Neurotoxicology and Teratology 71 (2019) 41–49

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

Toxic stress history and hypothalamic-pituitary-adrenal axis function in a social stress task: Genetic and epigenetic factors

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ARTICLE INFO

Keywords:
Early life stress
Discrimination
Cortisol
Mitochondria
FKBP5

ABSTRACT

Histories of early life stress (ELS) or social discrimination can reach levels of severity characterized as toxic to mental and physical health. Such toxic social stress during development has been linked to altered acute hypothalamic-pituitary-adrenal (HPA) response to social stress in adulthood. However, there are important individual differences in the size and direction of these effects. We explored developmental, genetic, epigenetic, and contextual sources of individual differences in the relationship between ELS, discrimination, and adult responses to acute social stress in a standard laboratory test. Additional measures included perceived status, social support, background activity of HPA axis, and genetic variants in aspects of the stress response system. Participants (n = 90) answered questions about historical and ongoing stress, provided a DNA sample to examine genetic polymorphisms and epigenetic marks, and underwent the Trier Social Stress Test (TSST) during which three saliva samples were collected to assess HPA function. Individuals who reported high levels of childhood adversity had a blunted salivary cortisol response to the TSST. Childhood adversity, discrimination experiences, and FKBP5 genotype were found to predict pretest cortisol levels. Following up on recent observations that the glucocorticoid receptor directly interacts with the mitochondrial genome, particularly the NADH dehydrogenase 6 (MT-ND6) gene, individuals who reported high childhood adversity were also found to have higher percent methylation across six CpG sites upstream of MT-ND6. These findings suggest multiple contributions across psychological, genetic, epigenetic, and social domains to vulnerability and resilience in hypothalamic-pituitary-adrenal axis regulation. Further study to examine how these multiple contributors affect developmental endpoints through integrated or independent pathways will be of use.

1. Introduction

Stress history can affect hypothalamic-pituitary-adrenal (HPA) axis regulation with implications for mental health, physical health, and quality of life (McEwen, 2003). Activation of the HPA axis results in elevated levels of glucocorticoids (GC), particularly Cort (cortisol in humans and corticosterone in rodents), which regulate the physiology of several target tissues, including the immune system, sympathetic nervous system, metabolism, and central nervous system (Juster et al., 2010). Toxic stress, such as abnormal developmental stress and major or chronic social stress in adulthood, is a well-established source of enduring modification to HPA regulation. HPA dysregulation from such sources has been linked to increased vulnerability for a wide range of negative physical and psychological health outcomes (Borges et al., 2013; Bradley and Dinan, 2010; Brindley and Rolland, 1989; Dennison et al., 1999; Dhabhar and McEwen, 1997; Jansen et al., 1998; Sapolsky et al., 1986; Seeman et al., 2010). Importantly, there are individual differences in the size and direction of the effects of toxic stress on HPA function, suggesting other contributing factors in HPA regulation. These other factors may interact with one another and the stress system at various time points to affect likelihood of manifest physical and psychological illness as allostatic load accumulates over the life cycle (McEwen and Wingfield, 2003; Seeman et al., 2010). For example, genetic variants and stress history may interact to contribute to vulnerability and HPA dysregulation, driven in part through epigenetic changes. An emerging theme in stress neurobiology is that both resilience and susceptibility to stress are determined by multiple interrelated contributions from the social environment, metabolic factors, and individual genetic and epigenetic differences (McEwen et al., 2015; Tronick and Hunter, 2016).

The Adverse Childhood Experiences (ACE) study is one of the most well-known studies demonstrating the connection between early stressful experiences and illness later in life. ACE scores reflect the number of distinct categories of adverse events (e.g. physical abuse or neglect) an individual experienced during the first 18 years, without considering the frequency or duration of any event(s). Incremental

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https://doi.org/10.1016/j.ntt.2018.01.011
Received 30 July 2017; Received in revised form 24 January 2018; Accepted 29 January 2018
Available online 21 February 2018
0892-0362/

increases in ACE score are correlated with corresponding increases in risk for mental illness, medical illness, and risky behaviors (Anda et al., 2006; Anda et al., 2010; Felitti et al., 1998). A positive relationship exists between ACE scores and risk for depressed affect, anxiety, hallucinations, and panic reactions as well as diagnosis for mental disorders including depression, anxiety, post-traumatic stress disorder (PTSD), and suicide attempts (Anda et al., 2006, 2008; Brown et al., 2005; Cabrera et al., 2007; Chapman et al., 2004; McCauley et al., 1997). Individuals with ACE scores of four or higher are especially vulnerable to poor stress-related mental health disorders (Cabrera et al., 2007).

ACE scores largely reflect intra-familial interactions with caregivers, but social interactions with others become increasingly important throughout adolescence and adult life. Social interactions can be a significant source of stress, particularly for individuals of low social status, who are at greater risk for stress-related illness (Kirschbaum et al., 1993; Marmot et al., 1991; Sapolsky, 2005; Seeman et al., 2010). Discrimination stress is a type of social stress that leads to negative emotional responses and physiological arousal (Armstead et al., 1989; Harrell et al., 2003; Sawyer et al., 2012). Specific types of discrimination, including racial discrimination, may be uncontrollable and unpredictable. Repeated discrimination experiences can contribute to increased allostatic load over time (Mays et al., 2006; Sellers et al., 2003). Flattened diurnal cortisol rhythm and high evening cortisol levels are associated with high self-reported discrimination in numerous studies (Berger and Sarnyai, 2015; DeSantis et al., 2007; Huynh et al., 2016; Martin et al., 2012; Skinner et al., 2011; Zeiders et al., 2012). Frequency of discrimination experiences has also been linked to higher total daily cortisol secretion in adolescents (Huynh et al., 2016). Moreover, daily and lifetime experiences with discrimination are associated with chronic cortisol levels deposited in hair, a chronic measure of HPA function (O'Brien et al., 2017). Coupled with childhood adversity, social stress through discrimination could produce a vicious cycle where the lessons learned in childhood about the unpredictability of social connections continue to be reinforced by discrimination experiences later in life and become deeply embedded in our biology.

In addition to toxic stress history, several genetic polymorphisms in HPA-related genes are associated with stress endophenotypes. Variations in the 5-hydroxytryptamine transporter long polymorphic region (5HTT-LPR), and single-nucleotide polymorphisms (SNPs) including the FK506 Binding Protein 5 (FKBP5) gene rs1360780 and glucocorticoid receptor (GR) gene rs41423247 have been linked to increased vulnerability to psychiatric disorders related to stress, including depression, anxiety, and PTSD (Ebner and Singewald, 2017; Goldman et al., 2010; Melas et al., 2013; Norrholm and Ressler, 2009). For instance, obese subjects who carry the “G” variant of the GR (NR3C1) rs41423247, which helps terminate physiological stress response via a negative feedback system, have higher waist-to-hip ratio, higher systolic and diastolic blood pressure, and higher insulin and glucose compared to obese non-carriers (Srivastava et al., 2011). Similarly, the short allele of 5HTT-LPR is associated with increased and prolonged salivary cortisol levels in response to an acute stressor (Gotlib et al., 2008; Miller et al., 2013). Finally, the carriers of the “T” allele variant of FKBP5 rs1360780 gene, which alters the negative feedback loop of the glucocorticoid receptor complex by promoting transcription of the FKBP5 gene itself, has been linked to glucocorticoid insensitivity, elevated cortisol levels, and interacts with early life stress to increase vulnerability of stress-related disorders (Binder, 2009; Binder et al., 2008).

The stress response is fundamentally about marshaling resources, energetic or otherwise, needed to adapt to a threat to homeostasis. The metabolic role of GCs in gluconeogenesis is incorporated into the name, so it is unsurprising that stress and GCs have been shown to have numerous interactions with mitochondrial function in the brain and elsewhere (Jeanneteau and Arango-Lievano, 2016; Picard et al., 2014; Picard and McEwen, 2014). Perhaps more surprisingly, we and our collaborators have shown that the GR translocates into neuronal mitochondria with stress and with GC exposure, where GR interacts directly with the mitochondrial genome to control transcript levels and mitochondrial physiology (Hunter et al., 2016). In terms of transcriptional effects, GR binding to the mitochondrial genome appears to have the greatest effects on the levels of the mt-nd6 gene, the sole protein-coding gene located on the light inner strand of the mitochondrial genome (Du et al., 2009; Hunter et al., 2016). Given this and recent findings that environmental toxicants can alter mitochondrial DNA methylation in early life, it is possible that toxic social stress during early life could affect the methylation status of the mitochondrial genome (Byun et al., 2013; Janssen et al., 2015).

The associations between early life stress, chronic stress, and genetic polymorphisms on HPA regulation have each been examined in isolation and few studies have looked at the interaction of these factors in one sample of participants. We examined developmental and contextual influences, including social status, perceived stress levels, experiences with discrimination, FKBP5 rs1360780 genotype, 5HTT-LPR genotype, NR3C1 rs41423247 genotype, and childhood adversity and HPA activity before and following exposure to an acute psychosocial stressor in young adults. In keeping with previous reports, we predicted that individuals with a history of childhood adversity would have altered HPA regulation. We also examined methylation of five CpG sites in the MT-ND6 promoter and mitochondrial DNA copy number (MTDNAcn) in participants who reported no childhood adversity and participants who experienced high levels of childhood adversity to determine if the interactions between the GR and the mitochondria can have lasting effects in humans with a history of toxic stress.

2. Methods

2.1. Participants

This study was approved by the Institutional Review Board at the University of Massachusetts Boston. Participants ($n = 90$, 48.4% female, M age = 32.12 ± 15.10) were recruited from the UMass Boston community and the greater Boston area. Participants received $15 compensation or course credit. Participants were prescreened and were excluded if they were pregnant, breastfeeding, or taking medication related to hypertension, blood pressure, inflammation, arthritis, or medication that alters hormone levels (except oral contraceptives). Among participants taking oral contraceptives, type and dose of oral contraceptive were not found to be related to cortisol levels (data not shown).

2.2. Experiment protocol

Participants were asked to complete a series of surveys online with Psychdata at least 24 h prior to coming into the laboratory (Psychdata, LLC, State College, CA, USA). All participants completed the Adverse Childhood Experiences (ACE) survey (Felitti et al., 1998), Lifetime Discrimination Survey (Williams et al., 2008), Daily Discrimination Survey (Williams et al., 1997), and demographic questions. Fifty-nine participants were also asked to complete the Perceived Stress Scale (PSS; Cohen et al., 1983), City Stress Index (Ewart and Suchday, 2002), and additional questions related to demographics and social status. Subjective social status was measured using a single item in which the participant is asked to indicate where he or she would place themselves on a ladder that represents where they stand relative to others in their community.

Participants were asked to avoid caffeine, vigorous exercise, and dairy products on the day of their appointment and were asked to avoid eating for at least 30 min prior to their appointment. Appointments were scheduled between 10 am and 4 pm to limit the effect of circadian rhythm on cortisol levels. Participants were not allowed to drink water during the appointment. After obtaining informed consent, participants

were asked questions regarding their overall health and well-being that day. Participants were then asked to give a buccal sample and a pretest saliva sample.

2.2.1. Trier Social Stress Test

A modified version of the Trier Social Stress Test (TSST), a task that reliably induces a strong stresss reponse, was used to assess acute stress response to a psychosocial stressor (Kirschbaum et al., 1993), (Dickerson and Kemeny, 2004). Participants were informed that he or she would give a 3 min speech in front of a panel of two evaluators who are trained to assess body language, followed by a 3-min arithmetic task to assess working memory capacity. The two evaluators were instructed to maintain neutral affect during the speech and prompted the participant to continue if the participant stopped speaking for 15 s by asking scripted questions (e.g. “Can you talk about your personality?”). Following the speech, participants were instructed to serially subtract seven from 2023 as fast and as accurately as possible for 3 min. When the participant made a mistake, they were corrected and asked to restart by the evaluators. The appointment was discontinued in one case where the evaluators knew the participant.

2.2.2. Stress recovery

Following the TSST, the participant was instructed to watch a nature video until the last saliva sample was collected. The second saliva sample was collected 20 min after the start of the speech task, as cortisol levels have been previously shown to peak in saliva 15 to 20 min after stress (Kirschbaum and Hellhammer, 2000). The final saliva sample was collected 40 min after the start of the speech task to capture recovery from the stressor. Finally, participants were debriefed, thanked, and compensated for their time.

2.3. Cortisol

2.3.1. Saliva samples

Salivary free cortisol was measured to assess HPA axis response to the acute stressor. Salivary cortisol reflects the unbound portion of cortisol in blood and provides a more direct measure of biologically active cortisol (Levine et al., 2007). Saliva samples were collected with salivettes (Sarstedt, Germany). Participants were instructed to chew the salivette gently for two minutes before placing it back in the tube. Salivettes were immediately placed on ice until the end of the experiment and then stored at −80 °C. Prior to analysis, samples were thawed on ice then spun down at 1800 ×*g* at 4 °C for 10 min.

2.3.2. Cortisol quantification

Quantification of salivary cortisol was determined using a competitive Enzyme-linked immunosorbent assay (ELISA) (Enzo Life Sciences) as specified by the manufacturer's instructions. A micro-plate reader was used to measure the optical density at 405 nm. The concentration of cortisol in the samples was calculated using a 4-parameter logistic curve fitting program (PRISM Graphpad). The sensitivity of the assay was 56.72 pg/mL, the intra-assay coefficient of variation was 7.3–10.5%, and the inter-assay coefficient of variation was 8.6–13.4%. The specificity was 100% for cortisol and cross reactivity was 3.64% for progesterone and < 1% for testosterone and estradiol.

2.4. Genotyping

2.4.1. DNA collection and extraction

Cytobrushes (Coopersurgical Inc.) were used to collect buccal cells. Participants were instructed to brush the swab 30 times against the inside of their cheek while slowly rotating the swab. Swabs were immediately placed on ice and stored at −80 °C until DNA extraction. Buccal samples were extracted using a Zymo Quick DNA Universal Kit per the manufacturer's instructions (Zymo Research). DNA yield from buccal samples ranged from 0.48 μg to 14.4 μg of DNA. Extracted DNA was stored in molecular biology-grade water at −80 °C until genotyping analysis.

2.4.2. 5HTT genotyping

Genotyping for 5-HTTLPR polymorphisms was determined using PCR and gel electrophoresis (adapted from Smith et al., 2004). 25 μL PCR contained 2.5 ng of DNA and 0.15 μM of forward primer (FW5′ TGA ATG CCA GCA CCT AAC CC 3′) and reverse primer (RV 5′TTC TGG TGC CAC CTA GAC GC 3′). DNA amplification was achieved used the following programming: Initial denaturation was run for 11 min at 95 °C, followed by 40 cycles of 45 s at 95 °C, 45 s at 60 °C, 45 s at 72 °C, and a final elongation step of 72 °C for 10 min. The two amplicon products were 515 base pairs (long allele) and 471 base pairs (short allele), and were visualized by running the DNA samples on a 1.5% agarose gel stained with 1.5% Ethidium Bromide. Heterozygous genotypes were visibly detected by the presence of two bands in the lane approximately 44 base pairs apart. Participants were categorized as Long/Long (L/L), Long/Short (L/S), or Short/Short (S/S) for analysis.

2.4.3. NR3C1 genotyping

Real-time 5′ nuclease TaqMan SNP genotyping was used to determine the NR3C1 rs41423247 genotype (C/G), using uniquely designed forward and reverse primers (FW: 5′- GTAGCGAGAAAAGAAA CTGG-3′ and RV: 5′-CAGTGGATGCTGAACTCTTGG-3′). Genotypic amplification was achieved using the StepOne Plus Real-Time PCR System (Applied Biosystems). PCR programming was as follows: initial denaturation at 95 °C for 10 min, followed by 42 cycles of 95 °C for 15 s and 60 °C for 1 min. Participants were categorized as C/C or G carriers for analysis.

2.4.4. FKBP5 genotyping

TaqMan SNP genotyping was used to determine the FKBP5 single nucleotide polymorphism rs1360780 (C/T). 25 μL PCR reactions were performed using a pre-designed 1× Taqman allelic discrimination assay (Applied Biosystems, USA; assay number: C_8852038_10). PCR programming was as follows: initial denaturation at 95 °C for 10 min, followed by 42 cycles of 95 °C for 15 s and 60 °C for 1 min. Participants were categorized as C/C or T carriers for analysis.

2.5. Mitochondrial DNA measurements

Due to our previous observations that the MT-ND6 gene appears to be highly regulated by stress and corticosteroids (Hunter et al., 2016), we chose to examine five CpG sites upstream of this gene in addition to CpG sites in the D-Loop control region. An additional 68 participants were recruited for the mitochondrial DNA portion of the study. During the lab visit, these participants completed the ACE survey, Lifetime Discrimination Survey, Daily Discrimination Survey, and Demographic information and provided a buccal sample as described above. MT-ND6 methylation and MTDNAcn measurements were conducted on samples from participants from both pools who had an ACE score of 0 (n = 43) or an ACE score of 4 or above (n = 42).

2.5.1. MT-ND6 methylation

Bisulfite pyrosequencing for targeted DNA methylation in MT-ND6 gene (GRCh38/hg38 14,921–14,970) was measured by EpigenDX (Hopkinton, MA).

2.5.2. MTDNAcn

Relative MTDNAcn was measured as previously described (Bersani et al., 2016; He et al., 2002). TaqMan Copy Number assay was run with primers (FW 5′-CCCTAAAACCCGCCACATCT-3′, RV 5′-AGCGATGGTG AGAGCTAAGGT-3′] and a FAM probe [5′ FAM-CATCACCCTCTACATC ACCGCCC-TAMRA-3′] to detect levels of NADH-ubiquinone oxidoreductase chain 1using Ribonuclease P RNA component H1 as a reference gene (TaqMan® Copy Number Reference Assay, RNase P, cat#

4403328, Applied Biosystems).

2.6. Statistics

The Statistical Package for the Social Sciences (SPSS) was used for statistical analysis. All tests are 2-tailed with significance values below $p = .05$ reported as statistically significant. All data are expressed as means ± SD unless otherwise noted. Consistent with previous studies that collapse individuals with four or more ACEs into one group, participants were assigned to one of three ACE groups: ACE 0 ($n = 31$), ACE 1–3 ($n = 33$) and ACE 4+ ($n = 26$) (Dube et al., 2003). All cortisol data were non-normal and was therefore log-transformed prior to statistical analysis. In the case that log transformed data still did not meet the assumption of normality, as for percent change in cortisol from pretest levels, a Kruskal-Wallis nonparametric tests was also run. In all cases, parametric and non-parametric tests were in agreement.

Due to high variability in pretest cortisol, cortisol measures were normalized to pretest levels by dividing by pretest cortisol. A 2 × 3 between subjects mixed measures ANOVA was used to evaluate percent change in cortisol at the three time points with ACE group as the between-subjects factor. Mauchly's test indicated that the assumption of sphericity had been violated; therefore Huynh-Feldt estimates of sphericity were used to correct for degrees of freedom. One-way ANOVA was used to test differences in pretest cortisol between ACE groups, percent change in cortisol between ACE groups, and differences in cortisol for 5HTT genotype. A *t*-test was used to analyze pretest cortisol levels between FKBP5 (C/C and T carriers) and NR3C1 genotype (G/G or C carriers). Survey data violated the assumption of normality, thus Spearman correlations were used. Dimension reduction using principal component analysis with oblique rotation was used for FKBP5 genotype, ACE score, Lifetime Discrimination, and Daily Discrimination since these three factors were found to be associated with pretest cortisol levels. The two emerging factors were then used in a hierarchical multiple regression to predict pretest cortisol while controlling for age, sex, nicotine use, diagnosis with a mood disorder, stage of menstrual cycle, diagnosis with diabetes, and time of appointment. Mann-Whitney *U* test for continuous variables was used to assess non-normal MTND-6 DNA methylation data between ACE 0 and ACE 4+ groups.

3. Results

3.1. Description of participants

Frequency of ACE scores of all participants followed a similar distribution as reported in previous studies, except that the number of individuals who reported experiencing four or more ACEs was slightly higher in our diverse sample (Fig. 1; cf. Anda et al., 2006; Felitti et al., 1998). Self-reported overall stress, wellbeing, and sleep on the day of the appointment were not different between ACE groups (all $p > .05$) (Table 1).

3.2. Salivary cortisol responses to TSST

Due to high variability in pretest cortisol, cortisol reactivity (+20 min) and recovery (+40 min) measures were normalized to pretest cortisol. A 3-within × 2-between subjects mixed measures ANOVA was used to analyze salivary free cortisol response to stress with ACE group as the between-subjects factor. Mauchly's test indicated that the assumption of sphericity had been violated, $X^2(2) = 11.21$, $p = .004$, therefore Huynh-Feldt estimates of sphericity were used to correct for degrees of freedom ($\varepsilon = 0.89$). The results show a significant main effect for time, $F(1.86, 157.75) = 39.45$, $p < .001$, $\eta_p^2 = 0.31$, with cortisol peaking after the stressor as expected (Fig. 2). There was also a main effect for ACE group, $F(1, 85) = 26.69$, $p < .001$, $\eta_p^2 = 0.23$. Finally, there was a time x ACE group interaction $F(3.71, 157.75) = 6.32$, $p < .001$, $\eta_p^2 = 0.13$ with the high ACE group showing a blunted cortisol response to the TSST. Contrast tests were performed comparing percent change in salivary cortisol levels at different time points across ACE groups.

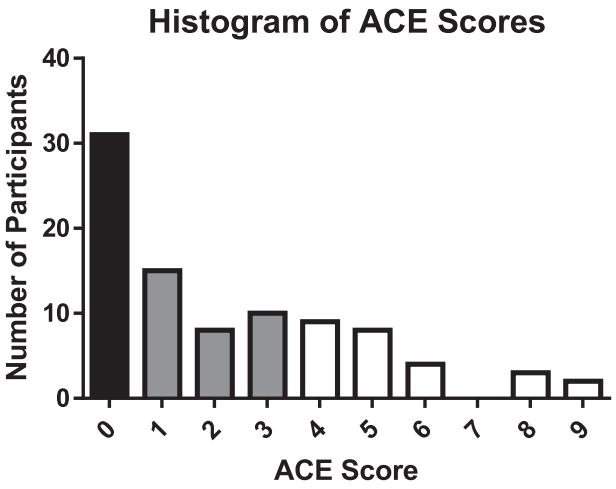

Fig. 1. Histogram of ACE scores. Individuals who had an ACE Score of 0 were categorized in the ACE 0 group ($n = 31$), individuals with ACE Scores of 1 to 3 were categorized in the ACE 1–3 group ($n = 33$), and individuals with ACE scores of 4 or higher were categorized in the ACE 4+ group ($n = 26$).

The mean percent increase in cortisol from five minutes before the TSST to 20 min post-stress was 189.13 ± 53.80% for the ACE 0 group, 155.42 ± 36.75 for the ACE 1–3 group, and 26.16 ± 11.46% for the ACE 4+ group. Percent change in cortisol from pretest levels to 20 min post-stress was significantly different between ACE groups, $F(2, 86) = 11.92$, $p < .001$, $\eta^2 = 0.22$, and Tukey HSD post-hoc analysis revealed significantly smaller change in cortisol levels from pretest levels in ACE 4+ group compared to the ACE 0 group ($p < .001$) and the ACE 1–3 group ($p < .001$).[1]

3.3. Genotype and cortisol measures

There was a significant difference in pretest cortisol between FKBP5 genotypes, $t(86) = 2.96$, $p < .01$. There was also a significant association between FKBP5 genotype and pretest cortisol, with higher cortisol levels associated with the "T" allele $r_s = 0.28$, $p < .01$ (Table 2; $n = 88$), but no other associations between FKBP5 genotype and cortisol reactivity and recovery measures were found (all $p > .05$). There was no relationship between pretest, reactivity, or recovery salivary cortisol between L/L, L/S, and S/S groups for 5HTT genotypes or between NR3C1 G/G individuals and C carriers (all $p > .05$).

3.4. Survey measures (Table 2)

3.4.1. Survey measures and cortisol

There was a significant association between pretest cortisol and Lifetime discrimination, $r_s = 0.307$, $p = .003$ ($n = 89$) and between cortisol and Daily discrimination, $r_s = 0.214$, $p = .044$ ($n = 89$). There were no other significant associations between survey measures and salivary cortisol measures.

3.4.2. ACE scores and discrimination surveys

There was a significant association between Lifetime Discrimination and ACE score, $r_s = 0.249$, $p = .018$ ($n = 90$), between ACE score and Daily Discrimination, $r_s = 0.222$, $p = .036$ ($n = 90$) and between ACE

[1] The percent change of Log-transformed cortisol measure was significantly non-normal, $D(89) = 0.10$, $p = .017$. An independent-samples Kruskal-Wallis Tests was run and was significant, $p < .001$.

Table 1
Participant demographic information.

	Sex		Race/ethnicity					Age (mean ± SD)	Diagnosis with mental disorder (n)	
	Male	Female	White	African American	Asian	Hispanic	Other/multiple		Yes (%)	No
ACE 0	17	14	19	6	5	0	1	33.03 ± 16.13	4 (19.05%)	17
ACE 1–3	11	22	15	6	5	4	3	28.18 ± 12.55	6 (27.27%)	16
ACE 4+	17	9	12	8	3	2	1	36.04 ± 16.15	6 (37.5%)	10
Total	45	45	46	20	13	6	5	32.12 ± 15.10	18 (29.5%)	43

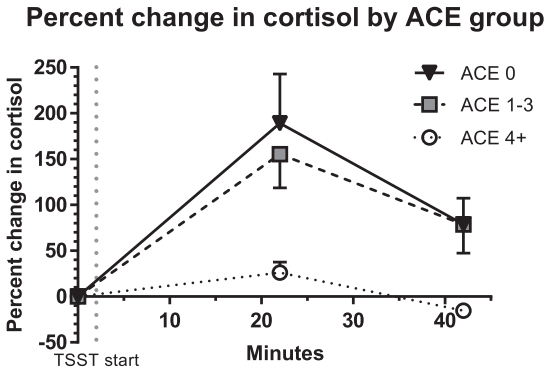

Fig. 2. Percent change in cortisol by ACE group (mean ± SEM). There was a time x ACE group interaction $F(3.71, 157.75) = 6.32$, $p < .001$, $\eta_p^2 = 0.13$ with the high ACE group showing a blunted cortisol response after the TSST. Contrasts were performed comparing percent change in salivary cortisol levels at different time points across ACE groups. (mean ± SEM).

Lifetime Discrimination and Subjective Social Status, $r_s = -0.266$, $p = .042$ ($n = 59$). There was a significant association between Subjective Social Status and Daily Discrimination, $r_s = -0.264$, $p = .043$ ($n = 59$).

3.5. Pretest cortisol

Pretest cortisol levels were significantly different between ACE groups, $F(2, 86) = 13.35$, $p < .001$, $\eta_p^2 = 0.24$, and Tukey HSD post-hoc analysis revealed significantly higher pretest cortisol levels in ACE 4+ group compared to the ACE 0 group ($p = .001$) and the ACE 1–3 group ($p < .001$) (Fig. 3).

Principal component analysis (PCA) with an oblique rotation was used on the four measures that were found to be significantly associated with pretest cortisol: FKBP5 genotype, ACE Score, Lifetime Discrimination, and Daily Discrimination. The KMO measure of sampling adequacy was 0.60 and Bartlett's test of sphericity was significant ($\chi 2$ (6) = 46.78, $p < .001$). Two factors with eigenvalues above 1

Table 2
Correlations between survey measures and pretest cortisol.

		Pretest cortisol	ACE score	Daily discrimination	Lifetime discrimination	Perceived stress scale	City stress index	Social status
ACE score	Correlation coefficient	0.34**						
	N	89						
Daily discrimination	Correlation coefficient	0.23*	0.26*					
	N	89	90					
Lifetime discrimination	Correlation Coefficient	0.22*	0.45***	0.47***				
	N	89	90	90				
Perceived stress scale	Correlation coefficient	0.09	0.13	0.25	0.15			
	N	58	59	59	59			
City stress index	Correlation coefficient	0.02	0.16	0.29*	0.38**	0.17		
	N	59	59	59	59	59		
Social status	Correlation coefficient	−0.10	−0.26*	−0.22	−0.32*	−0.28*	−0.21	
	N	58	59	59	59	59	59	
FKBPS genotype	Correlation coefficient	0.28**	0.14	−0.06	−0.11	0.02	−0.06	−0.05
	N	88	89	89	89	59	59	59

* $p < .05$.
** $p < .01$.
*** $p < .001$.

score and Subjective Social Status, $r_s = -0.320$, $p = .013$ ($n = 59$).

3.4.3. Social status, social support and stress correlations

There was a significant association between PSS and Subjective Social Status, $r_s = -0.266$, $p = .042$ ($n = 59$). There was a significant association between Lifetime Discrimination and City Stress Index, $r_s = 0.382$, $p = .003$ ($n = 59$). There was a significant association between City Stress Index and Daily Discrimination, $r_s = -0.291$, $p = .0226$ ($n = 59$). There was a significant association between

emerged (Table 3). Social stress measures (ACE Score, Daily Discrimination, and Lifetime Discrimination) loaded onto factor 1 (0.4 or above) and FKBP5 genotype loaded onto factor 2 (Table 3).

Stepwise multiple regression analysis revealed that Factor 1 and FKBP5 genotype significantly predict pretest cortisol levels after controlling for age, sex, nicotine use, diagnosis with a mood disorder, stage of menstrual cycle, and diagnosis with diabetes ($F(2,86) = 8.52$, $p < .001$, $R^2 = 0.43$; Table 4). In this model, Factor 1 accounted for 32% of the variance in pretest cortisol and FKBP5 genotype accounted

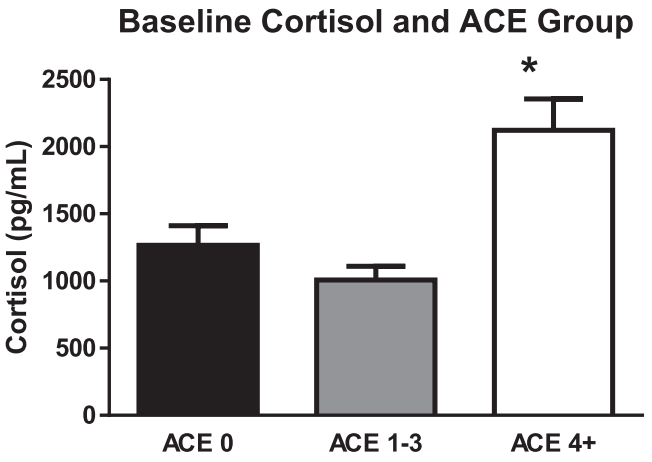

Fig. 3. Pretest cortisol by ACE group (mean ± SEM). Pretest cortisol levels were significantly different between ACE groups, $F(2, 86) = 13.35$, $p < .001$, $\eta^2 = 0.24$, and Tukey HSD post-hoc analysis revealed significantly higher pretest cortisol levels in ACE 4+ group compared to the ACE 0 group ($p = .001$) and the ACE 1–3 group ($p < .001$). * significantly different from ACE 0 group and ACE 1–3 group.

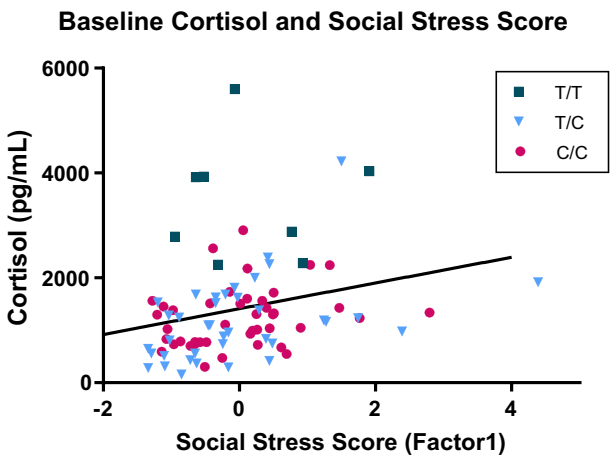

Fig. 4. Pretest cortisol and Social Stress Score (Factor 1 from PCA analysis). A stepwise multiple linear regression was calculated to predict pretest cortisol based on Social Stress Score and FKBP5 genotype. A significant regression was found ($F(2,86) = 8.52$, $p < .001$), $R^2 = 0.43$.

Table 3
Exploratory dimension reduction using principal component analysis.

	Factor 1	Factor 2
Daily discrimination	0.79	−0.26
Lifetime discrimination	0.85	0.02
ACE score	0.67	0.38
FKBP5 genotype	−0.04	0.93
Eigenvalues	1.8	1.08
% Variance	45.1	27.07

Table 4
Multiple regression analysis using Factor 1 and FKBP5 genotype to predict pretest cortisol. Stepwise multiple regression analysis revealed that Factor 1 and FKBP5 genotype significantly predict pretest cortisol levels after controlling for age, sex, nicotine use, diagnosis with a mood disorder, stage of menstrual cycle, and diagnosis with diabetes.

	B	B SE	β	Sig.
Step 1				
Constant	1569.66	140.91		$p < .001$
Factor 1	454.996	175.97	0.32	$p = .012$
Step 2				
Constant	2414.89	371.7		$p < .001$
Factor 1	450.17	168.67	0.32	$p = .012$
FKBP5 genotype	533.03	218.29	0.29	$p = .012$

Note: $R^2 = 0.32$ for Step 1, $\Delta R^2 = 0.11$ for Step 2.

for an additional 11% of the variance, suggesting more than half of the total variance in pretest salivary cortisol is explained by factors not measured in our study. A visual representation of this regression is shown in Fig. 4.

3.6. MT-ND6 mitochondrial DNA methylation and MTDNAcn

Mitochondrial DNA percent methylation data across five CpG sites near the MT-ND6 gene ranged from 0% to 2.1% across 85 samples (1.2 ± 0.55) and data were significantly non-normal, $D(79) = 0.24$, $p < .01$. Therefore, the nonparametric Mann-Whitney U test was used to compare MT-ND6 methylation levels between ACE 0 and ACE 4+ groups. Average methylation levels of MT-ND6 in the ACE 0 group ($Mdn = 1.22$) were significantly lower than the ACE 4+ group ($Mdn = 1.40$), $U = 670.50$, $z = -2.05$, $p < .05$, $r = 0.23$ (Fig. 5). There was no difference in MTDNAcn between ACE 0 and ACE 4+ groups ($p > .05$). No differences were observed in the methylation of the D-loop region.

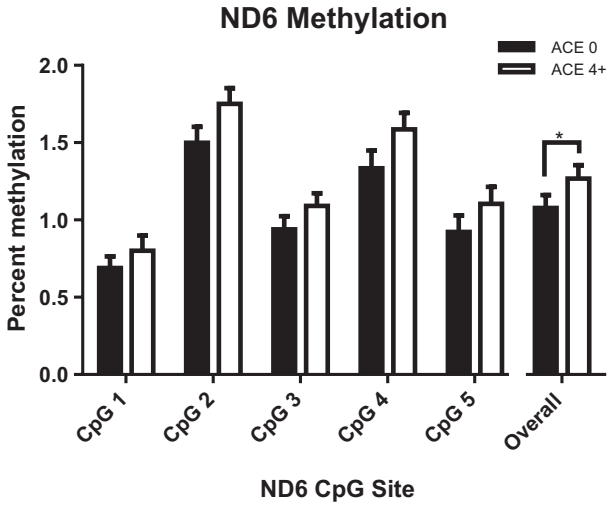

Fig. 5. Methylation of ND6 (mean ± SEM). Methylation levels of ND6 across 5 CpG sites in the ACE 0 group ($Mdn = 1.22$) were significantly lower than the ACE 4+ group ($Mdn = 1.40$), $U = 670.50$, $z = -2.05$, $p < .05$, $r = 0.23$. *$p < .05$.

4. Discussion

4.1. Effects of predictors on HPA response to an acute social stress test

Our results support previous work demonstrating enduring changes in HPA function in individuals with a history of childhood adversity (Carpenter et al., 2007; Carpenter et al., 2011; Heim et al., 2003; Klaassens et al., 2009; Kuhlman et al., 2015; Simmons et al., 2016). Specifically, we replicated studies showing a blunted cortisol response to the TSST among individuals who experienced childhood trauma (Carpenter et al., 2007, 2011; Elzinga et al., 2008) and other stressors (Klaassens et al., 2009; Voellmin et al., 2015). However, in contrast to previous reports that found no difference in pretest cortisol or lower pretest cortisol levels in individuals who experienced ELS, individuals with a history of high childhood adversity score in our study had significantly elevated pretest salivary cortisol and cortisol levels at 20 and 40 min post TSST comparable to ACE 0 and ACE 1–3 groups (Supplemental Fig. 1; cf. Carpenter et al., 2007; Elzinga et al., 2008; Klaassens

et al., 2009; Voellmin et al., 2015). These "arrival effects" have been reported by other labs and provide context for the blunted cortisol response observed in the ACE 4+ group in response to the TSST (Ruttle et al., 2011; Atkinson et al., 2013). Cortisol can continue to accumulate in saliva for up to 20 min after experiencing a stressor, meaning contextual factors just prior to the experiment include mild stressors associated with the hassle of getting to the laboratory, such as unpredictable traffic or late public transportation, or the novelty of being in a research lab (Kirschbaum and Hellhammer, 2000). Assuming these experiences were equal across ACE groups, elevated cortisol levels during our pretest measured in the ACE 4+ group could be interpreted as hyperresponsivity to a mild stressor or a general perturbation in HPA function. This interpretation is supported by work showing that childhood trauma may lead to hypervigilance and changes in brain areas involved in threat perception, leading to a lower threshold for HPA activation resulting in increased cortisol secretion (Dannlowski et al., 2012; Pine, 2003). Future work could examine this notion with a second pretest salivary cortisol measure to ensure that all groups have reached similar pretest levels before assessing cortisol reactivity to stress. It is important to note that self-reported well-being, stress levels, and sleep on the day of the appointment were not significantly different between ACE groups despite the physiological difference in salivary cortisol.

Exclusion protocols may be a second source of lab differences in pretest cortisol levels. Our participants were relatively heterogeneous in terms of psychological and physical health relative to other studies: we did not exclude participants because of diagnosis with mental disorder or diagnosis with a medical disorder (unless they were taking medication known to affect cortisol levels). Individuals with four or more ACEs are more susceptible to chronic physical and mental illness, so excluding participants with chronic illness may have disproportionally excluded individuals in the ACE 4+ group in other studies (cf. Anda et al., 2006, 2008; Chapman et al., 2004).

While childhood stress is a well-established determinant in development of HPA function, stressful experiences often persist after this developmental sensitive period into adulthood. In our study, both developmental stress and discrimination stress were related to pretest cortisol levels, building on previous work showing adult chronic stress can similarly have detrimental consequences for HPA function and health (Juster et al., 2010). Subjective Stress over the last three months and City Stress scores were not associated with absolute cortisol levels or cortisol changes in response to the TSST. However, self-reported Daily Discrimination (micro aggression-type discrimination) and Lifetime Discrimination (major events) were positively associated with pretest salivary cortisol. This is consistent with previous work showing abnormal HPA diurnal patterns in individuals who report more discrimination experiences (Berger and Sarnyai, 2015; DeSantis et al., 2007; Huynh et al., 2016; Martin et al., 2012; Skinner et al., 2011; Zeiders et al., 2012). This has meaningful implications for health disparities between minority and non-minority individuals and supports the hypothesis that chronic discrimination stress may lead to increased vulnerability to medical and mental illness through changes to the HPA axis. Perceived discrimination stress resulting in HPA dysregulation, here manifesting as elevated pretest cortisol, may explain these health disparities for stress-related medical and mental illness between minority and non-minority individuals (Green and Darity, 2010; Williams, 1999; Williams et al., 2003; Williams et al., 1997). Importantly, we did not inquire as to the specific kind of discrimination experienced, so it is unknown whether a specific type of discrimination, (e.g. racial discrimination), is responsible for the relationship with pretest cortisol found here. Whether specific types of discrimination, including discrimination based on gender, sexual orientation, physical appearance, or age are also associated with HPA function remains to be determined.

It is of interest that both discrimination variables and ACE Score loaded onto Factor 1 in our principal component analysis, suggesting that individuals who experience high levels of childhood adversity are also likely to report high levels of daily and lifetime discrimination. This finding could be due to environmental circumstances that make it likely that the same individuals exposed to social stress in the home also experience more discrimination outside the home. In our sample, subjective social status was negatively related to ACE score, lifetime discrimination, daily discrimination, and PSS score, suggesting that those who perceive themselves as being of lower social status within their community also have the highest levels of perceived stress across multiple domains (Table 2). Alternatively, individuals who experience high levels of adversity early in life may be more likely to interpret actions by others as discriminatory or events as stressful. These explanations are not necessarily mutually exclusive. In either scenario, negative social experiences, first with caretakers and family during early life then in other contexts in the form of discrimination, may be reinforced across the lifespan to have a significant impact on HPA function. Future work should seek to disentangle these hypotheses.

We found that the "T" allele of FKBP5 (rs1360780) was associated with higher pretest cortisol levels before the TSST but was not related to cortisol levels after the TSST or to change in cortisol during the experiment. Others have reported exaggerated plasma cortisol response to the TSST in T carriers (Buchmann et al., 2014; Höhne et al., 2015) and in infants after separation from their parents (Luijk et al., 2010), although these studies found no association between genotype and pretest cortisol levels. FKBP5 genotype explained 11% variance in pretest cortisol above and beyond that explained by the social stress measurements (Factor 1). Therefore, FKBP5 genotype seems to have a unique contribution to cortisol levels but no influence on Cort response to TSST. On the other hand, NR3C1 and 5HTT-LPR genotype were not associated with cortisol levels at any time point or to changes in cortisol after the TSST. This contrasts with previous studies that found homozygous S allele carriers to have elevated pretest cortisol levels and increased and prolonged cortisol levels in response to an acute stressor (Dougherty et al., 2010; Gotlib et al., 2008; Miller et al., 2013; Way and Taylor, 2010). Our sample size of 90 participants may not have provided sufficient power to detect influences of NR3C1 and 5HTT-LPR on cortisol.

4.2. Epigenetic pathway in mitochondria for enduring effect of ELS

We report provocative evidence of differential methylation near MTND-6 for adults with high levels of adverse childhood experiences. This extends recent findings that environmental pollutants are associated with differential methylation of the mitochondrial genome (Byun et al., 2013; Janssen et al., 2015) and is therefore the first evidence that psychological stress may contribute to epigenetic changes to the mitochondrial genome. It is of interest that the MT-ND6 gene, which shows the greatest level of regulation by stress and corticosteroids in animal models (Du et al., 2009; Hunter et al., 2016), is differentially methylated in individuals who report the highest levels of childhood adversity with no difference in mitochondrial copy number. Whether this differential methylation is due to the activity of the GR or through another mechanism cannot be established from our data. Further, it is worth noting that cytosine methylation levels in the mitochondrial genome are very low (1–2%) and therefore it seems likely that the changes we see are secondary to another process rather than functional in and of themselves (Liu et al., 2016). Future experiments should clarify the mechanistic links between early life stress and changes in mitochondrial DNA methylation.

5. Conclusions

We assessed several factors across domains of current and historical stress levels, genetic polymorphisms, and epigenetic factors, providing an integrated view of social, psychological and molecular effects on HPA regulation. We found that individuals who reported four or more ACEs had a blunted salivary cortisol response to the TSST. This effect

appears to be driven by elevated pretest cortisol in these individuals relative to those who reported have 1–3 ACEs or zero ACEs. Our study supports work demonstrating that social stress (specifically childhood adversity and perceived discrimination) and FKBP5 genotype have unique contributions to HPA activity. These factors influenced the pretest salivary cortisol measure. The pretest levels in our study may reflect a true baseline or an exaggerated response to a mild stressor, but either explanation suggests that these individuals are likely to have higher levels of chronic cortisol which may contribute to deleterious health outcomes over time. The final regression model only accounted for 43% of the variance in pretest cortisol, suggesting, as in the literature, that there are other influences not measured in this study. We also identified a new line of research with potential utility for understanding the lasting effect on early life stress over the life cycle. Overall percent methylation across five CpG sites in the MT-ND6 promoter was elevated in individuals who reported high childhood adversity compared to those who reported none, providing evidence that early experiences may lead to lasting epigenetic changes in the mitochondrial genome.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ntt.2018.01.011.

Conflicts of interest

The authors declare no conflicts of interest.

Transparency document

The http://dx.doi.org/10.1016/j.ntt.2018.01.011 associated with this article can be found in the online version.

Acknowledgements

This project was supported by a grant from the College of Liberal Arts at the University of Massachusetts Boston.

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