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Social subordination impairs hypothalamic-pituitary-adrenal function in female rhesus monkeys

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Abstract

Linear dominance hierarchies organize and maintain stability in female rhesus macaque (*Macaca mulatta*) social groups regardless of group size. As a consequence of their low social status, subordinate females suffer from an array of adverse outcomes including reproductive compromise, impaired immune function, and poor cardiovascular health. However, data that differentiate limbic-hypothalamic-pituitary adrenal axis (LHPA) parameters between dominant from subordinate female monkeys are inconsistent, bringing into question whether social subordination alters the LHPA axis in female macaques. One difficulty in examining LHPA function in macaques may be the confounding effects of cycling ovarian steroids that are known to modulate LHPA activity. The current study used ovariectomized dominant and subordinate female rhesus monkeys to examine the effect that social subordination has on LHPA function by measuring morning and diurnal serum cortisol levels, dexamethasone (Dex) suppression of cortisol, metabolic clearance of Dex, and ACTH stimulation of adrenal cortisol release and cortisol response following exposure to acute social isolation. Compared to dominant females, subordinate females showed diminished morning peak cortisol secretion, weakened glucocorticoid negative feedback, and decreased adrenal cortisol response to an ACTH challenge as well as a restrained cortisol response following social isolation. However, the metabolism of Dex did not account for differences in Dex suppression between dominant and subordinate females. These results indicate that the ability to mount and limit glucocorticoid release is significantly reduced by psychosocial stress in female rhesus macaques, suggesting a hyporesponsive LHPA phenotype which resembles that observed in several human psychopathologies.

Keywords

social subordination; dexamethasone; cortisol; ACTH; psychosocial stress; monkeys

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Declaration of Interest

The authors have no conflicts of interest to disclose.

Introduction

The psychogenic component of chronic stress is implicated in the development of a number of adverse health outcomes in human beings including depression and anxiety illnesses, drug addiction and obesity (Juster et al.; McEwen, 2008; Oroszi and Goldman, 2004; Pasquali and Vicennati, 2000; Sinha, 2008). Exposure to psychogenic stressors involves activation of cortico-limbic circuits that modulate both sympathetic and limbic-hypothalamic pituitary-adrenal (LHPA) responses (Choi et al., 2008; Herman et al., 2003; Jankord and Herman, 2008; Ulrich-Lai and Herman, 2009). Whereas acute physical or psychological stress initiates a coordinated sequence of responses to manage energy resources and ultimately restore homeostasis (McEwen; Schulkin et al., 1994), the chronic unpredictable nature of certain stressors can overwhelm these reactive and restorative mechanisms and result in the dysregulation of central and peripheral circuits regulating stress and behavioral responses (McEwen, 1998).

Many paradigms involving laboratory animals have been developed to examine how chronic stress may produce negative health outcomes. However, many of these animal models lack a psychosocial component and/or consist of repetitive and predicable procedures in which animals eventually adapt and do not continue to exhibit stress hormone or behavioral responses to that stressor (Armario, 2006 Jaferi, 2006; Bhatnagar and Dallman, 1998; Bhatnagar and Vining, 2003; Bhatnagar et al., 2004; Bhatnagar et al., 2006; Jaferi and Bhatnagar, 2006). Therefore, while interesting from a functional perspective, these paradigms do not reproduce the effects of chronic stress as it is most often experienced by human beings, that being psychosocial and uncontrollable or unpredictable in nature (Tamashiro et al., 2005). For instance, the experiences of people in war and in low socio-economic conditions are two examples that typify the human experience of chronic stress. Certainly, these two examples of chronic stress that affect people are related to a range of psychopathologies [reviewed in (Breslau, 2001; Gallo et al., 2004; Isovaara et al., 2006; Lemaire et al., 1994; Lemstra et al., 2009; McEwen, 2000)]. Hence, animal models developed to approximate chronic stress as experienced by humans, and intended to study the etiology of human psychopathologies, should have a strong psychosocial component (Anisman and Matheson, 2005; Huhman, 2006), and produce the type of stress similar to that encountered by people (Tamashiro et al., 2005).

Socially housed rhesus monkeys provide an ethologically relevant opportunity to study the impact of chronic psychosocial stress on a number of health-related outcomes (Sapolsky, 2005). When housed socially, female rhesus or cynomolgus macaques organize themselves into a linear dominance hierarchy wherein subordinate members are under constant harassment by more dominant animals (Bernstein and Gordon, 1974; Bernstein et al., 1974; Shively and Kaplan, 1984) and have less control over their social and physical environments (Bernstein, 1976; Sapolsky, 2005), mimicking the matrilineal hierarchy in free-ranging groups (Altmann, 1962; Sade, 1967). However, as observed even in free-ranging groups, this harassment is most often unpredictable and thus largely psychogenic in nature (Silk, 2002). Furthermore, in small experimentally formed groups where social support characteristic of large groups is lacking (Brent et al., 2011), subordinate animals suffer from an array of stress-related maladies, including reproduction compromise (Kaplan et al., 2010; Michopoulos et al., 2009); emotional feeding that includes increased calorie consumption when a high fat, high carbohydrate diet is available (Arce et al., 2010; Michopoulos et al., 2012b), immune dysfunction (Paiardini et al., 2009); altered reward pathways and psychostimulant self-administration (Grant et al., 1998; Morgan et al., 2002); altered gene expression (Tung et al., 2012); and cardiovascular health (Kaplan and Manuck, 1999). However, a number of LHPA parameters do not consistently differentiate dominant versus subordinate females, notably morning cortisol (Czoty et al., 2009; Gust et al., 1993b;

Stavisky et al., 2001), diurnal cortisol (Arce et al., 2010; Collura et al., 2009) or the response to adrenocorticotropic hormone (ACTH) (Riddick et al., 2009; Shively, 1998; Shively et al., 1997b). On the other hand, other studies have defined LHPA dysregulation in subordinate animals by increased adrenal size (Shively and Kaplan, 1984; Shively, 1998) and decreased glucocorticoid negative feedback following dexamethasone (Dex) injection (Collura et al., 2009; Jarrell et al., 2008; Kaplan et al., 2010; Shively, 1998; Shively et al., 1997b; Wilson et al., 2008). Thus, questions remain as to whether social subordination in macaque females is, in fact, a chronic stressor.

It is possible, however, that decreased glucocorticoid feedback in subordinate females can be explained apart from any central or pituitary dysregulation affecting LHPA activity. For example, metabolic clearance of Dex may be slower in dominant females, resulting in enhanced exposure and greater feedback inhibition. A second possible confounding factor may be the presence of ovarian hormones. Because decreased glucocorticoid negative feedback has been related to activity of estradiol in subordinate female macaques (Wilson et al., 2005), and because sex steroids can modulate adrenal morphology and function (Kasprzak et al., 1986; Malendowicz, 1986; Malendowicz and Jachimowicz, 1982) and the diurnal release of cortisol (Smith and Norman, 1987; Toufexis et al., 1999) the use of cycling female macaques in previous studies comparing feedback inhibition, diurnal and ACTH-induced cortisol release between dominant and subordinate females (Riddick et al., 2009; Shively, 1998; Shively et al., 1997b) may have contributed to variable results in tests measuring both of these parameters.

The goal of this study was to define parameters of LHPA regulation of cortisol secretion in female rhesus monkeys as a function of social status position within their dominance hierarchy. To eliminate the confound produced by changing ovarian steroid levels, we measured morning cortisol, diurnal cortisol rhythm, the cortisol response to a dexamethasone (Dex) suppression test, ACTH-stimulation of cortisol release, and response to an acute stressor in ovariectomized (OVX), socially-housed female rhesus macaques. As a part of the Dex suppression test, we assessed whether the metabolic clearance of Dex differed by social status that could explain differences in Dex sensitivity. Additionally, by using several small social groups wherein the social dominance hierarchy was experimentally predetermined, we were able to examine the effect of psychosocial stress on LHPA activity apart from any genetic predisposition towards social status or social support from family members characteristic of more naturalistic social group housing that may mitigate adverse effects of social subordination. We hypothesized that subordinate females will show decreased peak morning cortisol levels, blunted diurnal cortisol levels, diminished glucocorticoid negative feedback, decreased ACTH responsivity, and reduced cortisol response to an acute stressor, compared to dominant females. We predict that results will substantiate the contention that chronic social stress dysregulates the LHPA axis in this primate species.

Material and Methods

Animals

Adult subjects (n=40) were long-term ovariectomized adult female rhesus monkeys (*Macaca mulatta*) housed in small social groups consisting of six animals each (5 females and 1 male) at the Yerkes National Primate Research Center (YNPRC) Field Station. The eight small groups were experimentally established as previously described (Jarrell et al., 2008) prior to the beginning of this study. Briefly, multiparous females, ranging in age from 11 to 17 yr (mean ± SEM: 13.5 ± 0.38 yr), were removed from multi-male, multi-female breeding groups at the Yerkes NPRC Field Station. Unfamiliar, unrelated females were added to a new group over a one-week period. Several related females were in separate groups.

Dominance ranks were quickly established with minimal contact aggression. Groups were housed in adjacent indoor-outdoor runs, with each area measuring 3.8 by 3.8 by 3.8 m. In the months prior to new group formation, all females were ovariectomized as a part of NIH-funded studies to determine the effects of psychosocial stress, mediated by social subordination on a number of behavioral, metabolic and reproductive outcomes (Collura et al., 2009; Jarrell et al., 2008; Michopoulos et al., 2009; Michopoulos et al., 2011b; Michopoulos et al., 2012b; Michopoulos and Wilson, 2011) that required brief replacement therapy with estradiol and/or progesterone. Just prior to the initiation of this study, a single adult male was introduced to each group without incident and did not change the female hierarchy. Each male became the alpha member of the group. Animals were fed Purina monkey chow (PMI 5038) twice daily and received daily supplements with seasonal fruits or vegetables. All animals had ready access to monkey chow as it was available *ad libitum* both in the outdoor and indoor area, minimizing competition for food. The only time food was unavailable was during the one hour daily cleaning of the runs. Water was available at all times *ad libitum* in two locations within each housing unit. Social groups had been stable for four years prior to the initiation of this study. The Emory University Institutional Animal Care and Use Committee approved all procedures in accordance with the Animal Welfare Act and the US Department of Health and Human Services “Guide for the Care and Use of Laboratory Animals.”

Behavioral data and social status determinations

Behavioral data using an established ethogram were collected for 30 min twice weekly during the six weeks of the initial phase as described previously (Jarrell et al., 2008). Observational sessions were done several hours after feeding between 1300 and 1400 hr, thus eliminating competition for food during the observational periods. Affiliative behavior was comprised of proximity and grooming; aggression was defined by threats, slaps, grabs, and bites; and submissive behavior was characterized by withdrawals, grimaces, and screams. Anxiety-like behavior consisted of body shakes, yawns, self-scratching, and self-grooming (Troisi, 2002). Data were recorded on a notebook computer using a data acquisition program that records behavior in an actor – behavior – recipient format (Graves and Wallen, 2006). Dominance ranks were determined from dyadic interactions observed during the focal observations described above. A female was defined as being subordinate to another animals if she unequivocally emitted a submissive gesture (grimace, withdrawal, or scream) to another animal (Bernstein, 1976). The most subordinate female, number five, submits to all other group members while the most dominant, alpha female submits to none. Inter-observer reliability was greater than 92%.

Morning basal cortisol, Diurnal cortisol levels, and Dexamethasone Suppression Test (DST) Protocol

Subjects were evaluated under two different doses of Dex, 0.125 mg/kg, IM or 0.25 mg/kg, IM in a counterbalanced manner separated by a one-month interval. Pre Dex serum samples were collected at 0800, 1100, and 1730 hr. Morning baseline cortisol was accessed from the 0800 hr samples while all pre DEX samples obtained at 0800, 1100, and 1730 hr allowed us to evaluate social status differences in diurnal cortisol levels. Immediately following the sample at 1730 hr, females received the Dex injection. Post Dex samples were collected the following morning at 0800 and 1100 hr for the analysis of serum cortisol and Dex. A subordinate female failed to respond to the low dose Dex and a dominant female failed to respond to the high dose Dex, likely due to a faulty injection. These two females were excluded from the DST analysis.

Adrenal response to ACTH—In addition to the two DST’s, animals were also evaluated by an ACTH stimulation test. Following a previously described protocol (Shively, 1998),

subjects received an injection of dexamethasone (0.5 mg/kg, IM) at 0800 immediately following the collection of baseline plasma sample. Four hours later, at 1200 hr, a second serum sample was obtained followed immediately by an IV bolus of ACTH (10 ng/kg). Cortisol was assayed in additional samples collected 15 and 30 minutes following ACTH administration.

Social separation as an acute stressor

The brief removal and isolation of a monkey from her social group is a potent acute psychosocial stressor (Collura et al., 2009). To determine subordinate females show a reduced response to a social separation, each female was removed from her group and a serum sample immediately collected (time 0). She was the confined in a transfer box (18 × 15 × 26 in.) for a 40-minute duration after which a second sample was obtained, and then the female was returned to her group (Arce et al., 2010). The social separation for each female occurred between 0900 and 1100 hr. Samples were assayed for cortisol. One dominant female was excluded from this analysis as she was being treated for a gastrointestinal infection at the time of the study.

Sampling and assay methods—All subjects were habituated to being removed from their group for conscious venipuncture using procedures in place in this lab for over 35 years (Bernstein and Gordon, 1977; Blank et al., 1983; Walker et al., 1982). Briefly, animals were trained to move from the housing unit into a transfer box upon a cue from the research staff. For a given group, all females were removed from their housing within one minute. Once in the transfer box, an animal is placed in a specialized cage designed for venipuncture. The cage allows a monkey to voluntarily place a leg through one of two small openings in the front of the cage. The research staff holds the leg so that a blood sample can be obtained from the saphenous vein or an IV or IM injection administered. The order in which females in a group entered the cage was unrelated to rank. For a given assessment (DST or ACTH challenge), an entire group (n = 5) was sampled on the same day. Blood samples were obtained within 10 min from entering the animal area to minimize arousal. Females were back in their group within 10 minutes of the completion of the blood sampling procedure.

Assays were performed in the YNPRC Biomarkers Core Lab. Serum levels of cortisol were determined by radioimmunoassay (RIA) with a commercially available kit (Beckman-Coulter/DSL, Webster TX) as described previously for rhesus monkeys (Jarrell et al., 2008). Using 25 μl, the assay has a range from 0.5 to 60 μg/dl with an inter- and intra-assay CV of 4.9% and 8.7%, respectively. Dex quantification was achieved using liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) on a Thermo Scientific LTQ-Orbitrap mass spectrometer and Surveyor HPLC system. Calibrators of Dex-spiked rhesus macaque serum were employed to define the standard curve and assay range. All samples and calibrators were spiked with 20 ng of flumethasone and subjected to diethyl ether liquid-liquid extraction. Extracts were subsequently evaporated to dryness under nitrogen at 37 °C and resolubilized for LC-MS/MS analysis in 200 μL of 30:70 water/methanol. The LC-MS/MS analysis was performed by negative ion APCI with SRM (selected reaction monitoring) detection and reverse phase chromatography. Chromatographic separations were accomplished using a linear gradient from 40:60 5 mM ammonium acetate/methanol to 100% methanol on a Supelco Discovery C8 column (50 × 2.1 mm, 5 μm particles). Using 200 μl, the assay has a range from 1 to 50 ng/ml with an inter- and intra-assay CV of 3.32% and 4.09%

Statistical analyses

Using conventions in place for nearly 30 years (Kaplan et al., 1984), females ranked 1 and 2 were considered dominant and females ranked 3–5 were classified as subordinate. This

convention has been used by us and others to assess dominance status effects on a number of outcome measures in captive macaques (Kaplan et al., 1995; Kaplan et al., 1984; Kaplan et al., 2010; Kaplan and Manuck, 2008; Kaplan et al., 1982; Kaplan et al., 2002; Michopoulos et al., 2009; Michopoulos et al., 2011a; Michopoulos et al., 2010; Paiardini et al., 2009; Shively and Kaplan, 1984; Shively, 1998; Shively and Clarkson, 1994; Shively et al., 1997a; Shively et al., 1997b). The total number of subjects classified in this fashion was 16 dominant and 24 subordinate females. Data (Supplementary Table 1) were summarized as mean ± standard error of the mean (SEM). All test results with a $p \leq 0.05$ were considered significant.

Status differences in baseline peak morning cortisol were analyzed by a t-test. The diurnal pattern of serum cortisol was analyzed by repeated measures analysis of variance. The main effects of status (dominant vs. subordinate), time pre and post both doses of Dex, as well as their interactions were analyzed with analysis of variance (ANOVA) for repeated measures. Furthermore, the change in cortisol levels (pre- to post-Dex administration) due to the two doses of Dex was analyzed by repeated measures ANOVA. Repeated measure ANOVA was also used for the analysis of the status differences in the response across time to ACTH administration. In addition, the area under the curve (AUC) for the cortisol response to ACTH, reflecting ground cortisol as a measure of total cortisol output (Pruessner et al., 2003) was analyzed by a t-test. When interactions or main effect of time were significant, Fisher post-hoc tests were completed, with appropriate corrections for multiple comparisons. The response in serum cortisol to the acute social separation test was analyzed by repeated measures ANOVA. Finally, for these analyses the specific social group (1 – 8) each female was a member of was considered as a variable in the ANOVA. However, this factor did not significantly explain any variance in the outcomes ($p > 0.20$), and was thus not further described.

Pearson product moment correlations between the change in cortisol in response to Dex at 0800 hr, the AUC of cortisol and response to ACTH, and the change in cortisol resulting from the social separation were calculated with social status and total behavioral frequencies. These variables were also used as predictors in multiple linear regression analyses of outcome measures from the Dex and ACTH tests. In addition, stepwise multiple linear regression models were used to determine significant predictors for the suppression in serum cortisol by Dex as well as the area under the curve for the increase in serum cortisol following ACTH administration. Finally, boxplots were included showing variance in both of these outcome measures as a function of social rank.

Results

Social Status Categorization

The ranks within each of the 8 groups were linear; none were unequivocal or bidirectional. Figure 1 shows rates of aggression received and submissive behavior emitted for monkeys at each social dominance rank position. These data reflect agonistic behavior taken at six 30-minute observations throughout the study. As expected, there was a significant effect of rank on aggression received ($F_{4, 35} = 8.17$, $p<0.001$) and submissive behavior emitted ($F_{4, 35} = 4.11$, $p=0.008$) (Figure 1A). Categorizing females ranked 1 and 2 as dominant and those ranked 3 through 5 as subordinate results in a significant main effect of social status for submissive behaviors emitted ($F_{1,38} = 8.45$, $p=0.006$) and aggression received ($F_{1, 38} = 11.86$, $p=0.001$) (Figure 1B).

Morning cortisol levels

Peak basal cortisol was obtained from samples collected at 0800 prior to each dose of Dex administration. Since these represent two samples of cortisol at each time of day, samples were averaged for analysis. Morning baseline samples at 0800 hr were significantly higher in dominant compared to subordinate females (27.7 ± 1.33 vs. 23.9 ± 1.09 μg/dl, respectively; $t_{38} = 2.23$, $p = 0.032$).

Diurnal cortisol secretion

Diurnal changes in serum cortisol were obtained from samples collected at 0800, 1100, and 1730 hr, all prior to each dose of Dex administration (Table 1B). Because these represent two samples of cortisol at each time of day, samples were averaged for analysis. As shown in Table 1B, serum cortisol decreased significantly form 0800 through 1730 hr ($F_{2, 76} = 10.12$, $p < 0.001$), but this effect of time was not influenced significantly by social status ($p = 0.167$).

Glucocorticoid negative feedback

Serum cortisol levels varied significantly over time following Dex administration (Table 1A; $F_{4, 144} = 285.27$, $p < 0.001$). Cortisol levels at 0800 and 1100 post Dex administration were significantly lower than baseline levels of cortisol at 0800 ($p < 0.001$) and 1100 ($p < 0.001$) pre Dex administration (Table 1A). This main effect of time interacted significantly with dose of Dex administered ($F_{4, 144} = 6.52$, $p < 0.001$). Whereas cortisol values were not significantly different between the low (3.52 ± 0.40 μg/dl) and high dose (3.54 ± 0.40 μg/dl, $p = 0.63$) at the 0800 time following Dex, cortisol levels at 1100 post Dex were significantly higher following the low dose (4.92 ± 0.54 μg/dl) compared to the high dose (3.58 ± 0.40 μg/dl; $p = 0.004$).

There was a significant status by time from Dex interaction ($F_{4, 144} = 2.97$, $p = 0.021$) that was not influenced by dose ($p = 0.917$), as serum cortisol was significantly higher in subordinates (4.24 ± 0.43 μg/dl) compared with dominant females (3.54 ± 0.53 μg/dl) following Dex [even through, as described above, peak baseline early morning cortisol pre Dex was higher in dominant females (27.72 ± 1.26 vs. 23.27 ± 1.02 μg/dl)]. Consequently, the suppression in cortisol due to Dex administration was determined by calculating the change in cortisol from the pre Dex to post Dex levels at both 0800 and 1100 for both doses of Dex. There was a main effect of status, as suppression of cortisol by Dex was significantly less in subordinates (−18.57 ± 0.82 μg/dl) compared with dominant females (−21.50 ± 1.01 μg/dl; $F_{1, 36} = 5.08$, $p = 0.030$). The high dose of Dex induced a significantly greater change in serum cortisol (−21.49 ± 0.85 μg/dl) compared with the low dose (−18.58 ± 0.82 μg/dl; $F_{1, 36} = 7.69$, $p=0.009$) and this effect was similar for subordinate vs. dominant animals (no dose by status interaction; $p = 0.54$). However, this effect of status did vary significantly over time from Dex ($F_{1, 36} = 4.15$, $p = 0.049$). The change in serum cortisol was significant greater at 0800 following either dose of Dex in dominant (−24.54 ± 1.31 μg/dl) compared with subordinates (−19.39 ± 1.06 μg/dl; $F_{1, 36} = 12.55$, $p=0.001$), an effect that disappeared by 1100 ($p = 0.95$; Figure 3). Boxplots for the response to Dex (Figure 4A) show that dominant (rank 1 and 2) females were similar yet distinct from subordinates (ranked 3 – 5).

Multiple regression analyses were performed to determine how social status and serum levels of Dex resulting from the injections predicted serum concentrations of cortisol. For the low dose Dex, the change in serum cortisol at 0800 hr was significantly predicted by social status ($R = 0.45$, $p = 0.004$) while serum Dex failed to significantly improve predictability of the model ($p = 0.80$). Similarly, for the high dose Dex, the change in serum cortisol at 0800 hr was significantly predicted by social status ($R = 0.43$, $p = 0.008$) while

serum Dex also failed to significantly improve predictability of the model ($p = 0.73$). Neither social status nor serum Dex significantly accounted for variance in the change in serum cortisol at 1100 hr following the low or high dose Dex.

Adrenal responsivity to ACTH

Serum cortisol varied significantly over time during the ACTH stimulation test with highest values at 0800 prior to Dex suppression and nadir values four hours later prior to ACTH administration ($F_{3, 114} = 81.09$; $p < 0.001$). Subsequent to the ACTH injection, cortisol levels peaked at +15 min (Figure 3A). Importantly, time interacted with social status ($F_{3, 114} = 2.57$, $p = 0.05$) to influence cortisol responsivity as dominant animals showed greater elevations in cortisol levels following ACTH administration at +15 ($p = 0.021$) compared to subordinate females (Figure 3A). The analysis of area under the curve (AUC) of cortisol levels showed that dominant animals had overall increased cortisol levels in response to the ACTH compared to subordinate females (Figure 3B; $F_{1, 38} = 5.86$, $p=0.020$). Boxplots for response to ACTH (Figure 4B) show a more rank-related gradient for the response to ACTH whereas the response to Dex for females ranked 1 and 2 was similar yet distinct from those ranked 3 – 5 (Figure 4A).

Cortisol response to the social separation

The brief 40-minute separation of a female from her group significantly elevated serum cortisol (29.08 ± 1.48 μg/dl) compared with baseline (time 0) values (15.99 ± 0.82 μg/dl; $F_{1, 37} = 128.13$, $p < 0.001$). However, this response was unaffected by social status, as their was not a significant main effect of status ($F_{1, 37} = 0.32$, $p = 0.58$) or a status by time interaction ($F_{1, 37} = 0.17$, $p = 0.69$). Values of serum cortisol at time 0 and 40 minutes for dominant and subordinate females are shown in Table 1C.

Predictors of cortisol responsivity

Table 2 shows Pearson product moment correlations between social status, behavioral frequencies, and outcome measures of Dex suppression (change in serum cortisol at 0800 hr), ACTH stimulation (AUC), and the change in cortisol to the social separation. As expected, subordinate status was significantly related to aggression received ($p = 0.001$) from others and submission directed towards others ($p = 0.003$). Aggression directed towards others was not related to status, indicating that the highest-ranking females are not necessary the most aggressive. More dominant females more often received proximity from males while more subordinate females more often received proximity from other females. Importantly, there was a significant negative correlation of social status with Dex suppression ($p = 0.004$) and ACTH stimulation ($p = 0.02$), indicating these responses decrease with more subordination. Status was unrelated to the increase in serum cortisol in response to the social separation, suggesting it was a potent acute stressor for all females. Females that showed a greater suppression in serum cortisol in the DST (generally dominant females) more often directed affiliative behavior (grooming and initiation of proximity) to others ($p = 0.057$). In addition, the increase in cortisol following ACTH was positively related to the amount of aggression directed to others ($p = 0.026$) and negatively related to aggression received from others ($p = 0.027$) and submissive behaviors emitted ($p = 0.030$). Females that showed a bigger response to ACTH also received more proximity from the male ($p = 0.002$). Finally, there was also a significant negative correlation between Dex suppression and ACTH stimulation (Figure 5; $p = 0.009$), indicating that animals showing a reduced sensitivity to Dex suppression also showed reduced increases in response to ACTH. While the change in serum cortisol following the social separation was unrelated to cortisol following the ACTH challenge ($p = 0.18$), females showing a greater suppression following Dex showed a greater increase following the social suppuration ($p = 0.02$).

Stepwise multiple regression analyses were performed to determine how social status and behavioral frequencies predicted the change in serum cortisol following Dex, the AUC for cortisol following ACTH stimulation, and the increase in cortisol following the social separation. With respect to the average change in serum cortisol at 0800 hr following the low and high dose of Dex, more subordinate status ($\beta = 0.45$) predicted a smaller change serum cortisol ($R^2 = 0.21$ $p = 0.004$). No other predictors were entered into the equation. A similar analysis of the AUC for serum cortisol following ACTH stimulation revealed that the statistical combination of more proximity received from males ($\beta = 0.47$), more aggression directed to others ($\beta = 0.36$), and lower rates of anxiety ($\beta = -0.28$) significantly predicted a greater increase in serum cortisol following ACTH ($R^2 = 0.43$, $p = 0.02$). No variables entered the multiple regression equation for the increase in serum cortisol following the social separation.

Discussion

Results here show that social subordination in female rhesus monkeys leads to a disruption in feedback inhibition of the LHPA axis that occurs in the absence of ovarian hormones, and is not due to differences in dexamethasone metabolism following treatment. Moreover, the data suggest that adrenal responsivity is reduced in subordinate females resulting in a dampened cortisol response to ACTH. This reduced adrenal sensitivity may also contribute to the blunted diurnal peak of cortisol observed in subordinate females. In addition, data here show that even in response to the potent psychological stressor of social separation, monkeys with reduced negative feedback responses to dexamethasone (predominantly subordinate females) mount significantly reduced cortisol responses compared to those with more robust dexamethasone feedback inhibition (predominately dominant females). Furthermore, while delayed access to resources such as food and water in free-ranging groups may provide an additional source of stress for subordinate females (Sade, 1967), it is an unlikely stressor in these small, provisioned groups, as we have seen no social status difference in food intake of the typical low fat, high fiber Purina chow; rather subordinate females consume significantly more calories compared with dominant females when fed a high fat, high sugar diet (Arce et al., 2010; Michopoulos et al., 2012b). Thus, our data suggest that chronic exposure to psychosocial stress in female rhesus macaques, induced by rank-related differences in agonistic behaviors, produces a distinct LHPA phenotype in which the ability to both mobilize and curtail glucocorticoids is significantly compromised, and the overall efficiency of LHPA axis reactivity is considerably diminished.

Previous studies indicate that exposure to subordination in macaques (Collura et al., 2009; Jarrell et al., 2008; Kaplan et al., 2010; Shively, 1998; Shively et al., 1997b) and chronic exposure to stressors in rodents (Young, 1998) decreases glucocorticoid negative feedback similar to humans suffering from depression and other psychopathologies (Holsboer, 2001; Kalin et al., 1982; Raison and Miller, 2003). Rodents subjected to chronic stress often show increased basal glucocorticoid level; however, this is found only in a subset of human beings suffering from psychiatric disorders (Capuron et al., 2003). Dysregulation of glucocorticoid receptor (GR) signaling is more consistently implicated in human psychopathology (Raison and Miller, 2003). Diminished negative feedback inhibition of the LHPA axis in rodents is due to decreases of GR and mineralocorticoid receptors in the hippocampus and the hypothalamus (Aguilera and Rabadan-Diehl, 2000; Bhatnagar and Dallman, 1998; Kovacs et al., 2000). In monkeys, this resistance to dexamethasone suppression likely reflects a decrease in limbic GR expression (Brooke et al., 1994). This is also suggested to be the case in humans (Pariante and Miller, 2001). In rats, reduced glucocorticoid sensitivity is associated with chronic exposure to stressors is linked to increases of corticotropin-releasing hormone (CRH) in the central nucleus of the amygdala and the bed nucleus of the stria terminalis (Albeck et al., 1997; Keen-Rhinehart et al., 2009; Stout et al., 2000) and increases

arginine vasopressin (AVP) in the paraventricular nucleus of the hypothalamus (PVN) (Ma et al., 1999; Makino et al., 1995). Analysis of CRH and AVP in the cerebral spinal fluid from dominant and subordinate female macaques is planned to examine if these neuropeptides are also altered by chronic psychosocial stress.

In this study dominant females had significantly higher morning peak basal cortisol levels compared with subordinates but do not show a difference in diurnal cortisol levels. These results suggest a blunting of peak cortisol levels as a consequence of social subordination in rhesus macaques. This finding is important because blunting of morning cortisol levels is often present in psychopathologies (Bao et al., 2008; Rubin et al., 1987; Swaab et al., 2005), especially those in which women are over-represented. Consistent differences in peak morning cortisol due to social status in macaques species has not been reported in the literature thus far (Czoty et al., 2009; Gust et al., 1993b; Stavisky et al., 2001). It is possible these discrepancies are a result of the small sample size usual to studies in non-human primates. The effect of sex hormones in the control of diurnal cortisol secretion may be another factor. A previous study examining the effect of ovariectomy on circadian cortisol in female macaques found that absolute levels of cortisol were lower in OVX females, but there was no disruption of the circadian rhythm of cortisol (Smith and Norman, 1987). However, female monkeys in this investigation showed a rise in mid-day cortisol that may have been caused by the feeding or room-cleaning schedule that took place regularly at 12 p.m. each day. The rise in cortisol at mid-day may have activated negative–feedback mechanisms that would have caused a decline in evening levels of cortisol and masked accurate detection of basal cortisol. Studies with OVX rats replaced with estrogen found that the evening elevation in corticosterone was significantly enhanced by estrogen, and estrogen treatment reversed the blunting of the diurnal rise in corticosterone by Dex injection (Weiser and Handa, 2009). Hence, it may be that using a relatively large number of monkeys here, and/or by using only OVX females so as to preclude differences due to sampling females who are at different points in their ovarian cycles, unmasked the blunted morning peak cortisol release occurring in subordinate female monkeys.

The cortisol response to ACTH administration during an ACTH challenge has also been inconsistent in differentiating dominant from subordinate macaques (Riddick et al., 2009; Shively, 1998; Shively et al., 1997b). In this study dominant animals had increased cortisol levels in response to the ACTH compared to subordinate animals. Thus, despite the adrenal enlargement that is observed in necroscopic studies from subordinate macaques (Shively and Kaplan, 1984) and which we are assuming is the case in the subordinate females in this study, these females did not have enhanced cortisol release following ACTH. Chronic stress produces enlargement of the adrenal gland and hypercortisolemia in rats (Blanchard et al., 1993; Spencer and McEwen, 1990; Tache et al., 1978). Enlarged adrenals concomitant with increased secretion of cortisol are present in some populations of people with clinical depression (Nemeroff et al., 1992; Rubin and Phillips, 1993; Rubin et al., 1995; Stokes, 1995), and are thought to be predictive of ensuing depression for people undergoing stressful life events (Ising et al., 2005). However, studies of chronic stress effects on adrenal structure and function in laboratory rats are limited in duration and thus are probably not producing the same stage of chronic stress exposure as is present in our subordinate monkeys. One study in rodents that disambiguated adrenal reactivity (that is increased ACTH-stimulated corticosterone release due to enlargement of tissue and enhanced out-put) from increased sensitivity (increased ACTH-stimulated corticosterone due to increased adrenal cortical response to ACTH) suggested that 14 days of chronic variable stress increased adrenal reactivity and not sensitivity (Ulrich-Lai et al., 2006). Again, 14 days of stress, however intense, is unlikely to duplicate the months of chronic psychogenic stress experienced by subordinate female macaques living in groups for years. Indeed, we speculate that a similar change in adrenal size and output is occurring in the first weeks

following the onset of social subordination in monkeys and in the clinical studies on stressed people mentioned above. Certainly, the experimental imposition of social status used in this present study may have accelerated this effect on adrenal physiology. However, we believe that a more probable explanation for enlarged adrenals concomitant with reduced ATCH stimulated cortisol as seen in this present study is adrenal exhaustion. *In vitro* studies on extracted adrenal cortical tissue have shown that constant application of ACTH first causes tissue hypertrophy and increased glucocorticoid release followed by a drastic reduction in glucocorticoid secretion (Lamberts et al., 1987).

Previous studies in cynomolgus macaques assessing adrenal response to ACTH administration have shown either increased cortisol response to ACTH (Czoty et al., 2009) in subordinate males compared to dominant male monkeys or no social status differences in this LHPA measure in female monkeys (Riddick et al., 2009). While species differences could account for the differences in ACTH response, the simplest explanation is that in these studies, LHPA activity is modulated by hormonal status, as has been seen in rodents (Viau, 2002; Viau et al., 2005; Viau et al., 2001), monkeys (Stavisky et al., 2003; Wilson et al., 2005; Wood et al., 2004), and women (Burleson et al., 1998; Gudmundsson et al., 1999). Testosterone and estrogen have been shown to affect adrenal sensitivity to ACTH in male and female rats (Handa et al., 1994; Nowak et al., 1995; Sapolsky et al., 1983) as well as in men and women (Goel and Bale; Kudielka and Kirschbaum, 2005; Young, 1998). The study by Riddick and colleagues cited above (Riddick et al., 2009) tested adrenal responses to ACTH during several days of the follicular phase in ovarian intact female cynomolgus monkeys. Therefore, the differences in ACTH induced cortisol between this investigation and that prior study by could be specifically due to sampling affected by individual differences in changing levels of sex steroids that modulated the responsivity of the adrenal gland.

In this study we used brief period of social separation to determine the LHPA response of female monkeys to a potent species-specific stressor. The use of social separation is a powerful stressor for rhesus monkeys because they are both highly social and extremely xenophobic animals (Bernstein and Gordon, 1974; Gust et al., 1993a). Results showed that while subordinate and dominant females both responded to this strong psychogenic stress with significantly elevated cortisol, the magnitude of the response was predicted by the degree of cortisol suppression observed in the DSTs. Females with the greatest degree of cortisol suppression following the DSTs mounted the greatest cortisol response to social separation, and females with the weakest suppression of cortisol following the DSTs mounted the weakest cortisol response to social separation. In other words, the reduced glucocorticoid negative feedback following dexamethasone, observed significantly more often in subordinate females, was correlated with the lowest cortisol response in the social separation test. Results from the social separation test show that under circumstances that are particularly distressful, subordinate female monkeys can mount a sufficient LHPA response, but the level of that response is curtailed due to changes in LHPA reactivity that are status-dependent. It is important to note at this point that this study did not include a controlled injection of CRH. Such a treatment would act on the pituitary to stimulate ACTH release and would have revealed differences in pituitary corticotrope sensitivity due to social status. Compensatory changes in the responsivity of the pituitary due to chronic stress (De Goeij et al., 1992a; De Goeij et al., 1992b; Lee et al., 2011) and reproductive stage (Toufexis et al., 1999) have been suggested to occur in rodents, and in war veterans with PTSD (Golier et al., 2011). Similar compensatory changes may take place due to chronic psychogenic stress in subordinate monkeys. This compensatory change may permit an adequate glucocorticoid response in the face of a severe stressor like social separation to occur despite the overall lethargy of the LHPA axis. Future studies are planned to determine

if changes in pituitary responsivity to CRH and/or AVP exist in subordinate female monkeys.

The primary approach used in this present study was to quantify LHPA responsivity in females from multiple 5-member groups, categorizing dominants as females ranked 1 and 2 and subordinates as those ranked 3 – 5. This convention has been used for nearly 30 years to assess dominance status effects on a vast number of outcome measures in captive macaques (e.g., (Kaplan, 2008; Kaplan et al., 1995; Kaplan et al., 1984; Kaplan et al., 2010; Kaplan et al., 1982; Kaplan et al., 2002; Michopoulos et al., 2011b; Michopoulos et al., 2012a; Michopoulos et al., 2010; Paiardini et al., 2009; Shively and Kaplan, 1984; Shively, 1998; Shively and Clarkson, 1994; Shively et al., 1997a), an approach similar to the analysis of social stress effects in people (Marmot, 2006). The accepted rationale for this approach is to increase statistical power by comparing females who receive limited aggression and must submit less to those that receive proportionately more aggression and more frequently terminate these interactions by submitting more. Examination of the boxplots for both the response to dexamethasone and ACTH (Figure 4) shows a more rank-related gradient for the response to ACTH whereas the response to Dex for females ranked 1 and 2 was similar yet distinct from those ranked 3 – 5. The biological mechanism accounting for these two patterns of stress hormone responsivity are unexplored, but the data imply there is not a gradient in the loss of GR with increasing subordinate status.

In addition, the correlational analysis shows two key behaviors defining subordinate status, aggression received from others and submission emitted, are highly predicted from rank but not perfectly, suggesting, for example, that the lowest ranking female does not always received the most aggression nor are the most submissive in terms of frequency of behavior. As long as a female submits to every other monkey in her group, she would be classified as the most subordinate. However, the regression analysis showed that submissive behavior was the best predictor of the efficacy of Dex suppression. Because submissive behavior is emitted in response to perceived threats, it likely is a strong reflection of the number of attempts necessary to terminate potentially harmful interactions. While the use assessment of specific rank related differences in LHPA regulatory mechanisms would be informative, the present data nonetheless shows distinct phenotypes between more dominant compared with more subordinate females.

Overall, results here suggest that social subordination in female rhesus monkeys results in a diminished adrenal response to ACTH-induced stimulation, the absence of a morning peak in cortisol, a restrained capacity to mount a cortisol response to a severe psychogenic stressor, along with impaired inhibitory control following LHPA axis activation. Compilations of myriad studies in animals and humans have led to the conclusion that there is an optimum level of LHPA functionality that exists to deal with the metabolic and behavioral requirements of stress in the most advantageous fashion. Consequently, under or over activity of the LHPA system are both suboptimal and increase allostatic load on the organism and the propensity for dysregulation [for review see:(Chrousos, 2009)]. Dominant female monkeys here display a finely tuned LHPA system allowing an unconstrained response to stress as well as efficient inhibition subsequent to activation. Importantly, dominant monkeys here retain the ability to mount a robust cortisol response following dexamethasone suppression. In fact, the degree of suppression after Dex predicts the subsequent response to ACTH stimulation, further underscoring the flexibility of LHPA reactivity in dominant animals. Subordinate females in the current study were unable to mount a similar response in either direction. While these data suggest that the LHPA hyporesponsivity is the *consequence* of social subordination, it is possible that in more naturalistic environments this physiological phenotype is a trait that predisposes a particular animal towards becoming socially subordinate. A study in male vervet monkeys showed that

cortisol levels were the highest among males who eventually became dominant subsequent to the dominant males removal (McGuire et al., 1986), suggesting that a robust LHPA response is integral to establishing dominance. In baboons it has been shown that the level of LHPA axis activation may be conditional on the stability of the social dominance structure (Sapolsky, 1992). Thus, there are additional factors to be considered when examining the function of the LHPA axis in various primate species. This notwithstanding, a previous study that experimentally re-arranged the established ranks of female cynomolgus monkeys shows that LHPA differences emerge with current social status and are not due to individual characteristics (Shively et al., 1997b).

Several human pathologies are related to both under and over active LHPA responses (Chrousos, 2009) similar to those shown by subordinate females in the present study. Those related to hyporesponsive LHPA activity include: adult post traumatic stress disorder and seasonal affective disorder (Chrousos and Gold, 1992), premenstrual syndrome (Chrousos et al., 1998), chronic fatigue syndrome (Clauw and Chrousos, 1997), postpartum depression (Magiakou et al., 1996), fibromyalgia (Clauw and Chrousos, 1997), and perimenopausal depression (Chrousos et al., 1998). The particular deficits in the LHPA system observed in our subordinate female monkeys might be particularly relevant to the study of these human disorders. Indeed, considering the relationship that is believed to exist between stress and human illness (McEwen, 2000), many physiological as well as psychological similarities may exist between human beings and macaques given that psychosocial stress in these monkeys produces a related type of LHPA dysregulation.

In conclusion, the changes in LHPA activity characteristic of social subordination in rhesus females indicate that this non-human primate species represents an ethological valid animal model with which to study the mechanisms whereby chronic stress disrupts behavior and physiology in women. Furthermore, since individual traits, such as genetic variation, can modify individual responses to psychosocial stress exposure (Caspi et al., 2003), social subordination in macaque females can be used to identify genetic and trait differences that might be critical for understanding individual response to adverse psychosocial environments. Importantly, it must be emphasized that our experimentally constructed groups, while recapitulating the strict linear hierarchy of rhesus monkeys groups regardless of size (Bernstein and Mason, 1970), differs in many ways from free ranging populations of rhesus (Brent et al., 2011) and other old world monkeys (Wittig et al., 2008) in that the adverse effects of subordinate status may be mitigated by social support from group mates and maintaining distance from more dominant animals. It is important to point out that the variability present in the data presented here is predominantly due to differences in social status while the variability due to other factors, such as previous experimental interventions, early social experience, or genes, is undetermined. Future studies will elucidate the mechanisms by which subordination increases vulnerability to glucocorticoid signaling insufficiency and how this susceptibility is influenced by engaging in prosocial social behaviors with conspecifics. Nonetheless the present study shows that social subordination in rhesus females is an appropriate model to study how LHPA dysregulation may lead to chronic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- Social subordination alters stress axis activity in female rhesus monkeys
- Compared to dominant females, subordinates show blunted morning cortisol levels,
- Subordinates have diminished glucocorticoid negative feedback,
- And, subordinate females have decreased cortisol response to ACTH.
- Social subordination is an ethologically valid model of chronic stress exposure

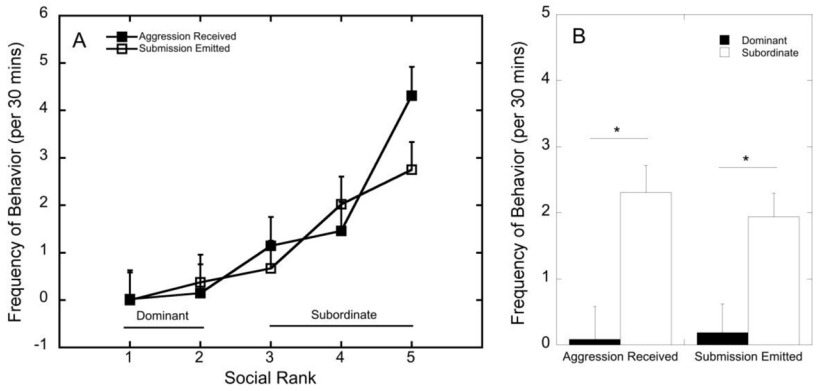

Figure 1.

Panel A. Rates (per 30 min) of aggressive behavior received and submission behavior emitted by females at each social dominance rank. Rates of behavior were averaged across the 12 observational sessions for a particular female. Rates were then averaged across females to generate the mean ± SEM rate for a specific rank. Panel B. Mean ± SEM rates of aggression received and submission towards others in females categorized as dominant rank 1 and 2, and subordinate (ranks 3 – 5). Rates of aggression received ($p = 0.001$) and submission emitted ($p = 0.003$) were higher in animals categorized as subordinate females (ranks 3 – 5) compared with those categorized as dominant (ranks 1 – 2).

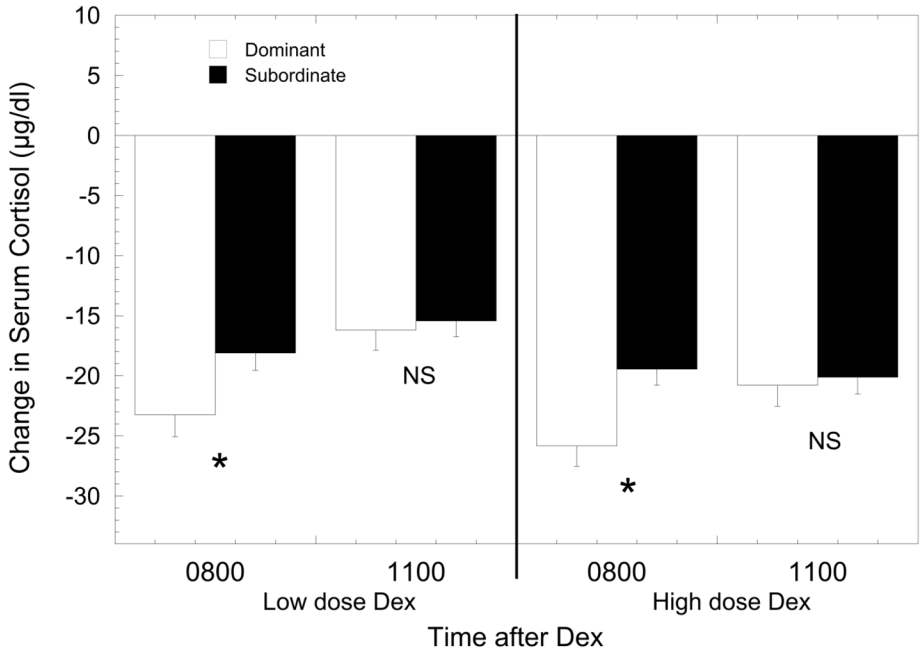

Figure 2.

Mean ± SEM change of serum cortisol at 0800 and 1100 hr following low (0.125 mg/kg) and high dose Dex (0.25 mg/kg) administered at 1730 hr the evening before for dominant and subordinate females. Asterisk denotes significantly greater decrease in cortisol at both doses in dominant compared to subordinate females ($p < 0.05$).

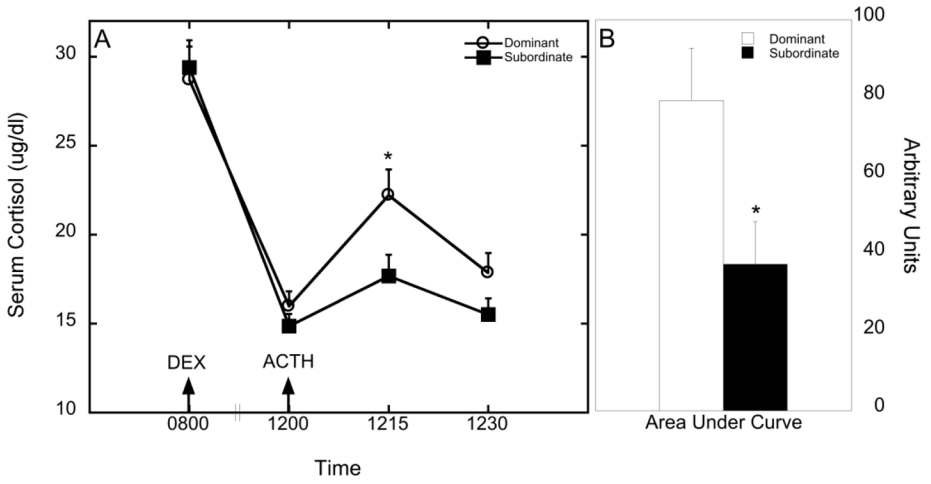

Figure 3.

Panel A shows the mean ± SEM levels of serum cortisol during the ACTH stimulation test for dominant and subordinate females. Dex (0.50 mg/kg) was administered following the sample at 0800 hr and ACTH (10 ng/ml) following the sample at 1200 hr. Panel B shows the mean ± SEM values for the area under the curve of cortisol levels following ACTH administration. Asterisks denote significant status differences in cortisol levels.

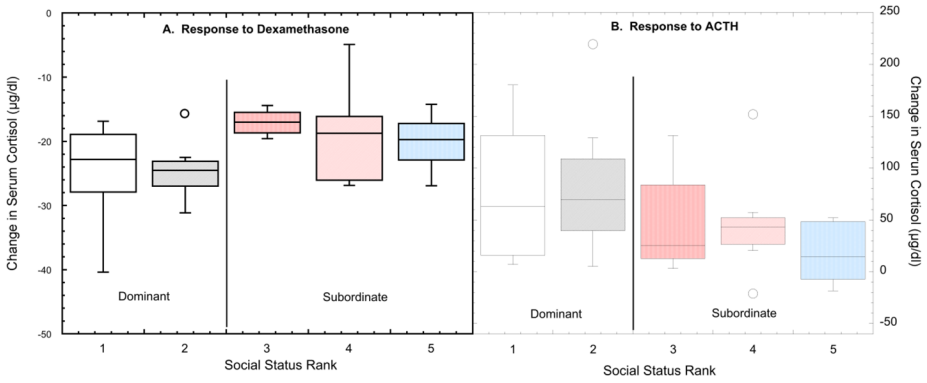

Figure 4.
Boxplots of the change in serum cortisol at 0800 hr following Dex suppression (A) and the area under the cortisol response curve following ACTH administration (B).

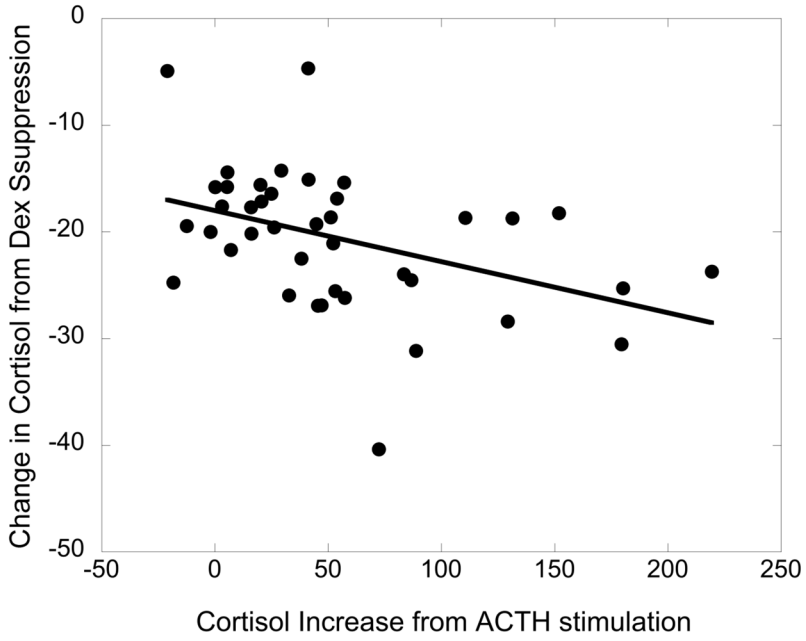

Figure 5.
Pearson product moment correlations between the cortisol area under the curve response to ACTH administration and the mean change in serum cortisol at 0800 hr following Dex suppression (averaged across low and high dose of Dex).

Table 1

(A) Mean ± SEM levels of serum cortisol (μg/dl) prior to and following two doses of dexamethasone (Low 0.125 and High 0.25 mg/kg) as a part of a DST. (B) Mean ± SEM levels of diurnal cortisol levels prior to Dex administration collapsed by Dex dose. (C) Serum cortisol before and following a 40-minute social separation from the group.

	Time	0800	1100	1730	0800	1100
A	**Low Dose**					
	Dominant	26.42 ± 1.53	20.66 ± 1.49	19.9 ± 1.56	3.17 ± 0.63*	4.45 ± 0.84*
	Subordinate	22.97 ± 1.24	21.16 ± 1.20	18.6 ± 1.26	3.87 ± 0.51*	5.87 ± 0.68*
	High Dose					
	Dominant	29.02 ± 1.53	24.07 ± 1.75	24.1 ± 1.67	3.18 ± 0.63*	3.36 ± 0.62*
	Subordinate	23.57 ± 1.23	23.52 ± 1.42	21.9 ± 1.35	3.89 ± 0.51*	3.80 ± 0.50*
B	**Diurnal Average**					
	Dominant	27.74 ± 1.33	22.92 ± 1.41	22.06 ± 1.47	-	-
	Subordinate	23.91 ± 1.09	22.91 ± 1.15	20.65 ± 1.20	-	-
C	**Response to social separation**	Time 0	Time 40			
	Dominant	16.35 ± 1.29[a]	29.90 ± 2.32[b]	-	-	-
	Subordinate	15.64 ± 1.02[a]	28.25 ± 1.83[b]	-	-	-

Asterisks denote significantly lower levels of cortisol following administration of Dex compared to baseline values prior to Dex. Differently letters denote serum cortisol was significantly higher at 30 minutes following social separation compared to baseline.

Table 2

Pearson product moment correlations between social status (dominant vs. subordinate), measures of the change in serum cortisol in response to dexamethasone suppression (Dex), ACTH stimulation, and response to social separation with rates of behavior.

Variable	Social Status	Dex Suppression	ACTH stimulation	Social Separation
Social Status	-	-	-	-
Dex suppression	0.45**	-	-	-
ACTH stimulation	−0.37*	−0.39*	-	-
Social separation	−0.07	−0.38*	0.22	-
Aggression - actor	−0.12	−0.07	0.35*	0.04
Aggression - recipient	0.49**	0.18	−0.35*	−0.04
Submission - actor	0.45**	0.19	−0.34*	0.17
Groom received from females	0.09	−0.11	−0.06	0.21
Groom received from male	−0.26	−0.14	0.15	−0.16
Prox received from females	0.40**	0.17	−0.16	0.20
Prox received from male	−0.37*	−0.25	0.47**	0.13
Affiliation directed to others	−0.29	−0.31[a]	0.15	0.13
Anxiety	−0.13	−0.02	−0.21	0.02

* $p < 0.05$;

** $p < 0.01$,

[a] $p < 0.06$.