Hair Cortisol and Salivary Cortisol Reactivity in Adolescents

John J. Salvatore

Tufts University

Harvard University

Children's Hospital Boston

Abstract

The HPA axis is initiated in response to stress, resulting in a hormonal cascade that culminates in the release of glucocorticoids. Glucocorticoids mediate many physiological changes in response to environmental challenges. Ultimately, they provide negative feedback to the brain regions that activate the HPA axis. When secreted chronically, however, glucocorticoids can damage neurons that regulate the axis. This possibility may be magnified during developmental critical periods, when the brain is most sensitive to environmental influences. Studies in animals suggest adolescents may be particularly vulnerable to the deleterious effects chronic stress. The present study hypothesized that adolescents exposed to chronic activation of the HPA axis would exhibit heightened reactivity to the Trier Social Stress Task (TSST). The accumulation of cortisol in hair was used as a biomarker of chronic activation of the HPA axis in order to investigate its relationship with subjective reports of stress and its impact on subsequent reactivity. Surprisingly, adolescent reports of high perceived stress significantly predicted decreased hair cortisol content.

Introduction

Every organism must survive in the face of myriad stressors throughout its lifespan. As a result, our ancestors evolved physiological tools to overcome countless environmental challenges and we have inherited those tools to deal with the challenges present today. When the task at hand demands 'fight or flight', the body can cut corners, favoring processes that make energy rapidly available to the muscles and brain at the expense of more ambitious physiological undertakings. For instance, reproduction, digestion, and immunity to disease become temporarily suppressed when an organism is faced with an immediate stressor (Sapolsky, 2000). Such physiological tools have been evolutionarily conserved, despite that the stressors we face as twenty-first century *Homo sapiens* seem to differ dramatically from those of our hunter-gatherer predecessors. However, finding an elusive parking spot or engaging in public speaking activate the same physiological machinery that evolved to handle stalking a mammoth meal or evading a persistent predator.

The hypothalamic-pituitary-adrenal (HPA) axis is a major neuroendocrine system that mediates physiologic flexibility in response to environmental perturbation (Korte et al., 2005). Despite this, human stress physiology did not evolve to remain activated indefinitely. The human brain, on the other hand, has an exquisite capacity to imagine myriad stressors. When the stress response is chronically active in the absence of an immediate threat, it can leave one vulnerable to disease. Numerous vulnerabilities have been associated with chronic HPA axis activation, including depression, post-traumatic stress disorder, heart disease, somatoform disorders, asthma, rheumatoid arthritis, alcoholism, and inflammatory skin disorders (Holsboer, 2000; Yehuda 1997; Brotman,

Golden, and Wittstein, 2007; Heim, Ehlert, and Hellhammer, 2000; Costa et al., 1996; Pereg et al., 2011; Buske-Kirschbaum and Hellhammer, 2003).

While it is known that chronic HPA axis activation can lead to disease in adulthood, less is known about *how* this occurs. Particularly little is known about the etiological impact of chronic HPA axis activation during adolescence. But why consider adolescence? For one, it is period of substantial brain development in the midst of psychological, social, and physical volatility (Spear, 2000; Paus, 2005; Casey, Jones, and Hare, 2008). This period of rapid neural and social development makes for a likely period during which chronic HPA axis activation can induce lasting changes in development and health. Additionally, a growing animal literature suggests that dramatic changes in HPA axis function occur between adolescence and adulthood (Romeo, 2010). Despite this, direct studies of chronic perturbation of the HPA axis during human adolescence and its impact on subsequent reactivity are lacking. In the present study, I set out to examine the consequences of chronic HPA axis activation in adolescents using a novel biomarker: the accumulation of cortisol in hair.

To understand how the normally adaptive HPA axis might go awry, we will first review how it functions in an adaptive manner under normal circumstances. Then, we will review what is known about what can go wrong. The following sections introduce the basic structure, function, and control mechanisms that regulate the HPA axis, with insight into how chronic activation can shape subsequent reactivity.

Hypothalamic-Pituitary-Adrenal Axis

One downstream consequence of HPA axis activation is the secretion of glucocorticoids. The neuroendocrine cascade responsible for this end result

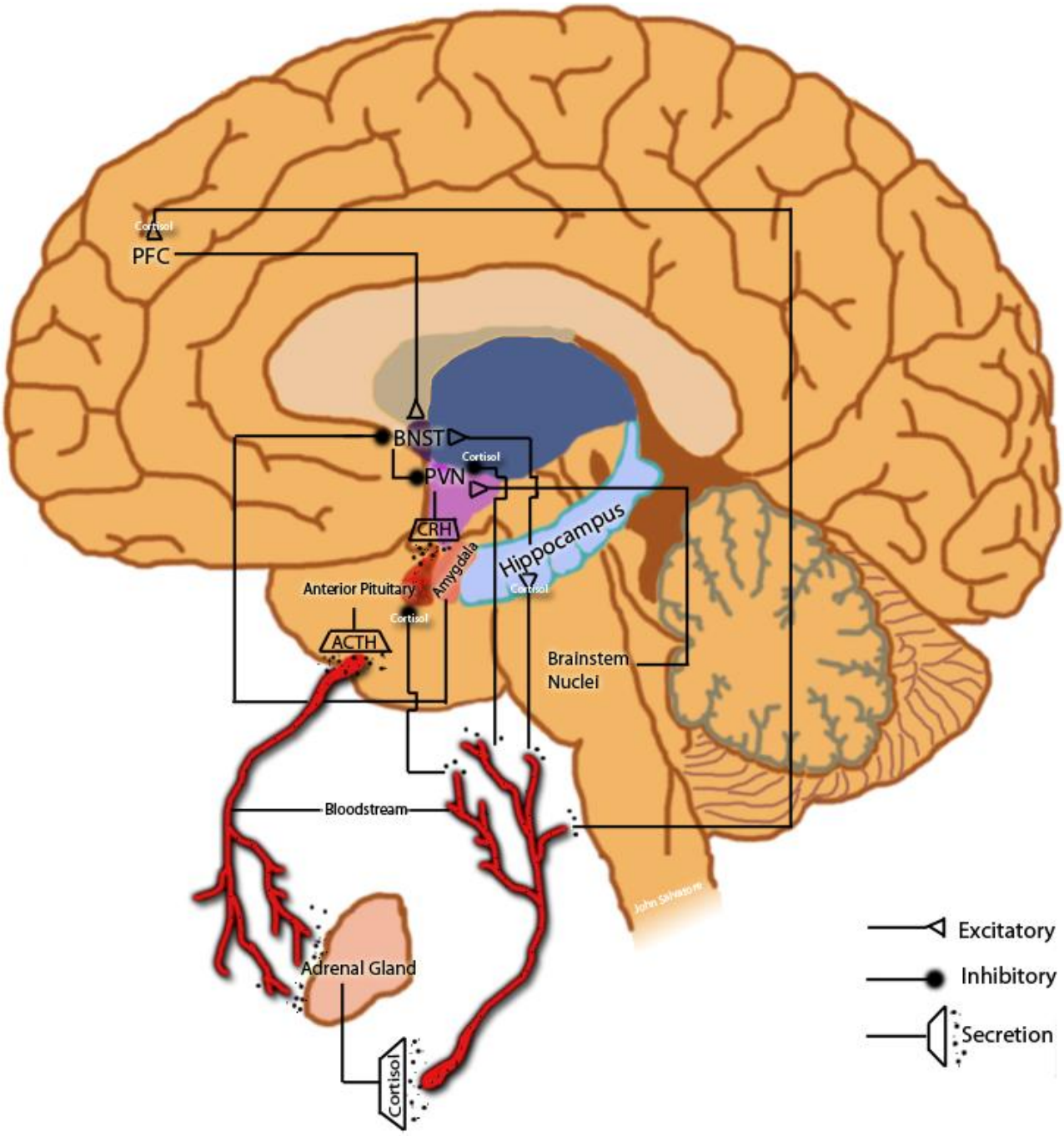

Figure 1. Basic schematic of the hypothalamic pituitary adrenal axis

PFC – prefrontal cortex; BNST – bed nucleus of the stria terminalis; PVN – paraventricular nucleus; CRH – corticotropin releasing hormone; ACTH – adrenocorticotropic hormone

begins in the paraventricular nucleus (PVN) of the hypothalamus (Figure 1). This structure contains a subpopulation of cells that secrete corticotropin-releasing hormone (CRH) into the vasculature of the hypophyseal portal system. The hypophyseal portal system is a series circuit of capillary beds that delivers hypothalamic secretions to the anterior pituitary (rev. Whitnall, 1993; Herman and Cullinan, 1997). Upon arrival of CRH, the anterior pituitary responds by secreting adrenocotricotropic hormone (ACTH) into the bloodstream. Other neurons in the PVN release arganine vasopressin (AVP), which can potentiate ACTH release by the anterior pituitary (Rivier and Vale, 1983). Once in circulation, ACTH makes its way to the adrenal cortex, where it stimulates the secretion of glucocorticoids. Cortisol, the byproduct of glucocorticoid breakdown, can be measured non-invasively in saliva.

When incoming information is recognized by an animal as threatening, the result is often activation of the HPA axis. Not all stressors are necessarily perceived the same way, however. There may be obvious differences between stressors, such as the perceptual modality by which they are detected. For instance, visual, auditory, tactile, or olfactory cues all have the potential to communicate threat and one might expect different signal transformations and neural pathways to underlie threat detection in each modality. There are likely subtler differences in signal properties that can distinguish a threatening from nonthreatening stimulus, potentially resulting in differential pathways leading to HPA axis activation.

For example, it is known that different classes of stressors activate CRH secretion by the PVN via separate neural pathways. For example, acute physical stressors such as hemorrhage, hypotension, and respiratory distress activate the CRH-secreting neurons of

the PVN directly via catecholaminergic synapses originating in brainstem nuclei (Plotsky, Cunningham, and Widmaier, 1989). This response is thought of as *reactive* due to the immediate physiological challenges that activate it (Herman et al. 2003). An *anticipatory* response, which depends on the organism's perception of a future threat, can also result in HPA axis activation. This pathway is mediated by amygdala inhibition of the bed nucleus of the stria terminalis (BNST) and several thalamic nuclei. These nuclei normally inhibit the secretion of CRH by the PVN, and the removal of that inhibition activates the HPA axis.

Once in circulation, glucocorticoids play an initially adaptive role in dealing with the metabolic, inflammatory, and cognitive demands required to deal with an acute stressor (Munck, Guyre, and Holbrook, 1984; Dhabhar, 2009; McEwen 2007). Maintenance of this response for prolonged periods of time becomes costly, necessitating feedback inhibition to restore equilibrium. Glucocorticoids themselves can participate in this restorative process by crossing the blood-brain barrier and binding to high-affinity mineralocorticoid receptors (MR) and low-affinity glucocorticoid receptors (GR) in the hippocampus and medial prefrontal cortex (Herman and Cullinan, 2007). In particular, the low-affinity GR mediate feedback inhibition of the HPA axis (de Kloet et al., 1998). In non-stress situations, circulating levels of glucocorticoids activate primarily higher-affinity MR. During stressful situations, glucocorticoid concentrations become elevated. In this case, MR are saturated and a significant proportion of GR become occupied as well, inhibiting further activation of the HPA axis.

The hippocampus and the prefrontal cortex are brain structures known to harbor high concentrations of GR and MR (Funder, 1994). Lesion studies have shown that heightened

HPA reactivity follows damage to either structure (Jacobson and Sapolsky, 1991; Diorio, Viau, and Meaney, 1993). This finding suggests both structures contribute to feedback inhibition in response to elevated glucocorticoids. Numerous additional structures play an important role in stress regulation through glucocorticoid-induced feedback inhibition, including the pituitary and the PVN itself (Whitnall, 1993; Figure 1).

Mechanisms of Dysregulation: Allostasis

Though stress exposure has been linked by evidence from epidemiology to various disease states, less is known about *how* stress pathology happens. In order to better understand its etiology, testable models have been developed to explain exactly how organisms cope with stressors. The allostasis model provides an account of the physiological mediators of homeostasis, or the maintenance of certain biological parameters within narrow life-sustaining ranges (McEwen and Wingfield, 2003). These mediators exhibit seasonal and daily fluctuations in response to environmental changes, and serve to keep critical parameters at equilibrium. The allostasis model posits an energy cost incurred as a result of this physiological upkeep.

Energy demands placed on homeostatic machinery are described as allostatic load (McEwen and Wingfield, 2003). Allostatic load occurs when environmental conditions are such that an organism struggles to meet the energy demands of homeostasis. It is characterized by increasingly drastic fluctuations in stress hormones in an attempt to restore physiological balance. The adaptive role of allostasis breaks down during allostatic overload, which can occur if mediators remain chronically elevated. In this state, the mediators of balance reach harmful levels, causing wear and tear on the organism. The allostasis model makes a clear prediction in the case of chronic HPA

activation: if elevated glucocorticoids can cause wear and tear on the cells that participate in HPA regulation, the entire axis can become dysregulated.

Researchers have investigated the mechanisms by which chronic exposure to stress can alter activity in the prefrontal cortex (PFC) and hippocampus. These structures have been targeted because both provide negative feedback to the HPA axis (Figure 1; McEwen 2005). For similar reasons, the amgydala has also been a popular structure to study. It plays a role in processing of emotional stimuli and activates the HPA (Davis and Whalen, 2001; Dedovic et al., 2009). These neural circuits function as biological switches and may be vulnerable to dysregulation under allostatic overload.

Allostatic Overload and Central HPA Circuits

Hippocampus and Prefrontal Cortex

The hippocampus undergoes morphological and functional changes in response to chronic HPA activation. For example, chronic restraint stress has been shown to impair hippocampal-dependent spatial memory in rats (Conrad et al., 1996). This behavioral impairment is mirrored by neurobiological impairments following chronic glucocorticoid exposure. Exposure to chronic stress and selective occupation of type II GR in rats both independently decrease hippocampal long-term potentiation (Palvides, Nivon, and McEwen, 2002; Palvides et al., 1995). The effect disappears following acute intraperitoneal injection of mifepristone, a type II GR antagonist that readily crosses the blood-brain barrier (Mailliet et al., 2008). These studies provide evidence that chronic stress can alter both cognitive and neurobiological function of the hippocampus through the action of glucocorticoids.

These functional impairments are accompanied by equally concerning structural changes. Hippocampal and medial prefrontal cortical cell morphology is observed to deteriorate following exposure to chronic stress. For example, rats subjected to chronic restraint or psychosocial stress show dendridic atrophy and reduced adult neurogenesis in the hippocampus and prefrontal cortex (Wantanabe, Gould, and McEwen 1992; Pham et al., 2003; Radley et al., 2004; Margarinos et al., 1996; Radley et al, 2006). The structural deterioration observed in response to exogenous stressors may be mediated by the biological effects of chronic HPA axis activation. In fact, many researchers find chronically elevated glucocorticoid levels to result in similar morphological restructuring, as well as enhanced neuronal cell death in the hippocampus of rodents and primates (Stein-Behrens et al., 1994; Sapolsky et al., 1990).

Amygdala

Prolonged exposure to glucocorticoids induces lasting changes in the amygdala as well. Chronic subcutaneous administration of corticosterone to rats increased CRH mRNA levels in the central nucleus of the amygdala, though it decreased CRH mRNA expression in the PVN (Makino, Golk, and Schulkin, 1994). These differential responses to corticosterone exposure among central circuits of the HPA axis underscore the necessity for a more targeted approach when investigating the effects of chronic stress on the brain. Though feedback mechanisms following chronic activation of the HPA axis serve to reduce subsequent responses, additional evidence suggests that amygdala activity may be potentiated by chronic HPA axis activation.

For example, intracerebroventricular infusions of CRH have been shown increase acoustic startle reflex in rats, and the effect requires an intact amygdala (Liang et al.,

1993; Liang et al., 1992). This suggests that glucorticoids can potentiate reactivity to subsequent stressors via their actions on the amygdala. Structural changes accompany these functional consequences. Neurons in the amygdala undergo increased dendridic complexity and arborization in response to a chronic and unpredictable stress paradigm (Vyas et al., 2002). Though these effects contrast with the damaging effects of stress on hippocampal and medial prefrontal neuronal populations, an enlarged amygdala may contribute just as much to dysregulation, given that the amygadala activates the HPA axis.

These studies suggest that chronic stress can initiate behavioral and neuronal changes in the central regulators of HPA reactivity in adult mammals. Chronic HPA activation may impair negative feedback via its damaging effects on hippocampal and medial prefrontal circuits and at the same time may potentiate subsequent HPA axis activation by enhancing amygdala circuitry.

Stress and the Adolescent HPA Axis

The influence of chronic stress and glucocorticoid exposure on neural plasticity is especially important to consider during critical periods of brain development, when neurons comprising the HPA axis may become more vulnerable to environmental influences. During these sensitive periods, environmental exposures can profoundly shape neural circuits (Knudsen, 2004). There is ample evidence that exposure to stressors during early postnatal life dramatically shapes adult responses to stress (rev. Sanchez, Ladd, and Plotsky, 2001). Much less is known about the effects of stress exposure during adolescence, a period of behavioral and hormonal changes thought to be an additional

period of vulnerability to the long-term consequences of stress exposure (McCormick et al., 2010; Romeo 2010).

When adult rats are exposed to chronic stress, they typically adapt to the habituated stressor, but show increased reactivity to a novel stressor (Fernandes et al., 2002). Adolescent rats exhibit patterns of reactivity that differ from those of adults; their responses are marked by increased duration of corticosterone and ACTH release following an acute stressor, but higher peak responses and faster return to baseline following chronic stress (Romeo et al., 2006). This adolescent pattern of facilitated HPA axis response to chronic stress contrasts with the adult-like habituation (Romeo, 2010).

Differential behavioral responses to chronic stress parallel differential neural responses between the adolescent and adult rat brain. For instance, c-Fos immunohistochemistry, a technique used to identify neurons active near the time of animal death, reveals neural differences between stressed adult and adolescent rats. Adolescents responded to both acute and chronic stress with a greater proportion of active CRH neurons in the PVN (Romeo et al., 2006). Differential patterns of reactivity between adolescent and adult rats suggests that the transition from adolescence to adulthood is a period of further maturation of the HPA axis.

This view is supported by evidence that the effects of chronic stress experienced in adolescence are not transient but can persist into adulthood. Adolescent rats subjected to a chronic-variable stress paradigm exhibited an extended corticosterone response to stressors as adults (Isgor, et al., 2004). This behavioral effect was accompanied by reduced expression of hippocampal GR. Studies of chronic social stress in adolescent mice also indicate decreased hippocampal GR and MR (Schmidt et al., 2007). This

finding illuminates a potential cross-species mechanism by which impaired hippocampal negative feedback of the HPA axis results in heightened adult reactivity.

Studies of recovery from prenatal and early postnatal adversity also provide evidence that adolescence is a period during which central HPA axis circuits exhibit heightened sensitivity to environmental conditions. Pregnant rats that undergo chronic restraint stress bear offspring that exhibit dysregulated circadian HPA axis functioning as adults (Koehl et al., 1999). However, when these offspring are housed in an enriched environment during puberty, basal levels of corticosterone in adulthood normalize relative to their peers housed in standard laboratory cages (Morley-Fletcher et al., 2003; Laviola et al., 2004). Similarly, rat pups subjected to early postnatal maternal separation exhibit heightened fearful behavior, decreased hippocampal GR expression, and decreased feedback inhibition of the HPA axis as adults (Francis et al., 2002). All of these effects were reversed by housing the pubertal rats in an enriched environment. These studies highlight the importance of examining adolescence as a critical period of maturation of the HPA axis, during which its underlying circuitry may be particularly sensitive to environmental adversity and intervention.

Investigation of HPA Axis Function in Humans

Little is known about the impact of chronic stress on reactivity of the HPA axis in humans, and even less so in adolescents. One reason for this is that observation of behavioral and neuronal changes following experimentally induced chronic stress is not an ethically appropriate paradigm for studying humans. As a result, most studies of chronic stress-induced changes in the human brain are complicated by their associative nature. Additional complicating factors include the presence of diurnal rhythyms and

interpersonal variation in patterns of cortisol secretion. Finally, all subjective reports of stress given by humans are subject to recall and reporting biases (Smyth et al., 1997; Gow et al., 2010). In addition to these factors, salivary and urinary hormonal measures are unlikely to be suitable indicators of chronic stress, as they only provide a snapshot into levels of stress hormones circulating at the time of sample collection.

A novel technique that can circumvent some of these shortcomings uses subjects' hair to detect long-term exposure to hormones (Gow et al., 2010; Gaillard, Vaysette, and Pepin, 2000). When hair samples are collected from the vertex posterior of the scalp, each 1 cm segment serves as a window into approximately 1 month of accumulated material (Wennig, 2000). This long-term record provides a means of examining a biological memory of chronic HPA axis activation. Importantly, the assay can be tailored to examine any specific hormonal exposure and may not suffer from the physiological ambiguity and biases that plague questionnaire assessments of chronic stress.

The use of hair to assay cortisol was first validated as a means to retrospectively examine exposure to chronic stress in rhesus macaques, and has since been used to effectively discriminate between patients experiencing chronic pain and healthy controls, long-term unemployed individuals and employed controls, and patients admitted to hospitals for acute myocardial infarction versus control patients (Davenport et al., 2006; Van Uum et al., 2008; Dettenborn et al., 2010; Pereg et al., 2011).

The objective of the present study was to investigate hair cortisol as a biomarker of chronic activation of the HPA axis in adolescents. Unpredictable, social evaluative stressors have been shown to reliably elicit HPA activation across a number of studies in adults, and social support has been shown to exert a protective effect on stress reactivity

(Dickerson and Kemeny, 2004; Kubzansky et al., 2009; Heinrichs et al., 2003; Uchino, Cacioppo, and Kiecolt-Glaser, 1996). As a result, I hypothesized that increased peer-related social evaluative stress and perceived uncontrollable stress would be accompanied by elevated hair cortisol.

Given the suggested shortcomings of subjective, retrospective reports of stress, we additionally sought to measure the relationship between hair cortisol and subsequent reactivity of the HPA axis directly. To this aim, we induced a stress response by administering the Trier Social Stress Task (TSST). The TSST is the standard laboratory-induced stress paradigm used in humans. It involves confronting participants with an uncontrollable, social evaluative threat in a laboratory setting (Kirschbaum et al., 1993). It has been found to reliably and robustly activate the HPA, as well as autonomic and cardiovascular responses (Dickerson and Kemeny, 2004). The task requires participants to give a speech in front of an unreceptive audience, followed by a mental arithmetic task. This paradigm was used to induce a stress response in participants of the present study.

Little is known of the effects of chronic activation of the HPA axis on subsequent reactivity in human adolescents. The animal literature reviewed suggests potential mechanisms of impaired feedback and facilitated activation of the HPA axis in response to chronic activation. We therefore hypothesized that elevated hair cortisol would predict heightened salivary cortisol reactivity in response to the TSST.

Methods

Subjects

20 adolescents (12 females, 8 males, M_{age} = 14.95 years, *SD* = 1.40 years, age range: 13-17 years) participated in the study. Recruitment was conducted through the Laboratories of Cognitive Neuroscience at Children's Hospital Boston, online ads, and flyers placed in and around Boston, Massachusetts. Each subject and his or her guardian were required to give written informed consent to participate in the study. Participants who did not provide consent to collect a hair sample were excluded from the present study. All subjects were adolescents between the ages 13 and 17, and were recruited as part of a broader study examining the influences of socioeconomic status and early life adversity on stress reactivity. Participants were given $50.00 for their participation in the study. All study procedures were approved by the institutional review board at Harvard University associated with the Faculty of Arts and Sciences.

Study Protocol

Scheduling

In order to minimize the impact of diurnal variability in hormonal samples, participants were scheduled at the same time each day throughout the study (4:00PM EST unless deviation was absolutely necessary). Participants were asked not to eat within a half hour prior to their arrival.

Consent

Upon arrival, the experimenter explained provided an overview of the experimental tasks and measures to be collected during the study. Caregivers were then asked to provide written informed consent for their adolescent to participate. A battery of psychometric questionnaires were administered to participants and their caregivers to probe participants' exposure to different types of stressors, socioeconomic status, and

evidence of psychopathology. A hair sample was collected from the vertex posterior scalp of participants. Baseline autonomic and saliva samples were collected after the first adolescent questionnaire packet assessing general health and social functioning of adolescents was completed.

Trier Social Stress Task (TSST) - Preparation

The experimenter met briefly with the participant to explain that the speech task would be audio and video recorded. The two evaluators, one male and one female, then met with the participant. Participants were told to prepare a five-minute speech on the meaning of friendship without taking notes. The evaluators then left the room and the participant was given five minutes to prepare alone, during which autonomic data were recorded.

Trier Social Stress Task (TSST) - Speech Task

Evaluators entered the room and told the participant to begin the five-minute speech. The participant was given five minutes, during which evaluators were instructed to provide neutral-negative feedback via facial cues (e.g. appearing bored, disinterested, annoyed) and body language. Throughout the speech, video, audio, and autonomic data were recorded.

Trier Social Stress Task (TSST) - Math Task

Following the speech task the evaluators led the participant in a five-minute backward serial subtraction task, during which subjects were told to count backward from 758 in steps of 7. Participants were asked to start over if they made a mistake. Throughout the five minutes of the task, video, audio, and autonomic data were collected. Upon completion, a second saliva sample was taken.

Trier Social Stress Task (TSST) - Recovery

Participants were given a five minute recovery period during which autonomic data were collected.

Frustration Task Part I: Computer Game

A separate two-part frustration task, each lasting three minutes, consisted of a computer reaction-time task during which subjects were required to press the spacebar in a timely manner following an on-screen cue. In the first task, performance feedback in the form of visual and audio cues (a bell indicating success and a buzzer indicating failure) depended upon participants' performance. Participants were instructed to continue for the full three minutes until they came to a screen that indicated they should wait for further instruction. Autonomic data were collected continuously during the epoch. This task was engaging but not difficult and served as a baseline for the subsequent computer game.

Frustration Task Part II: Defective Computer Game

The second, more frustrating task was identical to the first except that failure cues were presented despite successful performance. Autonomic data were collected continuously throughout the task. A final saliva sample was taken following removal of the autonomic electrodes and sensors.

Debriefing

Subjects and caregivers were debriefed following completion or withdrawal from the experiment. Adolescents and their guardians were given full disclosure of the study aims and met with the evaluators and a clinical psychologist if necessary.

Materials

Facilities

Adolescents and their caregivers completed all procedures at the Laboratory for Clinical and Developmental Research in the Harvard Psychology Department. The experimental tasks were conducted in a room equipped with audio-visual equipment for remote observation and recording. The room also housed an MP100 data acquisition system for measurement of impedance cardiography, blood pressure, electromyography, photoplethysmography, skin temperature, and skin conductance. A separate room contained four computers running AcqKnowledge 3.8.2 software for digital signal recording and Mediacruise 2.24 software for video recording. A third room stored saliva samples at -20˚C and hair samples at room temperature.

Data Collection

A broad dataset was collected, including self-report measures of participants' general health, socioeconomic background, past stressful experiences, and psychopathological symptoms. Additionally, many physiological parameters were measured during the experimental tasks. Some measures were part of a larger study, and are reported in the present study but were not included in the analysis.

Peer Victimization Questionnaire (PVQ)

The PVQ was adapted from the Peer Experiences Questionnaire, in order to assess both peer victimization and peer support (Prinstein, Boergers, and Vernberg, 2001; Vernberg, Jacobs, and Hershberger, 1999). The scale included 18 items, 13 of which assessed victimization while the remaining five assessed pro-social interactions. Participants were asked to report an estimate of the occurrence of each behavior on a five point Likert scale (1 = never, 2 = once or twice, 3 = a few times, 4 = about once a week, 5

= a few times a week). Four subscales (overt, relational, reputational, pro-social) were constructed to classify the types of peer behavior received. Each subscale score was derived from the sum of its containing items, corrected for reverse scoring.

Perceived Stress Scale (PSS)

The PSS used in the present study was a 10 item questionnaire that has previously been shown to be a valid and reliable ($\alpha = .85$) measure of the degree to which an individual perceives life events as uncontrollably stressful (Cohen, Kamarck, and Mermelstein, 1983; Mimura & Griffiths, 2004). It is a widely used measure that has been related to physiological stress reactivity and psychometric measures of depression and anxiety (Ginty and Conklin, 2011; Chang, 1998). Participants rated how often (0 = never, 1 = almost never, 2 = sometimes, 3 = fairly often, 4 = very often) each event occurred over the past month. The total score was calculated as the sum of each item following correction of reverse scored items.

Adolescent Perceived Events Scale (APES)

The APES is a widely used probe for both daily and major life events that adolescents perceive as stressful (Compas et al., 1987). In the present study, 90 items representing specific events were included, and participants were required to indicate whether they had ever experienced each event. For events that had occurred, participants indicated on a nine point Likert scale the degree to which event was perceived as negative (-4 = extremely bad) or positive (+4 = extremely good). Subscale scores for major and daily stress were calculated as the sum of the appraisal scores for events reported as negative.

Screen for Adolescent Violence Exposure (SAVE)

In order to assess exposure to violent stressors, the SAVE was included in the battery. The SAVE is a 32 item probe for exposure to violence, and has been validated as a reliable (α = .58 to .91) measure, with three subscales of violence (traumatic, indirect, verbal/physical) identified by confirmatory factor analysis (Hastings and Kelley, 1997). Participants estimated the frequency of violent events on a five point Likert scale (1 = never, 2 = hardly ever, 3 = sometimes, 4 = a lot, 5 = almost always), and subscale scores were calculated as the sum of their constituent items.

Childhood Trauma Questionnaire (CTQ)

The CTQ is a validated and reliable (α = 0.80 to 0.97) retrospective measure of child abuse (Bernstein et al., 1997; Fink et al., 1995). Five separate subscales (emotional abuse, physical abuse, sexual abuse, physical neglect, emotional neglect) represent unique categories of maltreatment (Bernstein et al., 1994). Participants were asked to report childhood maltreatment frequency (1 = never true, 2 = rarely true, 3 = sometimes true, 4 = often true, 5 = very often true), and scores were calculated as sums of constituent items corrected for reverse scoring.

Children's Depression Inventory (CDI)

The CDI is a 26 item self report measure designed to reflect symptomatology of major depressive disorder (Kovacs, 1992). It is modeled on the Beck Depression Inventory (BDI) and has proven to be a valid and reliable measure with a strong relationship to clinical diagnosis in adolescence (Beck et al., 1961; Saylor et al., 1984; Craighead, Curry, and Ilardi, 1995). Participants rated each item based on subjective report of frequency, and a total score was calculated as the sum of each item after correction for reverse scoring.

Multidimensional Anxiety Scale for Children (MASC)

The MASC is a validated, reliable, and widely used self-report questionnaire that intends to measure anxiety disorders (March, 1997; March et al., 1997; March et al., 1999). Participants are asked to rate the occurrence of each item on a four point Likert scale (0 = never true, 3 = often true), and four subscale scores (physical symptoms, social avoidance, harm avoidance, and separation anxiety) were computed as the sum of their constituent items. A total score was calculated as the sum of the individual subscale scores.

Child Behavior Checklist (CBCL)

The CBCL is a instrument given to participants' caregives, who answered 140 Likert style items designed to measure participants' problematic behaviors (Achenbach, 1991; Greenbaum and Dedrick, 1998). Depression and anxiety subscales were used to complement psychopathology measures based on participants' self report.

Socioeconomic Status

Questions were included to directly assess parents' highest level of formal educational attainment (number of years of high school, college, or graduate school). Based on the reported highest achievement of either parent, families were assigned a single education variable (0 = did not graduate high school, 1 = high school graduate, 2 = some college, 3 = college graduate).

Parents were asked to report their subjective social status within the United States and within their communities using the MacArthur Subjective Social Status Scale (Goodman et al., 2001; Adler et al., 2000). This measure requires participants to label their position

along a 10 step ladder representing a spectrum of socioeconomic status (1 = lowest, 10 = highest).

The income needs ratio (INR) is a measure of how well a family is able to meet basic economic needs given its total annual income. The measure was calculated using families' reported annual income divided by the poverty threshold for a family of the reported number of adults and children (US Census Bureau, 2010).

Hair

A pencil-width segment of hair was cut near the vertex posterior scalp with scissors. Hair samples were stored in a folded sheet of aluminum foil within a labeled envelope.

DNA

Saliva samples were collected for DNA analysis either directly following consent, or, if participants had eaten 30 minutes prior to arrival, following hookup to autonomic equipment. For this sample, participants were asked to spit into a test tube. An additional DNA sample was taken prior to debriefing.

Hormones

After subjects completed the first questionnaire packet, a baseline hormonal saliva sample was collected. Participants were asked to drool into a collection tube. A second (reactivity) sample was collected directly after the math task of the TSST. A final (recovery) sample was collected after participants were unhooked from the autonomic recording equipment, approximately 15 to 20 minutes after the end of the TSST.

Autonomic Recordings

Subjects were hooked up to electrodes and sensors that measured impedance cardiography, blood pressure, electromyography, photoplethysmograph, skin

temperature, and skin conductance. Following a brief signal check, participants were asked to remain still while a first autonomic baseline was collected for five minutes. A second, identical baseline was taken following completion of the second questionnaire packet.

Autonomic measurements were recorded continuously during six epochs of five minutes each (initial baseline, second baseline, speech preparation, speech task, math task, recovery), and two epochs of three minutes each (computer game and defective computer game). During the five minute epochs, blood pressure was recorded at the start and again at the fourth minute of the baseline recording. During the three minute epochs, blood pressure was recorded only once, one minute into the epoch.

Hormonal Analyses

All hormonal assays were shipped to the Department of Psychology at the University of Dresden in Dresden, Germany, where they were analyzed for cortisol concentration using the methods described below. Duplicate samples for two individuals were included to estimate test-retest reliability of the assay procedure.

Hair

Hair was cut into 3cm segments, up to 9cm total, beginning with the segment closest to the scalp. At least 50mg of hair was collected and used per 3cm segment from each subject. Each segment was assayed for cortisol concentrations, which was used as a measure of chronic HPA activation over each 3 month period, assuming approximately 1cm of hair growth per month (Wennig, 2000).

Cortisol was extracted from hair according to a modified version of the protocol used by Davenport et al. (2006). A brief summary of the assay is provided, though a more

detailed outline can be found elsewhere (Kirschbaum et al., 2009). Hair segments of 3cm each were washed three times in an isopropyl alcohol solution and was gently mixed. The solution was decanted between each wash. Samples were dried for at least 12 hours and subsequently pulverized in a ball mill. Fifty milligrams of powdered hair were weighed, and pure methanol was added for steroid extraction for over 24 hours. The mixture was microcentrifuged to remove hair debris and the supernatant solution containing cortisol in methanol was separated. The methanol was evaporated completely, and a phosphate buffer was added. Cortisol concentrations were measured using a commercial immunoassay using chemiluminescence detection for quantification.

Saliva

All saliva samples were assayed for cortisol, dehydroepiandrosterone (DHEA), and testosterone using commercially available immunoassays using chemiluminescence for quantification of concentrations.

Statistical Analysis

Data were pre-screened for outliers prior to analysis. Any data points that were greater than three standard deviations above the mean or less than three standard deviations below the mean were excluded from the analyses. Using these criteria, an outlier SAVE score was removed because it was greater than three standard deviations above the mean score. Significance cutoffs for all significance tests were also established prior to analyses ($\alpha = .05$).

Two hypotheses were directly tested in the present study. First, multiple linear regression models were used to test the hypothesis that uncontrollable, social evaluative stressors explain variance in hair cortisol. In this analysis, hair cortisol was the dependent

variable, while questionnaires thought to reflect chronic social evaluative (PVQ) and uncontrollable (PSS, PES, SAVE) stress were included as independent variables. Each model also included age and gender as independent variables to control for their effects. One model was constructed for each questionnaire assessment.

The second hypothesis was that elevated hair cortisol would predict greater salivary cortisol reactivity during the TSST. In order to test this, a linear regression model was conducted with hair cortisol as the independent variable and salivary cortisol reactivity as the dependent variable. Salivary cortisol reactivity was calculated as baseline subtracted from peak salivary cortisol concentrations (to capture hypothesized positive change in cortisol concentrations from baseline). Because the number of subjects was severely limited due to the exploratory nature of the data collection and analysis, a post-hoc power analysis was run using G*Power software (Faul et al., 2009). Test-retest reliability of the hair cortisol analysis was estimated using the average coefficient of variation between two samples collected from the same two individuals. The coefficient of variation between cortisol levels in these hair samples was higher than anticipated (C_v =14%).

Results

Participants' descriptive statistics and socio-demographic information are presented in Table 1. Results of the multiple regression models assessing the relationship between stress questionnaire scores and hair cortisol are summarized in Table 2. The PSS, PES, and PVQ all significantly predicted hair cortisol regardless of age or gender (Table 2). Surprisingly, the directions of these relationships were all opposite the predicted directions (Figure 2). Hair cortisol concentrations were not significantly predicted by SAVE scores.

Table 1.
Descriptive Statistics

Measure	*N*	*Mean*	*SD*	Min	Max
Gender					
Male	8				
Female	12				
Age (years)	20	15.0	1.4	13	17
Cortisol					
Hair (pg/mg)	20	17.11	8.08	6.20	27.51
Salivary Baseline (nmol/L)	13	4.71	4.73	0.58	18.29
Salivary Reaction (nmol/L)	13	7.34	7.89	-1.23	25.60
SES					
Subjective Status					
Community	19	6.3	1.5	2.0	9.0
National	19	5.7	1.9	0.0	9.0
Income to Needs Ratio	19	4.73	3.12	0.28	9.02

Table 2.

Regression models of chronic unpredictable or social stress, age, and gender as predictors of hair cortisol

Model	R^2	*F*	D_f	*p*	*Predictor*			*Age*			*Gender*		
					β	*t*	*p*	*β*	*t*	*p*	*β*	*t*	*p*
Uncontrollable Chronic Stress													
PSS*	.34	2.76	16	.076	-.54	-2.54	**.022**	.34	1.64	.122	-.21	-.97	.345
PES*	.37	3.11	16	.056	-.58	-2.73	**.015**	.30	1.52	.148	-.24	-1.14	.269
SAVE	.08	.44	16	.730	.00	.01	.991	.28	1.08	.297	-.05	-.18	.858
Social Evaluative Stress													
PVQ*	.35	2.90	16	.068	-.54	-2.61	**.019**	.19	.91	.376	.07	.35	.729

*D_f - Degrees of freedom; * $p < .05$*

Also contrary to our expectations, elevated hair cortisol tended to yield lower salivary cortisol reactivity to the TSST, though the prediction was not significant, β = -.193, $t(11)$ = -.653, *ns* (Figure 3). Hair cortisol did not explain a significant portion of variance in salivary cortisol reactivity, R^2 = .04, $F(1, 11)$ = .43, *ns*. Post-hoc power analysis yielded a value of .28.

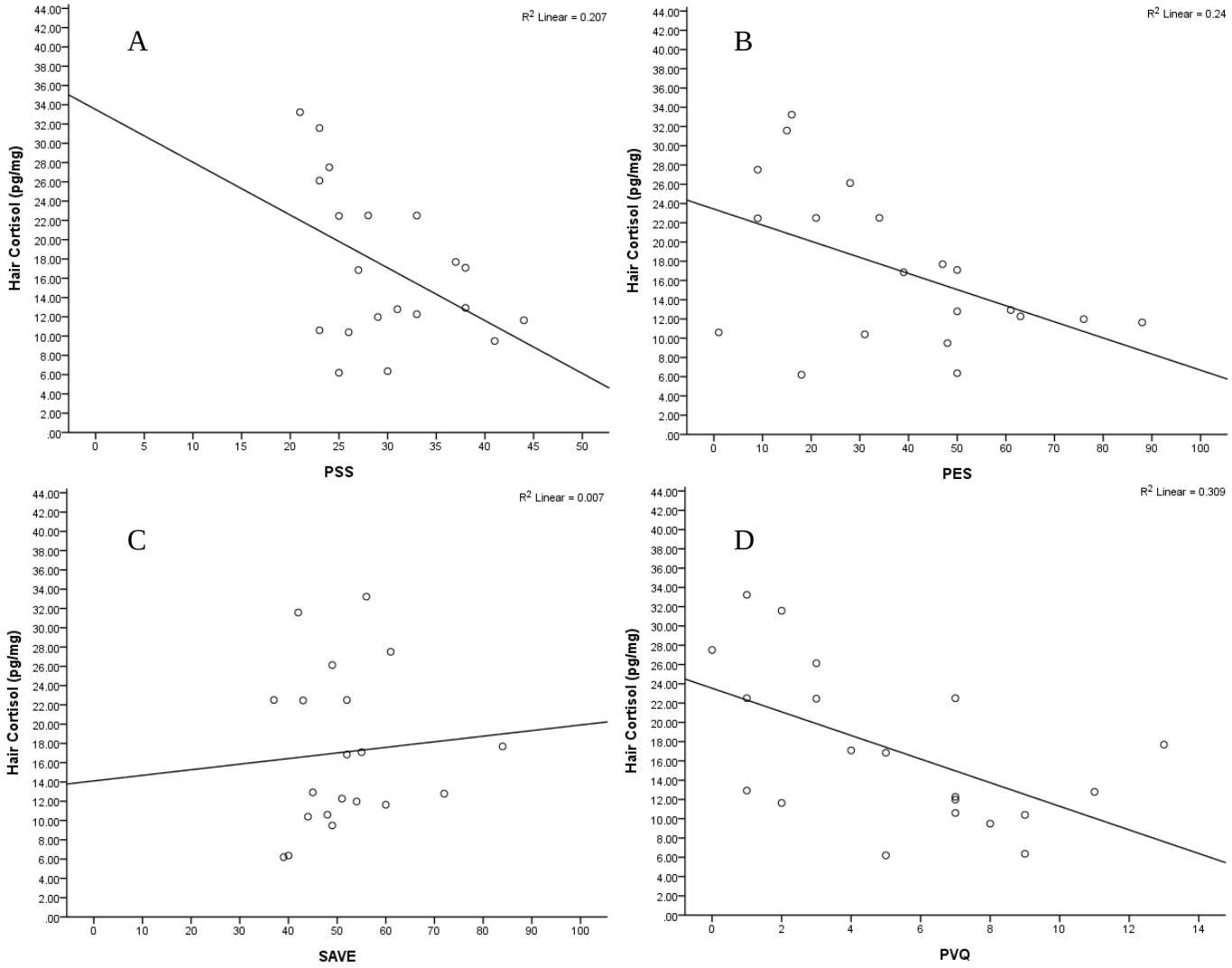

Figure 2. Scatter plots and best-fit line for A) PSS score vs. hair cortisol, B) PES score vs. hair cortisol, C) SAVE score vs. hair cortisol, and D) PVQ score vs. hair cortisol.

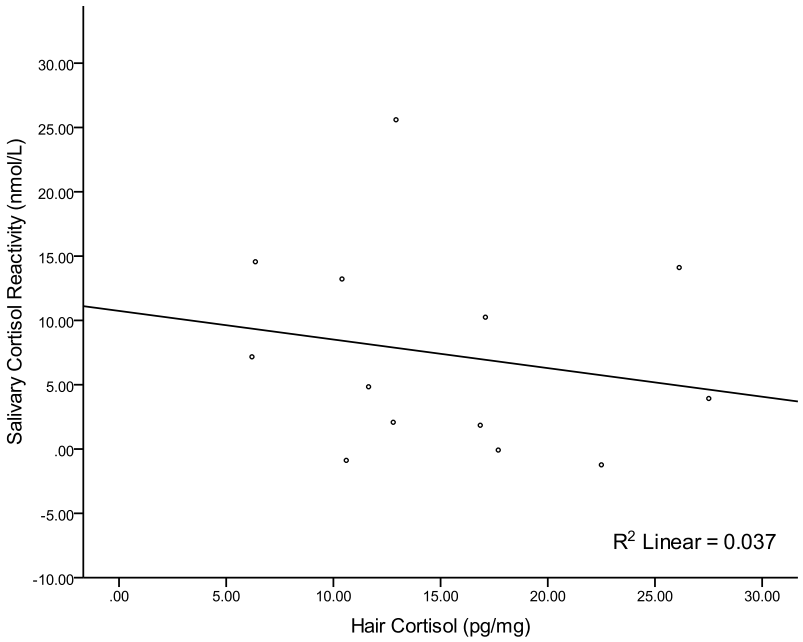

Figure 3. Scatter plot and best fit line for hair cortisol vs. cortisol reactivity

Discussion

The data support our first hypothesis that subjective reports of chronic stress predict hair cortisol concentrations in adolescents. However, the relationship was consistently opposite the hypothesized direction. Of the broad profile of subjective stress measures investigated, three showed significant negative relationships with hair cortisol (Table 2; Figure 2). These included the PSS, the PES, and the PVQ (Figure 2). The finding of a consistently negative relationship between subjective stress and hair cortisol is surprising, given that others have found hair cortisol concentrations to be significantly and positively correlated with PSS scores in adults (Kalra et al., 2007; Van Uum et al., 2008).

One interpretation of this finding is that there are differences underlying the neurophysiologic effects of chronic stress between the adult and adolescent brain. For example, glucocorticoid-mediated negative feedback on the HPA axis may be enhanced

by exposure to chronic stress in adolescents, rather than impaired. This appears to be the case in juvenile rats, which exhibit faster return to baseline following exposure to chronic stress compared to adults (Romeo et al., 2006). If negative feedback is not compromised following chronic stress exposure during adolescence, one might reasonably expect chronic stress to be accompanied by blunted patterns of diurnal cortisol secretion and decreased hair cortisol. Others have observed this pattern of blunted glucocorticoid secretion following chronic stress exposure in both humans and other species (Buske-Kirschbaum, Ebrecht, and Hellhammer, 2010; Van Den Eede et al., 2007; Rich and Romero, 2005).

The second hypothesis—that elevated hair cortisol would predict a more dramatic salivary cortisol response to the TSST—was not supported by the data (Figure 3). The possibility that adolescents do not exhibit impaired negative feedback following chronic stress may also account for the negative direction of this relationship. However, there are a number of additional methodological and theoretical problems illuminated by this null finding. For example, the hair segments analyzed in the present study were longer than those analyzed previously. Karla et al. (2007) analyzed 1 to 1.5 cm segments, and Van Uum et al. (2008) analyzed hair in 2 cm segments. In contrast, we analyzed hair in 3 cm segments in order to minimize the number of strands of hair collected from participants and still have enough hair to obtain hormonal data. If we use the standard estimation of 1 cm of hair growth per month at the vertex posterior scalp, our samples averaged hair cortisol over the 3 months prior to collection (Wennig, 2000). This temporal resolution may have been too coarse to detect more recent exposures to chronic stress that may have had a more immediate impact on salivary cortisol reactivity. Limited statistical power due

to the small sample size for the saliva assays (N = 13) may have also compromised our ability to uncover a significant relationship with hair cortisol, even if it does in fact exist. Follow-up studies should ensure that all target sample sizes are met.

The hypothesis of a positive relationship between chronic HPA axis activation and reactivity to the TSST was based on the idea of allostatic overload, which predicts damage to the mechanisms underlying negative feedback. One assumption implicit in this model is that the mechanisms themselves are fully formed and not subject to adaptive restructuring. Adults with fully developed brains facing chronic stress may indeed show elevated hair cortisol as a result of impaired negative feedback to the HPA axis (Kalra et al., 2007; Van Uum et al., 2008). However, adolescents might undergo adaptive neural changes that successfully cope with such damage. In fact, the HPA axis continues to develop throughout adolescence (Gunnar et al., 2009). It is possible, then, that increased neuroplasticity during this period offers resiliency to the deleterious effects of chronic HPA axis activation on subsequent reactivity. Alternatively, delayed effects of chronic stress exposure occurring in adolescence may require substantial time to manifest. A longitudinal study would be well suited to test these hypotheses directly.

A number of factors complicate the task of drawing clear conclusions from these data about the relationship between chronic activation of the HPA axis and subsequent reactivity in adolescents. For example, dramatic differences between individuals' diurnal fluctuations in circulating cortisol have been documented. Smyth et al. (2005) collected salivary samples from 120 people approximately every two hours over a period of two days and found no evidence of diurnal variation in salivary cortisol in 17% of subjects. Others exhibit a flattened diurnal cortisol cycle, marked by less dramatic fluctuations

(Stone et al., 2001). The existence of naturally occurring inter-individual variability may have contributed to noise in either salivary or hair cortisol concentrations, possibly both. Since diurnal patterns of hormonal secretion were not determined in the present study, we cannot rule out the possibility that our small sample may have included one or a few of these abnormal secretors, potentially magnifying their effects relative to the population at large.

Additional complications have been identified regarding extraneous sources of hair cortisol dilution. For instance, a recent study found that hair cortisol concentrations were significantly reduced in rhesus monkeys that regularly had their hair washed compared to those that did not (Hamel et al., 2011). The effect occurred regardless of whether or not shampoo was used. While these findings have not been replicated in humans, it is highly likely that a similar effect may have complicated the present analysis. Given that our hair segments capture a larger time window of cortisol accumulation than those of previous studies, the influence of washing may have been magnified. This would underestimate our true hair cortisol concentrations, with the most dramatic blunting evident in frequent washers. Since the Hamel et al. study was published while our data collection was ongoing; reports of hair washing frequency are not available.

The way in which compounds are incorporated into the hair shaft has not been agreed upon, and multiple parallel mechanisms of incorporation may exist. Some suggest that passive diffusion of blood-borne compounds into cells of the hair follicle results in incorporation into the growing hair shaft (rev. Kirschbaum et al., 2009). Others claim that deposition occurs following secretion of sweat and sebum carrying the compound. These mechanisms make sense if the substance in question is an exogenous drug, but might

become problematic when applied to substances of endogenous origin. For instance, a complex cutaneous endocrine system paralleling the central HPA axis has been discovered to exist within the human hair follicle itself (Ito et al., 2005). This has led some to postulate the existence of an independent, peripheral HPA axis (Arck et al., 2006). Hair cortisol concentration has been recently shown to exhibit diurnal fluctuation itself, and preliminary evidence suggests deviance between diurnal patterns of circulating and hair cortisol within individuals (Sharplet, Kauter, and McFarlane, 2010). The possibility of two independent HPA systems that may utilize different external or internal signals for phase entrainment poses a limitation on the use of hair cortisol as a tool to directly probe chronic HPA axis activation.

In the present study, the average coefficient of variation between hair samples collected from the same two individuals at the same time was surprisingly large (14%). Since two samples were collected from adjacent but non-identical vertex posterior scalp positions, variability across collection site may have inflated this estimation of test-retest reliability. One concern raised by this finding is that hair cortisol may reflect a localized response of the skin itself. In support of this possibility, one study found that cortisol levels in body hair responded to immersion in ice water locally and independent of circulating levels of cortisol (Sharpley, Kauter, and McFarlane, 2009). A similar effect may occur at the scalp. It is unclear how cortisol secreted by hair follicles responds to mechanical stresses such as massage of the scalp. A problematic possibility is that this behavior might systematically change in frequency with perceived stress. In order to use hair cortisol as a biomarker of stress wisely, further investigation into the development of stress reactivity of the peripheral and central HPA axis is needed.

Despite these complications, the data suggest there is utility in using hair cortisol as a biomarker of stress in adolescents. Three measures of subjective chronic stress significantly predicted hair cortisol, albeit contrary to the hypothesized direction. The discrepant direction between these and previous results is more likely a function of the different populations studied and limited explanatory power of the allostasis model rather than methodological artifact. Further studies are necessary to elucidate the impact that chronic HPA axis activation has on subsequent reactivity throughout human development. While the relationship between hair cortisol and salivary cortisol reactivity was not significant in the present study, our limited statistical power leaves open the door to discovery.

References

Achenbach, T.M. (1991). *Manual for the Child Behavior Checklist/4-18 and 1991 Profile*. Burlington, VT: University of Vermont, Department of Psychiatry.

Adler, N.E., Epel, E.S., Castellazzo, G., & Ickovicks, J.R. (2000). Relationship of subjective and objective social status with psychological and physiological functioning: Preliminary data in healthy white women. *Healthy Psychology*, *19*, 586-592.

Anacker, C., Zunszain, P.A., Carvalho, L.A., & Pariante, C.M. (2011). The glucocorticoid receptor: Pivot of depression and of antidepressant treatment? *Psychoneuroendocrinology*, *36*, 415-425.

Arck, P.C., Slominski, A., Theoharides, T.C., Peters, E.M.J., & Paus, R. (2006). Neuroimmunology of stress: Skin takes center stage. *Journal of Investigative Dermatology*, *126*, 1697-1704.

Beck, A.T., Ward, C.H., Mendelson, M., Mock, J., & Erbaugh, J. (1961). An inventory for measuring depression. *Archives of General Psychiatry*, *4*, 561–571.

Bernstein, D.P., Ahluvalia, T., Pogge, D., Handelsman, L. (1997). Validity of the childhood trauma questionnaire in an adolescent psychiatric population. *Journal of the American Academy of Child & Adolescent Psychiatry*, *36*, 340-348.

Bernstein, D.P., Fink, L., Handelsman, L., & Foote, J. (1994). Initial reliability and validity of a new retrospective measure of child abuse and neglect. *American Journal of Psychiatry*, *151*, 1132-1136.

Boyle, M.P., Brewer, J.A., Funatsu, M., Wozniak, D.F., Tsien, J.Z., Izumi, Y., & Muglia, L.J. (2005). Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 473-478.

Brotman, D.J., Golden, S.H., & Wittstein, I.S. (2007). The cardiovascular toll of stress. *Lancet*, *370*, 1089-1100.

Buske-Kirschbaum, A., & Hellhammer, D.H. (2003). Endocrine and immune responses to stress in chronic inflammatory skin disorders. *Ann. NY Acad. Sci*, *992*, 231-240.

Buske-Kirschbaum, A., Ebrecht, M., & Hellhammer, D.H. (2010). Blunted hpa axis responsiveness to stress in atopic patients is associated with the acuity and severeness of allergic inflammation. *Brain, Behavior, and Immunity*, *24*, 1347-1353.

Casey, B. J., Giedd, J. N., & Thomas, K. M. (2000). Structural and functional brain development and its relation to cognitive development. *Biol. Psychol.*, *54*, 241–257.

Casey, B.J., Jones, R.M., & Hare, T.A. (2008). The adolescent brain. *Annals of the New York Academy of Sciences, 28,* 62-77.

Chang, E.C. (1998). Does dispositional optimism moderate the relation between perceived stress and psychological well-being? A preliminary investigation. *Personality and Individual Differences, 25,* 233–240.

Cohen, S., Kamarck, T., & Mermelstein, R. (1983). A global measure of perceived stress. *J. Health and Soc. Behav., 24,* 385-396.

Compas, B.E., Davis, G.E., Forsythe, C.J., & Wagner, B.M. (1987). Assessment of major and daily stressful events during adolescence: The adolescent perceived events scale. *Journal of Consulting and Clinical Psychology, 55,* 534-541.

Conrad, C.D., Galea, L.A., Kuroda, Y., & McEwen, B.S. (1996). Chronic stress impairs rat spatial memory on the Y-maze, and this effect is blocked by tianeptine pretreatment. *Behav Neurosci, 110,* 1321–1334.

Costa, A., Bono, G., Martignoni, E., Merlo, P., Sances, G., Nappi, G. (1996). An assessment of hypothalamo-pituitary-adrenal axis functioning in non-depressed, early abstinent alcoholics. *Psychoneuroendocrinology, 25,* 263-275.

Craighead, W.E., Curry, J.F., & Ilardi, S.S. (1995). Relationship of children's depression inventory factors to major depression among adolescents. *Psychological Assessment, 7,* 171–176.

Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A., & Meyer, J.S. (2006). Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *General and Comparative Endocrinology, 147,* 255-261.

Davis, M., & Whalen, P. J. (2001). The amygdala: vigilance and emotion. *Mol. Psychiatry*, *6*, 13–34.

de Kloet, E.R., Vreugdenhil, E., Oitzl, M.S., & Joels, M. (1998). Brain corticosteroid receptor balance in health and disease. *Endocrine Reviews*, *19*, 269-301.

Dedovic, K., Duchesne, A., Andrews, J., Engert, V., & Pruessner, J. C. (2009). The brain and the stress axis: the neural correlates of cortisol regulation in response to stress. *Neuroimage, 47*, 864–871.

Dettenborn, L., Tietze, A., Bruckner, F., & Kirschbaum, C. (2010). Higher cortisol content in hair among long-term unemployed individuals compared to controls. *Psychoneuroendocrinology, 35*, 1404-1409.

Dhabhar, F.S. (2009). Enhancing versus suppressive effects of stress on immune function: Implications for immunoprotection and immunopathology. *NeuroImmunoModulation*, *16*, 300-317.

Dickerson, S.S., & Kemeny, M.E. (2004). Acute stressors and cortisol responses: A theoretical integration and synthesis of laboratory research. *Psychological Bulletin*, *130*, 355-391.

Diorio, D., Viau, V., & Meaney, M.J. (1993). The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. *Journal of Neurosci*, *13*, 3839-3847.

Faul, F., Erdfelder, E., Buchner, A., & Lang, A.G. (2009). Statistical power analyses using G*Power 3.1: Tests for correlation and regression analyses. *Behavior Research Methods, 41*, 1149-1160.

Fernandes, G.A., Perks, P., Cox, N.K.M., Lightman, S.L., Ingram, C.D., & Shanks, N. (2002). Habituation and cross-sensitization of stress-induced hypothalamic-pituitary-adrenal activity: Effect of lesions in the paraventricular nucleus of the thalamus or bed nuclei of the stria terminalis. *Journal of Neuroendocrinology*, *14*, 593-602.

Fink, L.A., Bernstein, D., Handelsman, L., Foote, J., & Lovejoy, M. (1995). Initial reliability and validity of the childhood trauma inteview: A new multidimensional measure of childhood interpersonal trauma. *American Journal of Psychiatry*, *152*, 1329-1335.

Francis, D.D., Diorio, J., Plotsky, P.M., & Meaney, M.J. (2002). Environmental enrichment reverses the effects of maternal separation on stress reactivity. *J. Neurosci.*, *22*, 7840–7843.

Funder, J.W. (1994). Corticosteroid receptors and the central nervous system. *The Journal of Steroid Biochemistry and Molecular Biology*, *49*, 381-384.

Gaillard, Y., Vaysette, F., & Pepin, G. (2000). Compared interest between hair analysis and urinalysis in doping controls. Results for amphetamines, corticosteroids and anabolic steroids in racing cyclists. *Forensic Science International*, *107*, 361-379.

Ginty, A.T., & Conklin, S.M. (2011). High perceived stress in relation to life events isassociated with blunted cardiac reactivity. *Biological Psychology*, *86*, 383-385.

Goodman, E., Adler, N.E., Kawachi, I., Frazier, A.L., Huang, B., & Colditz, G.A. (2001). Adolescents' perceptions of social status: Development and evaluation of a new indicator. *Pediatrics, 108*, e31.

Gow, R., Thomson, S., Rieder, M., Van Uum, S., & Koren, G. (2010). An assessment of cortisol analysis in hair and its clinical applications. *Forensic Science International*, *196*, 32-37.

Greenbaum, P.E., & Dedrick, R.F. (1998). Hierarchical confirmatory factor analysis of the Child Behavior Checklist/4-18. *Psychological Assessment, 10*, 149-155.

Gunnar, M.R., Wewerka, S., Frenn, K., Long, J.D., & Griggs, C. (2009). Developmental changes in hypothalamus – pituitary-adrenal activity over the transition to adolescence: Normative changes and associations with puberty. *Development and Psychopathology*, *21*, 69-85.

Hamel, A.F., Meyer, J.S., Henchey, E., Dettmer, A.M., Suomi, S.J., & Novak, M.A. (2011). Effects of shampoo and water washing on hair cortisol concentrations. *Clinica Chimica Acta*, *412*, 382-385.

Hastings, T.L., & Kelley, M.L. (1997). Development and validation of the screen for adolescent violence exposure (SAVE). *Journal of Abnormal Psychology*, *25*, 511-520.

Heim, C., Ehlert, U., & Hellhamer, D.H. (2000). The potential role of hypocortisolism in the pathophysiology of stress-related bodily disorders. *Psychoneuroendocrinology, 25*, 1-35.

Heinrichs, M., Baumgartner, T., Kirschbaum, C., & Ehlert, U. (2003). Social support and oxytocin interact to suppress cortisol and subjective responses to psychosocial stress. *Society of Biological Psychiatry*, *54*, 1389-1398.

Herman, J.P., & Cullinan, W.E. (1997). Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. *Trends in Neurosci, 20*, 78-84.

Herman, J.P., Figuiredo, H., Mueller, N.K., Ulrich-Lai, Y., Ostrander, M.M., Choi, D.C., & Cullinan, W.E. (2003). Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Frontiers in Neuroendocrinology, 24,* 151-180.

Holsboer, F. (2000). The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology, 23,* 477-501.

Isgor, C., Kabbaj, M., Akil, H., & Watson, S.J. (2004). Delayed effects of chronic variable stress during peripubertal-juvenile period on hippocampal morphology and on cognitive and stress axis functions in rats. *Hippocampus, 14,* 636-648.

Ito, N., Ito, T., Kromminga, A., Bettermann, A., Takigawa, M., Kees, F., Straub, R.H., & Paus, R. (2005). Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. *The FASEB Journal, 19,* 1332-1334.

Jacobson, L., & Sapolsky, R. (1991). The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocrine Reviews, 12,* 118-134.

Juruena, M.F., Cleare, A.J., Papadopoulos, A.S., Poon, L., Lightman, S., & Pariante, C.M (2006). Different responses to dexamethasone and prednisolone in the same depressed patients. *Psychopharmacology, 189,* 225-235.

Kalra, S., Einarson, A., Karaskov, T., Van Uum, S., & Koren, G. (2007). The relationship between stress and hair cortisol in healthy pregnant women. *Clin. Invest. Med., 30,* e103-e107.

Kirschbaum, C., Pirke, K.M., & Hellhammer, D.H. (1993). The 'trier social stress test' – a tool for investigating psychobiological stress responses in a laboratory setting. *Neuropsychobiology*, *28*, 76-81.

Kirschbaum, C., Tietze, A., Skoluda, N., & Dettenborn, L. (2009). Hair as a retrospective calendar of cortisol production-increased cortisol incorporation into hair in the third trimester of pregnancy. *Psychoneuroendocrinology*, *34*, 31-37.

Knudsen, E. I. (2004). Sensitive periods in the development of the brain and behavior. *J. Cogn. Neurosci*., *16*, 1412–1425.

Koehl, M., Darnaudery, M., Dulluc, J., Van Reeth, O., Le Moal, M., & Maccari, S. (1999). Prenatal stress alters circadian activity of hypothalamo-pituitary-adrenal axis and hippocampal corticosteroid receptors in adult rats of both gender. *J. Neurobiol.*, *40*, 302–315.

Korte, S.M., Koolhaas, J.M., Wingfield, J.C., & McEwewn, B.S. (2005). The Darwinian concept of stress: Benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neurosci Biobehav Rev*, *29*, 3-38.

Kovacs, M. (1992). *Children's Depression Inventory, CDI, Manual*. New York: Multi-Health Systems.

Kubzansky, L.D., Mendes, W.B., Appleton, A., Block, J., & Adler, G.K. (2009). Protocol for an experimental investigation of the roles of oxytocin and social support in neuroendocrine, cardiovascular, and subjective responses to stress across age and gender. *BMC Public Health*, *9*, 481.

Liang, K.C., Melia, K.R., Miserendino, M.J.D., Falls, W.A., Campaeu, S., & Davis, M. (1992). Corticotripin-releasing factor: Long lasting facilitation of the acoustic startle reflex. *J. Neurosci*, *12*, 2303-2312.

Liang, K.C., Melia, K.R., Miserendino, M.J.D., Falls, W.A., Campaeu, S., & Davis, M. (1993). Lesions of the central nucleus of the amydala, but not the paraventricular nucleus of the hypothalamus, block the excitatory effect of corticotripin-releasing factor on the acoustic startle reflex. *J. Neurosci*, *12*, 2313-2320.

Magarinos, A.M., McEwen, B.S., Flugge, G. & Fuchs, E. (1996). Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews. *J. Neurosci.*, *16*, 3534–3540.

Mailliet, F., Qi, H., Rocher, C., Spedding, M., Svenningsson, P., & Jay, T.M. (2008). Protection of stress-induced impairment of hippocampal/prefrontal LTP through blockade of glucocorticoid receptors: Implications of MEK signaling. *Exp. Neurol.*, *211*, 593-596.

Makino, S., Golk, W.P., & Schulkin, J. (1994). Corticosterone effects on corticotropin-releasing hormone mRNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus. *Brain Research, 640*, 105-112.

March, J. S. (1997). *Multidimensional Anxiety Scale for Children: Technical manual.* Toronto, ON: Multi-Health Systems.

March, J. S., Parker, J. D. A., Sullivan, K., Stallings, P. & Conners, C. K. (1997). The Multidimensional Anxiety Scale for Children (MASC): Factor structure,

reliability and validity. Journal of the American Academy of Child and Adolescent Psychiatry, 36, 554–565.

March, J. S., Sullivan, K. & Parker, J. (1999). Test-retest reliability of the multidimensional anxiety scale for children. Journal of Anxiety Disorders, 13, 349–358.

McCormick, C.M., Mathews, I.Z., Thomas, C., & Waters, P. (2010). Investigations of HPA function and the enduring consequences of stressors in adolescence in animal models. *Brain and Cognition*, *72*, 73-85.

McEwen, B.S. (2005). Glucocorticoids, depression, and mood disorders: Structural remodeling in the brain. *Metabolsim: Clinical and Experimental*, *54*, 20-23.

McEwen, B.S. (2007). Physiology and neurobiology of stress and adaption: Central role of the brain. *Physiol Rev*, *87*, 873-904.

McEwen, B.S., Wingfield, J.C. (2003). The concept of allostasis in biology and biomedicine. *Horm Behav*, *43*, 2-15.

Mimura, C.M., & Griffiths, P. (2004). A Japanese version of the perceived stress scale. *International Journal of Nursing Studies*, *41*, 379 - 385.

Morley-Fletcher, S., Rea, M., Maccari, M., & Laviola, G. (2003). Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. *Eur. J. Neurosci*., *18*, 1-8.

Munck, A., Guyre, P.M., & Holbrook, N.J. (1984). Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine Reviews*, *5*, 25-44.

Palvides, C., Nivon, L.G., & McEwen, B.S. (2002). Effects of chronic stress on hippocampal long-term potentiation. *Hippocampus, 12,* 245-257.

Palvides, C., Wantanabe, Y., Magarinos, A.M., & McEwen, B.S. (1995). Opposing roles of type I and type II adrenal steroid receptors in hippocampal long-term potentiation. *Neurosci., 68,* 387-394.

Paus, T. (2005). Mapping brain maturation and cognitive development during adolescence. *Trends in Cognitive Sciences, 9,* 60-68.

Pereg, D., Gow, R., Mosseri, M., Lishner, M., Rieder, M., Van Uum, S., & Koren, G. (2011). Hair cortisol and the risk for acute myocardial infarction in adult men. *Stress, 14,* 73-81.

Pham, K., Nacher, J., Hof, P.R., & McEwen, B.S. (2003). Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. *Euro. J. Neurosci., 17,* 879-886.

Plotsky, P.M., Cunningham, E.T., & Widmaier, E.P. (1989). Catecholaminergic modulation of corticotropin-releasing factor and adrenocorticotropin secretion. *Endocr Rev., 10,* 437-458.

Porter, K., & Hayward, L.F. (2011). Stress-induced changes in c-fos and corticotropin releasing hormone immunoreactivity in the amygdala of the spontaneously hypertensive rat. *Behavioral Brain Research, 216,* 534-551.

Prinstein, M.J., Boergers, J., & Vernberg, E.M. (2001). Overt and relational aggression in adolescents: Social-psychological adjustment of agressors and victims. *J. Clin. Child Psych., 30,* 479-491.

Radley, J.J., Rocher, A.B., Miller, M., Janssen, W.G.M., Liston, C., Hof, P.R., McEwen, B.S., & Morrison, J.H. (2006). Repeated stress induces dendritic spine loss in the rat medial prefrontal cortex. *Cereb. Cortex, 16*, 313–320.

Radley, J.J., Sisti, H.M., Hao, J., Rocher, A.B., McCall, T., Hof, P.R., McEwen, B.S., & Morrison, J.H. (2004). Chronic behavioral stress induces apical dendritic reorganization in pyramidal neurons of the medial prefrontal cortex. *Neuroscience, 125*, 1-6.

Rich, E.L., & Romero, L.M. (2005). Exposure to chronic stress downregulates corticosterone responses to acute stressors. *American Journal of Physiology – Regulatory Integrative and Comparative Physiology, 288*, R1628-R1636.

Rivier, C., & Vale, W. (1983). Interaction of corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin secretion in vivo. *Endocrinology, 113*, 39–42.

Romeo, R.D. (2010). Pubertal maturation and programming of hypothalamic-pituitary-adrenal reactivity. *Frontiers in Neuroendocrinology, 31*, 232-240.

Romeo, R.D., Bellani, R., Karatsoreos, I.N., Chhua, N., Vernov, M., Conrad, C.D., & McEwen, B.S. (2006). Stress history and pubertal development interact to shape hypothalamaic-pituitary-adrenal axis plasticity. *Endocrinology, 147*, 1664-1674.

Sanchez, M. M., Ladd, C. O., & Plotsky, P. M. (2001). Early adverse experience as a developmental risk factor for later psychopathology: evidence from rodent and primate models. *Dev. Psychopathol., 13*, 419–449.

Sapolsky, R.M. (2000). Stress hormones: Good and bad. *Neurobiology of Disease, 7*, 540-542.

Sapolsky, R.M., Uno, H., Rebert, C.S., & Finch, C.E. (1990). Hippocampal damage associated with prolonged glucocorticoid exposure in primates. *J. Neurosci*, *10*, 2897-2902.

Saylor, C.F., Finch, A.J., Spirito, A., & Bennett, B. (1984). The children's depression inventory: A systematic evaluation of psychometric properties. *Journal of Consulting and Clinical Psychology, 52*, 955–967.

Schmidt, M.V., Sterlemann, V., Ganea, K., Liebl, C., Alam, S., Harbich, D., Greetfeld, M., Uhr, M., Holsboer, F., & Muller, M.B. (2007). Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence. *Psychoneuroendocrinology, 32*, 417-429.

Sharpley, C.F., Kauter, K.G., & McFarlane, J.R. (2009). An initial exploration of in vivo hair cortisol responses to a brief pain stressor: latency, localization, and independence effects. *Physiol. Res.*, *58*, 757-761.

Sharpley, C.F., Kauter, K.G., & McFarlane, J.R. (2010). Diurnal variation in peripheral (hair) vs central (saliva) HPA axis cortisol concentrations. *Clinical Medicine Insights: Endocrinology and Diabetes*, *3*, 9-16.

Smyth, J.M., Ockemfels, M.C., Gorin, A.A., Catley, D., Porter, L.S., Kirschbaum, C., et al. (1997). Individual differences in the diurnal cycle of cortisol. *Psychoneuroendocrinology*, *22*, 89-105.

Smyth, J.M., Ockemfels, M.C., Gorin, A.A., Catley, D., Porter, L.S., Kirschbaum, C., Hellhammer, D.H., & Stone, A.A. (1997). Individual differences in the diurnal cycle of cortisol. *Psychoneuroendocrinology*, *22*, 89–105.

Spear, L.P. (2000). The adolescent brain and age-related behavioral manifestations. *Neuroscience and Bjobehavioral Reviews, 24*, 417-463.

Stein-Behrens, B., Mattson, M.P., Chang, I., Yeh, M., & Sapolsky, R. (1994). Stress exacerbates neuron loss and cytoskeletal pathology in the hippocampus. *J. Neurosci.*, *14*, 5373-5380.

Stone, A.A., Schwartz, J.E., Smyth, J., Kirschbaum, C., Cohen, S., Hellhammer, D., & Grossman, S. (2001). Individual differences in the diurnal cycle of salivary free cortisol: A replication of flattened cycles for some individuals. *Psychoneuroendocrinology, 26,* 295–306.

U.S. Census Bureau. (2010). Poverty thresholds by size of family and number of children. Retrieved April 1, 2011, from http://www.census.gov/hhes/www/poverty/data/threshld/thresh10.xls.

Uchino, B.N., Cacioppo, J.T., & Kiecolt-Glaser, J.K. (1996). The relationship between social support and physiological processes: A review with emphasis on underlying mechanisms and implications for health. *Psychological Buletin, 119,* 488-531.

Van Den Eede, F., Moorkens, G., Van Houdenhove, B ., Cosyns, P., & Claes, S.J. (2007). Hypothalamic-pituitary-adrenal axis function in chronic fatigue syndrome. *Neuropsychobiology*, *55*, 112-120.

Van Uum, S., Suave, B., Fraser, L.A., Morley-Forster, P., Paul, T.L., & Koren, G. (2008). Elevated ontent of cortisol in hair of patients with severe chronic pain: a novel biomarker for stress. *Stress, 11,* 483-488.

Vernberg, E. M., Jacobs, A. K., &Hershberger, S. L. (1999). Peer victimization and attitudes about violence during early adolescence. *J. Clin. Child Psych., 28*, 386–395.

Vyas, A., Mitra, R., Rao, B.S.S., & Chattarji, S. (2002). Chronic stress induces contrasting patterns of dendridic remodeling in hippocampal and amygdaloid neurons. *J. Neurosci*, *22*, 6810-6818.

Watanabe, Y., Gould, E. & McEwen, B.S. (1992). Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. *Brain Res., 588*, 341–345.

Wennig, R. (2000). Potential problems with the interpretation of hair analysis results. *Forensic Science International, 107*, 5-12.

Whitnall, M.H. (1993). Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. *Progress in Neurobiology*, *40*, 573-629.

Yehuda, R. (1997). Sensitization of the hypothalamic-pituitary-adrenal axis in posttraumatic stress disorder. *Ann. NY Acad. Sci., 821*, 57–75.

Young, E.A., Haskett, R.F., Murphy-Weinberg, V., Watson, S.J., & Akil, H. (1991). Loss of glucocorticoid fast feedback in depression. *Arch. Gen. Psychiatry, 48*, 693-699.