Effect of 3 days of shaker stress on plasma corticosterone (A) and oxytocin (B).
**p<0.01 - significant difference compared to controls, $^{++}$p<0.01 - significant difference compared to the light phase.

DISCUSSION

It is well established that stressors affect endocrine, cardiovascular and behavioral systems. We tested the effect of chronic stress in mice to determine whether there were diurnal changes in behavioral and endocrine responsiveness. The diurnal cycle had no effect on the stress-related behavioral responses but altered the posterior pituitary oxytocin system. There were no differences in open field behavior in mice tested under the light or dark periods. However, there was a marked difference in the light/dark pituitary stress response with an abolition of stress-induced oxytocin release in the dark period. Unlike oxytocin, the light/dark rhythm of stress responsiveness of corticosterone was not altered.

Shaker stress was originally described as an environmental model of stress (earthquake simulation), evoking appreciable neuroendocrine and cardiovascular responses (Nakata *et al*., 1993). Studies conducted in rats and mice showed that acute or chronic shaker stress increased blood pressure, heart rate and corticosterone, oxytocin and catecholamine secretion (Nishioka *et al*., 1998). We developed a similar paradigm in mice, using intermittent shaker stress with a specialized cage design that eliminates the need for animal handling (Bernatova *et al*., 2002b, 2004). The animals tolerated the stress well, showing only a small loss in body weight and a generalized good appearance. Likewise, in the present study, chronic stress produced a small decrease in body weight, which recovered over time. Evaluation of licking activity and food consumption showed that chronic stress was associated with an increase in water intake but not a change in food intake (Bernatova *et al*., 2002b). Thus, the decrease in body weight may be related to alterations in metabolism or nutrient absorption. Chronic mild and chronic restraint stress were found to decrease food and water intake in rats, resulting in weight loss (Hatcher *et al*., 1997; Momose *et al*., 1999; Harris *et al*., 2002). Our previous study also showed that chronic stress was associated with cardiovascular and endocrine changes, elevation of blood pressure, heart rate and corticosterone secretion. Plasma corticosterone was significantly increased in acutely as well as in chronically stressed mice, with a partial attenuation in the chronically stressed mice under both light conditions. Interestingly, the pressor responses showed a diurnal pattern with the greatest responses noted during the day when the animals are normally sleeping (Bernatova *et al*., 2002b). This diurnal pattern in cardiovascular stress responsiveness was replicated in a subsequent study using genetically modified mice (Bernatova *et al*., 2004).

Stress has been shown to cause a variety of behavioral responses in laboratory animals, with most studies conducted in rats. Long-term restraint stress (29 days) produced a slight decrease in body weight and reduced exploratory activity in the hole-board test (Armario *et al*., 1985). Acute restraint stress caused immobilization and a reduction in the number of head-dips when tested immediately after the stressful event (Armario *et al*., 1991). Acute exposure to a less traumatic stress

(noise and light) consistently increased open field activity, whereas, chronic stress reduced activity (Katz *et al*., 1981). A single session of social defeat in rats caused a marked reduction in open field activity, an effect which was still present days after the conflict (Meerlo *et al*., 1996). On the other hand, chronic social defeat in mice did not alter locomotor activity when tested during the light phase, while it increased activity during the dark phase (Keeney *et al*., 2001). A dose-dependent reduction of locomotor activity was seen after administration of a pharmacological stressor, interleukin-1β, in mice. The effect was dependent on the mice strain with greater effects observed in BALB/cJ than in C57BL/6J mice (Anisman *et al*., 2001).

Circadian rhythms are characterized by marked alterations in the neuroendocrine and neurochemical systems during the day/night periods (Lemmer *et al*., 1985; Kafka *et al*., 1986; Wirz-Justice, 1987). Thus, it is conceivable that there would be different responses to the same insult when evaluated under different light conditions. In a study using rats (Kelliher *et al*., 2000), time spent in escape-oriented behavior during the forced-swim test was significantly less when animals were tested in the dark phase. Similarly, chronic social defeat in male mice significantly increased locomotor activity only when it was tested in the dark phase (Keeney *et al*., 2001). In our study which used a different stress paradigm, there was no evidence of a circadian influence on the stress/behavioral interactions. There was a consistent habituation of the behavioral patterns which was not different if tested during the light or night phase. This suggests that the robust pattern of locomotor activity is not influenced by a relatively mild, well-tolerated stress.

In our study, corticosterone stress responsiveness expressed in absolute values was higher in the dark phase than in the light; however, the percentage changes were identical. There was a trend toward higher baseline corticosterone in the light which is well documented in nocturnally active animals (Kelliher *et al*., 2000, Barrett *et al*., 2000). This difference in baseline plasma corticosterone could explain the alteration in stress response. The finding is in contrast to the finding of increases in stress-induced corticosterone during the dark phase of the circadian rhythm (Retana-Marquez *et al*., 2003). In contrast to corticosterone, oxytocin was a more sensitive indicator of circadian rhythm-dependent stress responsiveness. While in the light phase there was a significant increase in oxytocin in response to chronic shaker stress, oxytocin stress responsiveness in the dark was abolished. Similar results were found by Bernatova *et al*. (2004) using oxytocin deficient mice suggesting that the endogenous oxytocin system is important in regulating the stress-induced responses. Animal as well as human studies show that oxytocin represents an important component of the stress axis. Oxytocin may be considered a typical stress hormone responding to osmotic as well as other stress stimuli (Morris and Alexander, 1989; Jezova *et al*., 1995; Forsling and Williams, 2002; Esch and Stefano, 2005). While the relationship between the pineal gland and pituitary function is controversial, the role of melatonin in the adaptation of the organism to the light/dark cycle is well recognized. Our results of the absence of an oxytocin stress response in the dark are in accordance with studies on human healthy volunteers. Forsling (2000) and Forsling and Williams (2002) found that melatonin produced dose-dependent changes in circulating oxytocin, the 0.5 mg dose being stimulatory, while 5.0 mg was inhibitory. Similarly, the nocturnal increase in oxytocin was absent after melatonin administration (Kostoglou-Athanassiou *et al*., 1998). We did not observe a light/dark rhythm in oxytocin levels in mice; however, there is evidence in rats for diurnal pattern of secretion (Forsling, 2000, Lightman *et al*., 2001). The lack of a circadian effect may be a methodological issue related to the limited blood samples (a single time point). In the dark phase of the circadian cycle, pineal and plasma melatonin levels were found to be elevated compared to the light phase (Ebihara *et al*., 1986; Kennaway *et al*., 2002). This increase in melatonin could abolish the oxytocin response to shaker stress in the dark period. The results indicate that melatonin modulates the neurohypophyseal responses to stressful stimuli. However, changes in oxytocin stress responsiveness in the dark period were not accompanied by measurable behavioral alterations.

Zone analysis revealed that mice tested in the dark phase traveled, spent, rested, entered as well as poked more frequently into the central zone of the open field. They also spent and rested less time in the peripheral zone than mice tested in the light phase. Overall, the results indicate a lower anxiety-like behavior of mice tested in the dark phase. We hypothesize that pineal melatonin and the hypothalamo-pituitary-adrenal axis (HPA) interactions might play a role in these changes. Administration of melatonin for 7 days was found to attenuate the HPA secretory responses to chronic stress and to decrease hypothalamic corticotrophin content (Konakchieva *et al*., 1997). A modulatory effect of melatonin on HPA function could in turn be one of the mechanisms by which it reversed depressive and anxiety-like behaviors induced by chronic stress (Kopp *et al*., 1999; Wong and Ong, 2001). This supports the idea that melatonin may be implicated in a homeostatic system that protects animals from behavioral changes induced by stress, e.g. a new environment or a predator. In contrast to decreased anxiety-like behavior, mice tested in the dark phase had higher emotional reactivity as expressed by the number of fecal boli. The increased defecation rate in the dark phase may be attributable to more eating and drinking by mice in their active phase.

Despite the lack of effect of stress on behavioral patterns in the present study, there is much published evidence to show that acute and chronic stress produce behavioral alterations in laboratory rodents. The intensity and range of these changes depends on the methodological approach and the species (rat vs. mice).

For example, novelty evokes different coping behavior in rats and mice. In a new environment, rats tend to freeze and decrease their locomotion while mice tend to escape or run and increase their activity (Pierce and Kalivas, 1997). The strain of mice also plays an important role in behavioral reactivity to stressful stimuli. C57/Bl/6J mice are generally characterized as non-emotional and less fearful then other strains (van Gaalen and Steckler, 2000). Thus, failures of several laboratories to replicate behavioral results indicate a need for behavioral test standardization (Wahlsten, 2001).

In conclusion, chronic stress in mice produced differential changes in behavior and endocrine secretion, as related to the light/dark cycle. In contrast to corticosterone, oxytocin stress responsiveness was abolished in the dark phase as compared to the light period. The results may suggest a modulatory effect of the increased nocturnal melatonin on the pituitary oxytocin in reaction to stress. Hormonal changes in stress response were not accompanied by any behavioral alterations.

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