

Nervous temperament in infant monkeys is associated with reduced sensitivity of leukocytes to cortisol's influence on trafficking

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ABSTRACT

There is growing evidence that temperament/personality factors are associated with immune function and health-related outcomes. Neuroticism, in particular, is a risk-factor for several diseases, many with a strong inflammatory component. We propose that neuroticism (or nervous temperament in monkeys) is related to dysregulation of immune function by glucocorticoids. The present study tested the hypothesis that animals with a nervous temperament would show no relationship between cortisol concentrations and leukocyte numbers in peripheral blood (an easily obtainable measure of glucocorticoid-mediated immune function), while animals low on this factor would show expected relationships. Infant rhesus monkeys ($n = 1507$) experienced a standardized testing procedure involving blood sampling, behavioral tests, and temperament ratings. Results confirmed the hypothesis: low-nervous animals showed the expected positive relationship between cortisol levels and neutrophil numbers, while high-nervous animals showed no relationship. High-nervous animals also showed elevated cortisol concentrations at most sample points, and responded to a human challenge with more negative emotional behavior. These data suggest that individuals with a nervous temperament show evidence of glucocorticoid desensitization of immune cells. Differences with other studies, including the specific types of leukocytes that are affected, are discussed, and implications for disease processes are suggested.

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1. Introduction

The idea that stable differences in behavioral dispositions are associated with health-related outcomes is an ancient one, dating back at least to Greek and Roman times, when imbalances in bodily humors were believed to be associated both with temperament and physical disease (Capitanio, 2008). While the humoral theory is no longer considered valid, empirical research has documented many links between personality or temperament traits (terms that I will use interchangeably), immune function, and health outcomes. Conceptually, the influence of such traits on health can occur via three broad, and not mutually exclusive, routes (Capitanio, *in press*). First, temperament can have an impact primarily through behavioral means. For example, the trait conscientiousness has long been known to be associated with longevity (Friedman, 2008), and one suggested mechanism for this relationship has been through the role of conscientiousness on health behaviors: conscientious people tend to take better care of themselves (Bogg and Roberts, 2004),

which could translate into a longer lifespan. The other two ways in which temperament may influence health are more directly physiological. On the one hand, it is possible that individuals of a particular personality type are “built” differently from others, in ways that could impact a disease process. Elsewhere, we have referred to this as a “main effects” model (Capitanio, *in press*), and recently, we suggested an example of such a phenomenon: adult rhesus monkeys that were low in Sociability (a major personality dimension in human and non-human primates) had greater sympathetic innervation of lymph nodes compared to high-Sociable monkeys, and innervation density was negatively related to a tetanus-specific IgG response (Sloan et al., 2008). An alternative to the main effects model is an interaction model: temperament can impact health via its role in affecting coping responses – in stressful circumstances, animals with different temperament characteristics may cope in ways that lead to differences in activation of stress-response systems that can then influence immune function and disease processes (e.g., Capitanio et al., 2008).

Several studies have recently suggested that glucocorticoid (GC) regulation of inflammation may be a mechanism by which individuals may be at risk for inflammation-mediated disease. It has been long-known that one important physiologic role of GCs is to regulate immune function, and in particular, to counter inflammation

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(Munck and Guyre, 1986). There is growing evidence, however, that the experience of stress, particularly over long time frames, is associated with development of glucocorticoid resistance in immune cells, which can result in a reduction in the anti-inflammatory capabilities of these steroid hormones. For example, *in vitro* incubation of blood with lipopolysaccharide will stimulate inflammatory cytokine production, which will be suppressed in a dose–response fashion when different molar concentrations of GC are added to the culture. Chronically stressed individuals, however, do not show the same degree of steroid-induced suppression as do non-stressed individuals (Miller et al., 2002, 2005) – their leukocytes are more resistant to the anti-inflammatory effect of GCs. Because a principal mechanism of action of steroids is to regulate gene transcription, such results suggest that stressed individuals should show alterations in transcription of GC receptor-mediated signaling molecules. In fact, Miller et al. (2008) have reported that, compared to controls, chronically stressed humans show a down-regulation of genes that express one or more glucocorticoid response elements, and an up-regulation of genes bearing NF- κ B response elements, which are components of a signaling pathway that is pro-inflammatory. Importantly, these differences were evident even in the absence of differences in cortisol concentrations. This stress-induced alteration in GC regulation of immunity may explain the seemingly paradoxical findings of stress often being associated with increased (or no change in) cortisol concentrations along with increased risk of inflammation-related disease.

While the evidence is clear that glucocorticoid resistance (GCR) can be associated with experiences such as chronic stress, it is not known whether such a mechanism might mediate temperament-related differences in disease risk, and if so, whether it is best considered a “main effect” or an “interaction effect.” There is some indirect evidence that GCR may be associated with temperament, however. One of the most-studied dispositions in humans reflects a heightened tendency to show negative emotion, such as anger and hostility. The broader trait that subsumes negative affect and emotion is usually referred to as neuroticism, and has significant health consequences, having been associated with inflammation-related physical diseases such as atopic dermatitis, asthma, and irritable bowel syndrome (reviewed in Lahey (2009)). Marsland et al. (2008), studying a healthy community sample of 855 adults, found that plasma levels of the inflammatory markers IL-6 and C-reactive protein were associated with both trait negative affect and a measure of the behavioral component of hostility. While this study did not include measures of circulating cortisol concentrations, other studies have revealed higher circulating concentrations of daytime cortisol among individuals that are high in hostility and neuroticism (Nater et al., 2010; Pope and Smith, 1991; Ranjit et al., 2009; Suarez et al., 1998). Taken together, these studies illustrate the paradox referred to earlier, namely higher concentrations of cortisol associated with higher plasma concentrations of inflammatory markers, suggesting dysregulation in the HPA-immune axis. These studies further suggest that GCR might mediate the relationship between neuroticism and inflammation-related disease.

A number of measures exist that could be used to study a relationship between negative emotionality and GCR in immune cells, including cell culture and measures of transcriptional activity, as described above. Another measure is based on the fact that GCs also affect leukocyte trafficking dynamics. Administration of exogenous glucocorticoids, for example, results in increased numbers of neutrophils and decreased numbers of lymphocytes and monocytes in peripheral blood (Fauci et al., 1976). Similarly, correlations have been found in humans between cell numbers and endogenous concentrations of cortisol: positive relationships for neutrophils, and negative relationships for lymphocytes and monocytes. Importantly,

socially-related stress appears to abrogate these relationships in all three cell types (Cole, 2008). We reported similar results recently from an experiment in which adult male rhesus monkeys were randomized either to non-stressful, stable social conditions, or to stressful, unstable social conditions. Animals in the stable conditions showed the expected negative relationship between cortisol concentrations and lymphocyte numbers (though no effects were found for neutrophils or monocytes), while animals in unstable social conditions showed no such relationship (Cole et al., 2009). Together, these data are consistent with the idea that chronic stress can lead to development of GCR in immune cells. While it remains to be demonstrated within a single study that leukocyte trafficking, *in vitro* cell culture as described above, and transcriptional analysis all display a consistent picture of stress-related GCR, the easy accessibility of the blood compartment makes the cortisol–leukocyte relationship an attractive and “low-tech” biomarker of this phenomenon.

The present study was undertaken to test the specific hypothesis that rhesus monkeys that show negative emotionality (which we refer to as being high in “negative temperament”) would show an attenuated correlation between circulating cortisol concentrations and leukocyte numbers, compared to animals judged to be low on this temperament dimension. We had no specific predictions that nervous temperament would be related to cortisol concentrations or to leukocyte numbers; rather our interest was in the relationship between the two sets of measures. Our focus was on the three principal leukocyte subsets (neutrophils, lymphocytes, and monocytes); we also examined two lymphocyte subsets in more detail to determine whether either or both subsets might be responsible for a hypothesized effect on the broader lymphocyte class. To test our hypothesis, we make use of data obtained from a multi-year study of the causes and consequences of variation in biobehavioral organization in infant rhesus monkeys (Capitanio et al., 2006). In addition, we present additional data showing that low versus high negative temperament is also associated with measures of HPA regulation that are consistent with the idea of high-nervous animals showing GCR.

2. Methods

2.1. Subjects and living arrangements

Subjects were 1507 (639 males, 42.4%) infant rhesus monkeys (*Macaca mulatta*) born to mothers that lived in any of 17 stable, outdoor, 0.2 hectare enclosures. Each enclosure contained up to 150 animals of all ages and both sexes, which approximated the composition of a troop of rhesus monkeys in the wild. Social groups were provisioned twice daily with commercial monkey chow, twice weekly with fruits and vegetables, and water was available *ad libitum*.

2.2. Procedures

At 3–4 months of age (mean = 107.5 days, range = 89–133 days) each animal participated in a biobehavioral assessment (BBA) program conducted at CNPRC that has been described in detail elsewhere (Capitanio et al., 2005, 2006; Golub et al., 2009). Briefly, cohorts of up to eight animals at a time were separated from their mothers and relocated to an indoor testing area at 0900 h. Each animal in a cohort was housed in an individual holding cage (60 cm \times 65 cm \times 79 cm, Lab Products, Inc., Maywood, NJ), containing a cloth diaper, a stuffed terrycloth duck, and a novel, manipulable object. Over the next 25-h period, behavioral data were collected in a variety of standardized situations (described in Golub et al. (2009) and Capitanio et al. (2006)) and blood was

drawn on four occasions. At the conclusion of this period, infants were returned to their mothers (who had been housed in a separate location out of sensory contact with their infants), and mother–infant pairs were returned to their outdoor enclosures. Three sets of measures from the BBA program were used in the present analysis.

2.2.1. Blood samples

Data obtained from blood samples were the principal outcome measures. Blood was sampled via femoral venipuncture following manual restraint on four occasions during the 25-h assessment period: two hours after maternal separation and relocation (1100 h: Sample 1); five hours later (1600 h: Sample 2); at 0830 h on Day 2 (Sample 3); and at 0900 h on Day 2 (Sample 4). Immediately following Sample 2, animals were injected with 500 µg/kg dexamethasone im, and immediately following Sample 3, animals received 2.5 IU ACTH im; thus, cortisol concentrations from Samples 3 and 4 reflect pharmacologic treatments designed to assess regulatory aspects of the hypothalamic–pituitary–adrenal axis. About 0.5 ml of whole blood from each sample was centrifuged for 10 min at 3000 rpm at 4 °C. Plasma was removed and decanted into tubes for storage at –80 °C. Samples were later assayed in duplicate using commercially available kits (Diagnostics Products Corporation, Los Angeles, CA). Inter- and intra-assay coefficients of variation were 5.8% and 7.9%, respectively. Cortisol values were available for all animals for Sample 1, but owing to missed samples, the full set of four values was available only for 1434 animals.

A second 0.5 ml aliquot of blood from Sample 1 was transferred to an EDTA tube, and hematology and flow cytometry were performed by CNPRC's Clinical Laboratory. Complete blood counts were performed using a Serono Baker Diagnostic System (Allentown, PA), and all electronic counts were verified by a manual differential. About 50 µl aliquots were directly labeled with phycoerythrin (anti-CD4-M-T477; BD Pharmingen), peridinin chlorophyll-alpha protein (anti-CD8-SK1; BD Pharmingen), and fluorescein isothiocyanate (anti-CD3-SP34; BD Pharmingen). A Coulter Q-prep (Coulter Corp., Miami, FL) was used to lyse the red blood cells and fix the samples in paraformaldehyde. Lymphocytes were gated by forward and side light scatter. A FACS Calibur flow cytometer (BD Pharmingen) was used to phenotype the lymphocyte subsets.

2.2.2. Temperament ratings

Measures of temperament provide the independent variables for the present analysis. At the end of the 25-h period, the observer that had observed, handled, and interacted with the animals during the various assessments rated each infant using a list of 16 trait adjectives (Golub et al., 2009), and a seven point Likert scale for each trait. Assessment of inter-rater agreement and reliability for the data collection has been described (Weinstein and Capitano, 2008). Briefly, mean inter-rater reliability for the 16 individual items, assessed using an intra-class correlation, was 0.53. Inter-rater agreement, assessed using chi-square (Lawlis and Lu, 1972), was significantly greater than chance ($p < 0.00001$) for each item, and the mean T index, a kappa-based measure indicating the magnitude of agreement (Tinsley and Weiss, 1975), was 0.64, when different observers' ratings were allowed to vary from each other by one point. Exploratory (using promax rotation) and confirmatory factor analyses were conducted to identify and confirm (respectively) the latent structure of the rating data. A four factor solution was found to provide a close fit to the data (details of factor analyses and all fit indices are described in Golub et al. (2009)). Factor scores were calculated by summing the z-scores (computed for each year of data collection) for all adjective items loading on a given factor, and then z-scoring each scale. Cronbach's alpha values for the scales ranged from 0.6 to 0.9. The four scales, named for the

adjective with the highest factor loading, were: Vigilant (vigilant, NOT depressed, NOT tense, NOT timid), Gentle (gentle, calm, flexible, curious), Confident (confident, bold, active, curious, playful), Nervous (nervous, fearful, timid, NOT calm, NOT confident). The traits preceded by the word “not” reflect a negative loading in the factor analysis. The four scales were significantly inter-correlated, with Nervous correlating with Confident ($r = -0.51$), Vigilant ($r = -0.57$) and Gentle ($r = -0.68$). The correlation coefficients among the latter three scales were positive and ranged from $r = 0.52$ to $r = 0.73$.

2.2.3. Human Intruder

Data from a Human Intruder test, which is designed to assess the responsiveness of the infant to standardized and graded conditions of challenge (Karere et al., 2009), were used to explore behavioral differences between high- and low-nervous animals. A technician dressed in protective clothing first sat approximately 1 m away and presented a profile for 1 min. She then moved to within 0.3 m and continued to present a profile for 1 min. Next the technician returned to the 1 m position and attempted to maintain direct eye contact for 1 min. For the last trial she again moved to within 0.3 m of the cage and continued to maintain eye contact for 1 min. These 4 trials were designated profile far, profile near, stare far and stare near, respectively. The 4-min test session was video-recorded and later scored using The Observer (Noldus, 1991), and an ethogram comprising behavioral categories reflecting activity states (e.g., locomote, sleep, hang) and events such as negative emotional behaviors (threats, fear grimaces), positive emotional behaviors (lipsmack, proximity to the intruder), and anxiety-related behaviors (scratch, yawn, self-groom). All behavior categories and definitions are listed in Golub et al. (2009). Interobserver reliability for behavioral categories was greater than 85%. Owing to slight variations in the lengths of each trial, durations were converted to the proportion of time observed, and the frequencies were converted to the rate per 60 s. The Human Intruder test was performed at 1400 h, five hours after the initial separation and relocation of the infants.

2.3. Data analysis

Our principal hypothesis was that low-nervous animals would show significant correlations between plasma cortisol levels from Sample 1 and leukocyte counts derived from the same sample, whereas high-nervous animals would show an attenuated correlation. To test this hypothesis, we first used hierarchical multiple regression. Leukocyte subset numbers were the dependent measures, and independent variables included nervous temperament and cortisol concentration on the first step and an interaction term defined as the product of temperament and cortisol on the second step (to reduce multicollinearity, cortisol was centered; temperament factors were already z-scored). If the step containing the interaction term was significant, this suggested that the relationship between a particular cell subset and cortisol was influenced by temperament. To further explore a significant interaction, the sample was divided into deciles based on temperament score, and Pearson product-moment correlation coefficients were calculated between cortisol concentration and leukocyte subset for each decile. To insure that any observed effects were specific to the factor “nervous,” the multiple regression was re-run with the other three temperament scales entered as covariates. Contrasts between high- and low-nervous animals (defined based on the decile analysis) were made to determine whether significant differences in cortisol concentrations existed across the four sample points. Finally, in order to confirm the behavioral correlates of high- versus low-nervous temperament, means were calculated for each behavior category across the four challenge conditions

in the Human Intruder test, and multivariate analyses of variance were performed. For all repeated measures analyses, when the assumption of sphericity could not be met, *df* were adjusted using the Huynh–Feldt correction.

3. Results

3.1. Nervous temperament and cortisol regulation of leukocyte numbers in peripheral blood

Neutrophil numbers were positively associated with cortisol concentrations as expected, but addition of the interaction term in the analysis showed that these effects were significantly attenuated in animals that were high in nervous temperament. Table 1 summarizes the multiple regression analyses. Animals with a more nervous temperament had higher numbers of neutrophils, lymphocytes, CD4+ T-cells, and CD8+ T-cells in peripheral blood. In addition, animals with higher cortisol concentrations had higher neutrophil and monocyte numbers. After statistical control of these main effects, however, the interaction term explained significant additional variance for neutrophils: the negative regression coefficient indicates that for animals high in nervous temperament, the slope of the line describing the relationship between cortisol and neutrophils is flatter than is the slope for animals that are lower in nervous temperament.

To further characterize the relationship between nervous temperament, neutrophil numbers and cortisol, the sample of 1507 was divided into deciles based upon temperament score; owing to tied values, each of the 10 groups comprised 149–152 animals. Within each decile, correlation coefficients between cell numbers and cortisol concentrations were computed and tested to determine whether the coefficients were significantly different from zero. Fig. 1, which shows decile group on the *x*-axis and correlation coefficient for each decile on the *y*-axis, shows significant or near-significant positive correlations between cortisol concentrations and neutrophil numbers for the six deciles with the lowest scores on nervous temperament, but coefficients that were not significantly different from zero for the four deciles with the highest scores for nervous temperament. Consistent with the results of the regression analysis, parallel analyses for the other cell subsets revealed no consistent pattern; for example, for lymphocytes, none of the 10 correlation coefficients were statistically different from zero. Together, the regression and decile analyses show that animals that are low in nervous temperament (60% of the sample, based on the decile analysis) show the expected positive relationship between cortisol concentrations and neutrophil numbers,

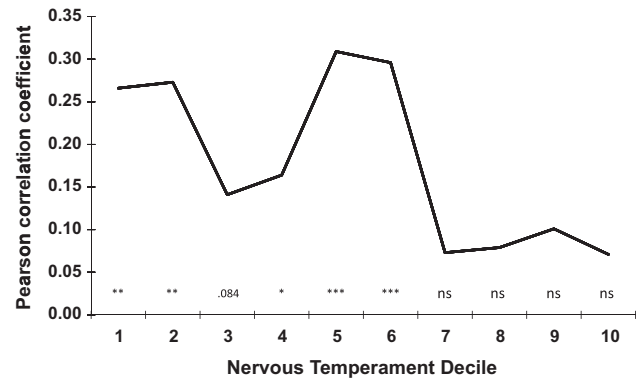


Fig. 1. Pearson product-moment correlation coefficients between plasma cortisol concentrations and neutrophil numbers for each decile of the nervous temperament factor. Sample sizes for each decile range from 149 to 152. Significance level is indicated above *x*-axis (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$).

while animals that are high in nervous temperament (the upper 40% of the distribution) show no such relationship. We depict these relationships in Fig. 2; owing to the large sample size we contrast animals in the lowest two deciles (Fig. 2A, low nervous, $n = 302$) with animals in the highest two deciles (Fig. 2B, high nervous, $n = 301$).

3.2. Specificity of effect for nervous temperament

Because of the significant inter-correlations among the four temperament scales, we examined the specificity of our result for nervous temperament by re-running the regression analysis with the other three temperament scales (gentle, vigilant, confident) included as covariates. For the neutrophil result, the step that included nervous temperament and cortisol concentration remained significant ($F(2, 1502) = 22.689$, $p < 0.001$), the step that included the three covariates was not significant ($p = 0.141$), and the step including the interaction term (nervous temperament \times cortisol) remained significant ($F(1, 1498) = 6.480$, $p = 0.011$). The standardized regression coefficient for the interaction term was unchanged from the original regression analysis ($\beta = -0.65$). This analysis demonstrates that the aforementioned relationship between nervous temperament, plasma cortisol, and neutrophil numbers is specific to the nervous temperament factor. Parallel analyses conducted with the other leukocyte subsets likewise revealed no differences in results compared to the original analyses shown in Table 1, and no significant effects for the step containing the covar-

Table 1
Standardized regression coefficients and summary of analysis.

Independent variables	Dependent variables				
	Neutrophils	Lymphocytes	Monocytes	CD4+ T-cells	CD8+ T-cells
Nervous temperament	0.053*	0.068**	-0.002	0.058*	0.069**
Cortisol concentration	0.161***	0.000	0.115***	-0.030	-0.023
Nervous \times cortisol interaction	-0.065*	-0.045	0.032	-0.029	-0.022
Step 1 statistics (nervous temperament and cortisol)					
<i>F</i> -Change value (2, 1504)	22.695***	3.538*	10.112***	3.136*	3.933*
Adjusted R^2	0.028	0.003	0.012	0.003	0.004
Step 2 statistics (interaction term of nervous temperament and cortisol)					
<i>F</i> -Change value (1, 1503)	6.476*	3.004	1.580	1.289	0.693
R^2 change from step 1	0.004	0.002	0.001	0.001	0.000

$n = 1507$ for all analyses.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

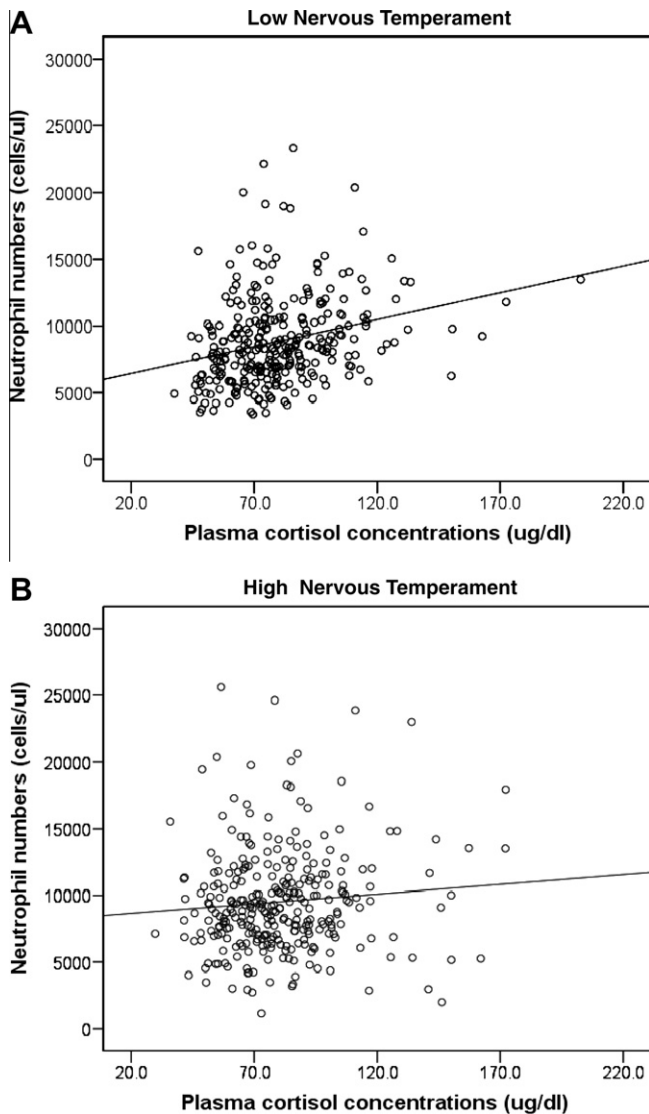


Fig. 2. Scatterplots showing the relationship between plasma cortisol concentrations and neutrophil numbers for low-nervous (A: top panel, lowest two deciles, $n = 302$) and high-nervous (B: bottom panel, highest two deciles, $n = 301$) infant rhesus monkeys.

iates, despite the substantial bivariate correlations between the four temperament scales.

3.3. HPA regulation

Our principal hypothesis, supported by the analyses described above, is that high nervous temperament is associated with altered glucocorticoid regulation of immune function, as indexed by leukocyte numbers in peripheral blood. One implication of this idea is that nervous temperament might also be associated with other measures of HPA regulation. To test this hypothesis, we contrasted low- and high-nervous animals on the four measures of cortisol taken during the 25-h biobehavioral assessment: the first sample taken at 1100 h (cortisol values from this sample are the ones used in all analyses so far), an afternoon sample at 1600 h, a dexamethasone-suppressed sample at 0830 on Day 2, and an ACTH-stimulated sample at 0900. To define low- versus high-nervous animals, we used the results of the decile analysis, shown in Fig. 1. Low-nervous animals were defined as those that were in the first six deciles (z -scores for nervous temperament were 0.257 or less), and high-nervous animals were in

the upper four deciles, with z -scores greater than 0.257. Repeated measures analysis of variance supported the prediction of differences in HPA regulation between low- and high-nervous animals. We found a significant main effect for Nervous temperament ($F(1, 1457) = 9.506$, $p = 0.002$) with high-nervous animals having higher cortisol overall. We also found a significant interaction of temperament status by sample ($F(1.93, 2811.28) = 5.48$, $p = 0.005$), and tests of simple effects revealed that high-nervous animals had significantly greater cortisol concentrations for the second (afternoon) sample ($F(1, 1457) = 11.04$, $p = 0.001$), for the third (dexamethasone-suppressed) sample ($F(1, 1457) = 8.15$, $p = 0.004$), and for the fourth (ACTH-stimulated) sample ($F(1, 1457) = 10.33$, $p = 0.001$) compared to low-nervous animals. Groups were not significantly different for Sample 1. Values for all samples are presented in Fig. 3.

3.4. Behavioral measures of nervous temperament

To confirm that the animals identified as high in nervous temperament also tended to respond with negative affect in stressful situations, we contrasted low- and high-nervous animals (defined as in the preceding section) on measures obtained during the Human Intruder test. Multivariate analysis of variance revealed that high-nervous animals displayed significantly higher rates of negative emotional behaviors (Pillai's Trace = 0.017, $F(3, 1493) = 8.59$, $p < 0.001$), lower rates of positive emotional behaviors (Pillai's Trace = 0.025, $F(2, 1500) = 19.05$, $p < 0.001$), and lower rates of anxious behavior (Pillai's Trace = 0.009, $F(3, 1499) = 4.79$, $p = 0.003$), but were not significantly different from low-nervous animals for durations of activity behaviors, such as active, crouch, and hang from the side of the cage ($p = 0.846$). Follow-up univariate tests showed that high- and low-nervous animals differed on the negative behaviors (Fig. 4A) threat ($p = 0.041$), fear grimace ($p < 0.001$), and bark vocalization ($p = 0.004$), on the positive behavior (Fig. 4B) time spent near the intruder ($p < 0.001$) but not lipsmack ($p = 0.744$), on the anxious behaviors (Fig. 4C) scratch ($p = 0.002$) and self-groom ($p = 0.045$) but not on the behavior yawn ($p = 0.174$).

4. Discussion

Our data show that infant rhesus monkeys with a nervous temperament respond to a stressful situation with frequent expressions of negative emotions and infrequent expressions of affiliation. High-nervous animals also display elevated cortisol

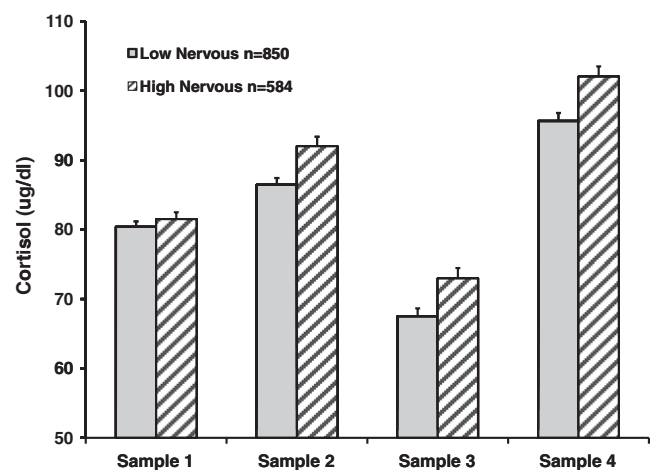


Fig. 3. Plasma cortisol concentrations for low- and high-nervous animals defined based on decile analysis (see text) at four time points during biobehavioral assessment. Significant group differences were evident for Samples 2, 3, and 4.

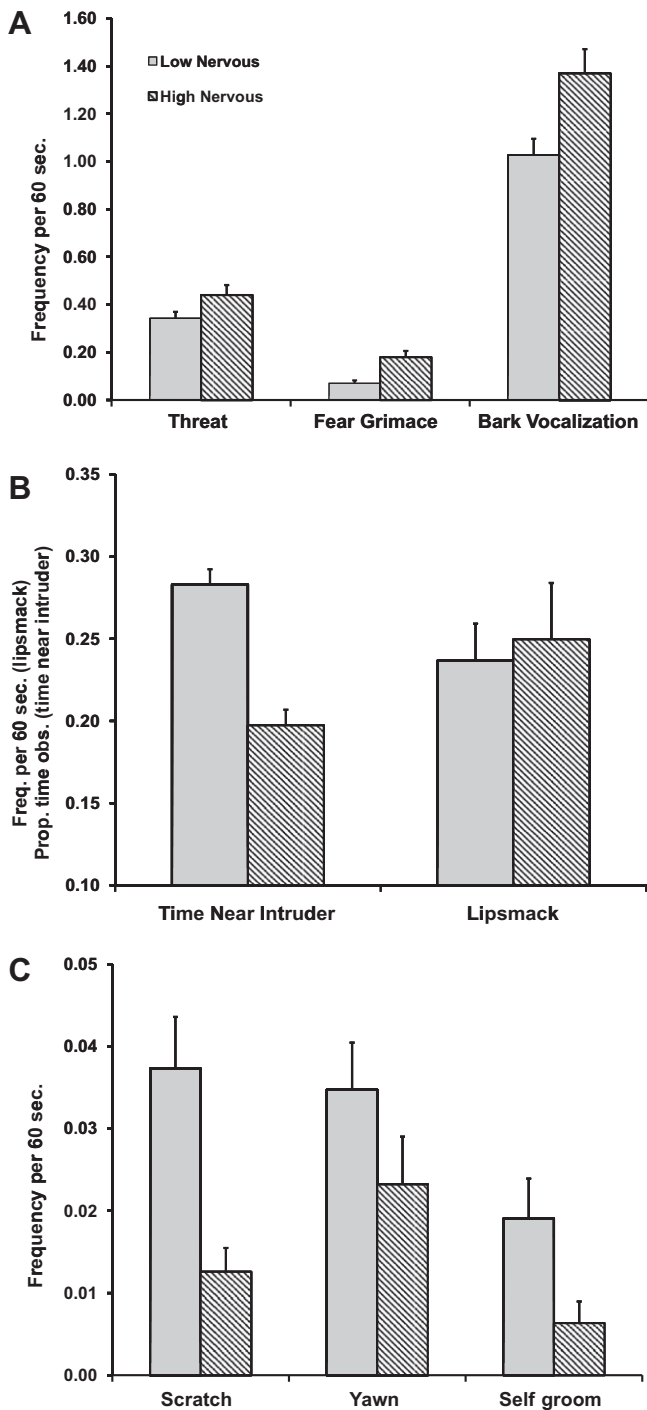


Fig. 4. Behavioral responses of low- and high-nervous animals in response to a Human Intruder: negative emotional behaviors (A), positive engagement (B), and anxious responses (C).

responses during the 25-h testing period, and show a disrupted relationship between plasma cortisol concentrations and neutrophil numbers in peripheral blood. This is in contrast to animals judged to be low in nervous temperament, which display less negative emotionality and more anxiety and affiliation, lower cortisol concentrations, and did show the expected positive relationship between cortisol concentration and neutrophil numbers. These results were specific to the temperament factor identified as “nervous.” Separate studies, described earlier, suggest that humans that are high in neuroticism and show negative affect, including

hostility, have elevated plasma markers of inflammation, and elevated cortisol levels, consistent with the idea of dysregulation of an HPA-immune axis. The present study confirms and extends those findings.

4.1. Nervous temperament and cortisol

Although we had no specific hypotheses about the role of temperament on cortisol levels, we found that nervous temperament was associated with elevated cortisol concentrations, as had been found in human studies. While many studies of neuroticism have assessed basal cortisol concentrations, some have focused on stressed values (Suarez et al., 1998) or values obtained in response to pharmacologic challenge (Zobel et al., 2004). In fact, our data closely parallel those found by Zobel et al. (2004), who studied a normal human population and conducted a dexamethasone suppression test and CRF challenge; in that study, individuals high in neuroticism had significantly higher cortisol levels than did individuals that were low on this dimension. Both studies suggest that the deficit within the HPA system is one of negative feedback, presumably as a consequence of reduced GC receptor number and/or efficiency.

While our analysis did show a main effect of temperament on cortisol concentrations, the significant interaction revealed that the low- and high-nervous groups did not differ for the first of the four samples. This was not a basal sample, but likely reflected the acute stress associated with separation from mother and relocation to an unfamiliar, indoor room. It is possible that the intensity of this experience overwhelmed any temperamental influences on the cortisol response; in fact, variation in the cortisol response to separation is often associated with variation in attachment security rather than temperament (e.g., Spangler and Grossmann, 1993). Nonetheless, as the BBA program continued, nervous temperament became increasingly important in mediating the biobehavioral responses to the situation, as shown in our Human Intruder data, and in the results for the other three cortisol samples.

We believe that the absence of a group difference in cortisol for Sample 1 highlights the idea that the difference between low- and high-nervous animals is one of regulation, and not differential responsiveness – members of both groups showed a similar initial HPA response to the separation (which would have begun immediately upon separation, two hours before our first blood sample was taken), but only low-nervous animals showed the expected relationship between cortisol levels and leukocyte numbers from that sample. This result suggests that it was not the acute stress response that caused the observed group difference; rather, the acute stressor was important in revealing a pre-existing difference in HPA-immune regulation between low- and high-nervous animals. We note that our failure to find group differences in cortisol concentrations while simultaneously finding differences in measures of HPA-immune regulation is consistent with other reports described above (Cole et al., 2009; Miller et al., 2008).

4.2. Specificity of the effect of nervous temperament on neutrophil trafficking

Our overall hypothesis was that nervous temperament was associated with glucocorticoid resistance as indexed by attenuated correlations between cortisol concentrations and the three major leukocyte subsets (neutrophils, lymphocytes, and monocytes), the trafficking of which are known to be affected by glucocorticoids (Fauci et al., 1976). Our hypothesis was supported, however, only for the neutrophil subset. The specificity of our result suggests to us three possibilities.

First, it is possible that our findings are an epiphenomenon; the specificity of our result might actually reflect a non-HPA response

to the stress of separation and relocation. Certainly such a stressor will activate a variety of physiological systems, each of which may operate in different time-frames, and which may be sensitive to different aspects of the stressful experience (e.g., Hofer, 1996); and different physiological systems can differentially influence leukocyte numbers (e.g., Capitanio et al., 1996; Landmann, 1992; Maisel et al., 1990; Ottaway and Husband, 1992, 1994). We consider this possibility unlikely, however, inasmuch as the cortisol data from our study, described above, is also consistent with nervous temperament being associated with glucocorticoid resistance – a standard dose of dexamethasone was significantly less effective in reducing plasma cortisol concentrations in high-nervous compared to low-nervous animals. Furthermore, an alternative mechanism to explain our results would have to (1) differentiate low- from high-nervous animals (or humans that are high versus low on negative affect) individuals, and (2) impact the regulation of leukocyte trafficking in the same way that cortisol does. While ACTH may be a possibility, it does not seem to discriminate between high- and low-neurotic people (Mangold and Wand, 2006), and we are unaware of studies demonstrating effects on trafficking. Catecholamines may be more likely candidates, in that they certainly affect trafficking, and are likely elevated in response to maternal separation. A recent meta-analysis, however, reveals that while neuroticism/negative affect may be related to cardiovascular reactivity, it does not appear to be related to more general sympathetic functioning (Chida and Hamer, 2008); consequently, while it can affect numbers of leukocytes, sympathetic nervous system activity cannot explain the difference in the *relationship* between cortisol and neutrophil numbers that we saw in low-nervous animals, but not in our high-nervous, animals. That is, while catecholamines clearly affect leukocyte trafficking, there is no reason to expect that such dynamics would occur differently in low- vs. high-nervous animals; any influence of catecholamine regulation of leukocyte trafficking that did exist between temperament groups would differentially affect correlations between catecholamine concentrations and circulating leukocyte counts in those groups, but would not affect the correlations between cortisol concentrations and leukocyte counts that were observed here.

A second possible explanation for the specificity of our results is that the glucocorticoid resistance suggested by the neutrophil and cortisol data for the nervous animals may also be evident for the other leukocyte subsets, but methodological issues may have prevented our finding the expected results for lymphocytes and monocytes. Earlier, we described a study that focused on subjective social isolation in humans (Cole, 2008). This study found expected relationships with cortisol for all three subsets in non-isolated individuals, but no such relationships in lonely individuals (Cole, 2008). In a study of adult monkeys, also described earlier, chronic social stress was found to abrogate the cortisol–leukocyte relationship for lymphocytes, but not for neutrophils or monocytes (Cole et al., 2009). The different pattern of results for those two studies plus the present study could reflect numerous methodological differences: the three studies differed by species of subjects (rhesus monkeys versus humans); type of sample from which cortisol measures were obtained (afternoon basal samples from plasma (Cole et al., 2009), 12-h overnight urine samples (Cole, 2008), morning, stressed samples from plasma [present study]); whether the cortisol values and leukocyte data were obtained at the same (Cole et al., 2009 and the present study) or different (Cole, 2008) time points; as well as the nature of the phenomenon under study (loneliness (Cole, 2008), chronic social stress (Cole et al., 2009), and temperament [present study]). While it is clear that administration of exogenous GC affects trafficking of all three leukocyte subsets (Fauci et al., 1976), the dynamics associated with morning versus afternoon, plasma versus urine, basal versus stressed, or contem-

poraneous versus sequential samples could make some results more obvious than others.

A third possible explanation for our results is intriguing, but is much more speculative. It is possible that this variation in the basic phenomenon – the regulation of leukocyte subset trafficking by glucocorticoids – might be a signature reflecting the influence of different types of stressors. This suggestion has its origins in seminal work by Mason (1971) challenging Selye's notion that physiological stress responses are non-specific across a range of stressors, as well as more recent empirical work supporting the non-specificity idea (e.g., Pacak and Palkovits, 2001; Bowers et al., 2008). In the present context, there is no reason to suspect that loneliness in humans (Cole, 2008), chronic, socially-induced stress in adult monkeys (Cole et al., 2009), or nervous temperament in infant monkeys (this study) should necessarily bear a direct relationship to each other. Furthermore, the complexity associated with leukocyte trafficking – involving separate processes of rolling, activation, and arrest; and a variety of molecules such as selectins, integrins, annexins, adhesion molecules, and chemokines that are themselves dynamically regulated (Butcher, 2005; Kinashi, 2005) – might result in other stress-associated molecules affecting these various receptors and mediators to differentially influence the regulation of the trafficking process in different cell subsets by glucocorticoids. This idea may not be far-fetched; after all, glucocorticoid administration to animals results in increases in neutrophil numbers, but decreases in lymphocyte and monocyte numbers; that is, even in the absence of a stressor of some specific type, glucocorticoids are already contributing to regulation of these cell types in different ways. At the molecular level, for example, L-selectin is responsible for the initial tethering and rolling of leukocytes. While *in vivo* studies show that GCs cause neutrophils to shed L-selectin (which may account for the GC-induced neutrophilia), *in vitro* studies suggest that annexin 1 (which is induced by GCs) is required for L-selectin shedding – but only in neutrophils and monocytes, not lymphocytes, which apparently do not possess annexin 1 receptors (Strausbaugh and Rosen, 2001). This example illustrates the complexity of the process of trafficking, and that characteristics of the leukocyte subsets themselves can contribute to differential responsiveness to GCs. Further testing of the specificity idea may be more easily accomplished using *in vitro* techniques, such as cell stimulation in the presence/absence of the dexamethasone to determine whether there are differential effects of regulation on different cell subsets based upon characteristics of individuals' dispositions or situations.

4.3. Implications for disease processes

Are there disease-related implications of which leukocyte subset is dysregulated by temperament or by chronic stress? In our earlier study (Cole et al., 2009), unstable social conditions were associated with desensitization of lymphocytes, but not neutrophils. Importantly, a separate set of animals was run in the larger study from which those data were derived, and members of this set were infected with SIV, the simian immunodeficiency virus. In that study (Capitanio et al., 1998), we found that median survival for animals in the unstable conditions was significantly shorter compared to animals in stable social conditions. How might glucocorticoid desensitization of lymphocytes in stressed animals be involved? Gene transcription in HIV-1 is regulated by a long terminal repeat (LTR) promoter that contains a glucocorticoid response element (Ghosh, 1992). While early research suggested that GCs promoted HIV replication *in vitro* (e.g., Markham et al., 1986), subsequent research has strongly suggested that GCs exert inhibitory effects (e.g., Mitra et al., 1993; Kilby et al., 1997). More recent studies have confirmed that GCs suppress tat activation of the HIV-1-LTR promoter in a cell-specific fashion, possibly through

disruption of NF- κ B's cooperation with tat to promote HIV gene transcription (Kino et al., 2000). If GCs do have an inhibitory effect on HIV-1 replication *in vivo*, then the GCR in lymphocytes resulting from unstable social conditions might be a contributor to the accelerated disease progression observed in our earlier study (Capitanio et al., 1998). In contrast, because nervous temperament is associated with GCR of neutrophils and not lymphocytes, we would predict that nervous temperament might have minimal association with HIV/SIV disease progression; rather, the effects of nervous temperament (or, in humans, neuroticism/negative affect) might be more evident in disease processes involving inflammation, or more generally, with markers of inflammation – which has, indeed, been reported (Lahey, 2009; Marsland et al., 2008; Nabi et al., 2008).

Finally, we note that our data extend previous results by demonstrating that temperament-associated dysregulation exists in infancy. Earlier we suggested that one way that temperament could affect health-related outcomes is through a main effects model, whereby animals of different temperament types are “built” differently, and we provided an example of how Sociability was associated with density of sympathetic innervation of lymph nodes (Sloan et al., 2008). We suggest that the present data provide a second potential example of a main effect. If this interpretation is correct, then we would predict that the influence of temperament on GC-related trafficking (or some other measure of GCR) should remain consistent across the lifespan. We would also expect that there should be health-related consequences of differences in nervous temperament in these animals, and that there should be some identifiable origins of variation in nervous temperament (e.g., genetic, prenatal, early postnatal or some combination of these). All of these questions are being examined in our laboratory.

Conflict of interest

All authors declare that there are no conflicts of interest.

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