Psychoneuroendocrinology 107 (2019) 261–269

Contents lists available at ScienceDirect

Psychoneuroendocrinology

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

Childhood maltreatment, behavioral adjustment, and molecular markers of cellular aging in preschool-aged children: A cohort study*

Kathryn K. Ridout[b], Stephanie H. Parade[b,c], Hung-Teh Kao[b], Stevie Magnan[a], Ronald Seifer[b,c], Barbara Porton[b], Lawrence H. Price[a,b], Audrey R. Tyrka[a,b,*]

[a] Mood Disorders Research Program and Laboratory for Clinical and Translational Neuroscience, Butler Hospital, Providence, RI, USA
[b] Department of Psychiatry and Human Behavior, Alpert Medical School of Brown University, Providence, RI, USA
[c] Bradley/Hasbro Children's Research Center, E. P. Bradley Hospital, East Providence, RI, USA

ARTICLE INFO

Keywords:
Early life stress
Maltreatment
Adversity
Behavior problems
Mitochondria
Telomere

ABSTRACT

Objective: Childhood maltreatment is a major risk factor for the development of behavioral problems and poor physical and mental health. Accelerated cellular aging, through reduced telomere length and mitochondrial dysfunction, may be a mechanism underlying these associations.

Methods: Families with (n = 133) and without (n = 123) child welfare documentation of moderate-severe maltreatment in the past six months participated in this study. Children ranged in age from 3 to 5 years, were racially and ethnically diverse, and 91% qualified for public assistance. Structured record review and interviews were used to assess a history of maltreatment and other adversities. Telomere length and mitochondrial DNA copy number (mtDNAcn) were measured from saliva DNA using real-time PCR. Measures were repeated at a six-month follow-up assessment. Repeated measures general linear models were used to examine the effects of maltreatment and other adversities on telomere length and mtDNAcn over time.

Results: Maltreatment and other adverse experiences were significant positive predictors of both telomere length and mtDNAcn over time. Internalizing and externalizing behavior problems were also both significantly associated with telomere length, but only internalizing symptoms were associated with mtDNAcn.

Conclusions: This is the first study to show that mtDNAcn is altered in children with stress and trauma, and the findings are consistent with recent studies of adults. Surprisingly, children who experienced moderate-severe levels of maltreatment in the prior six months had longer telomeres, possibly reflecting compensatory changes in response to recent trauma. Telomere length and mtDNAcn were also associated with behavioral problems, suggesting that these measures of cellular aging may be causally implicated in the pathophysiology of stress-related conditions.

1. Introduction

Childhood maltreatment is a major public health crisis; over 700,000 cases of childhood maltreatment are reported to child protective service agencies every year in the United States (Children's Bureau, 2012), and as few as 5% of cases are reported (Gilbert et al., 2009). Maltreatment exposure increases risk for a number of psychiatric conditions including major depressive disorder (MDD), post-traumatic stress disorder (PTSD), and internalizing behaviors (Brown and Anderson, 1991; Rich-Edwards et al., 2010; Scott et al., 2012). Such conditions exact costs in excess of $124 billion through suffering, disability, treatment, and loss of productivity over the lifespan (Fang et al., 2012). There is great interest in identifying biomarkers of both maltreatment and psychiatric disorder risk that would facilitate early intervention, disorder prevention and treatment.

Childhood maltreatment impacts the stress response system and an increasing body of evidence supports the validity of studying the impact of these exposures through a stress-related aging lens (Entringer et al., 2018; Ridout et al., 2016a, 2015). Stress exposure activates the neuroendocrine and autonomic stress response systems and, while short term activation of these systems is necessary to respond to stress, frequent or chronic stress exposure can result in physiologic damage due to increased metabolic and physiologic demands (Entringer et al., 2016; Epel, 2009; Ridout et al., 2016a, 2015). Such chronic strain is evident in

* This research was supported by grants R01 MH083704 (ART), R01 HD086487 (ART), R01 HD095837 (SHP). Dr. Ridout received support from R25 MH101076 (ART). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

* Corresponding author at: Butler Hospital, 345 Blackstone Blvd., Providence, RI 02906, USA.
E-mail address: Audrey_Tyrka@Brown.edu (A.R. Tyrka).

https://doi.org/10.1016/j.psyneuen.2019.05.015
Received 5 September 2018; Received in revised form 4 May 2019; Accepted 14 May 2019

biomarkers impacted by repetitive stress.

Telomere length is a well-accepted biomarker of aging that is influenced by stress exposure and reflects physiological and metabolic demands placed on an organism (Epel and Prather, 2018; Ridout et al., 2016a, 2015). Telomeres are repetitive DNA sequences found at the ends of linear chromosomes (Blackburn et al., 2015) that help protect coding genomic information by providing a buffer of non-coding DNA that naturally shortens over the lifespan with cellular division and replication (O'Sullivan and Karlseder, 2010). Many stress-related disorders are associated with shortened telomeres and telomere shortening may impact risk for poor health (Blackburn et al., 2015; Ridout et al., 2015). Shortened telomeres are seen with a number of chronic diseases, including asthma, diabetes, and cardiovascular disease (Ridout et al., 2015). Telomere attrition can be accelerated with exposure to repetitive stress (Blackburn et al., 2015; Epel et al., 2004; Ridout et al., 2015).

Recent meta-analyses from our group and others support the association of shorter telomere length with childhood adversity in the form of maltreatment, neglect, abuse, adversity, and low socioeconomic status (Hanssen et al., 2017; Li et al., 2017b; Ridout et al., 2017). The overall effect was small-medium in size, and several studies report no change with measures of early adversity (Adams et al., 2007; Boks et al., 2015; Brody et al., 2015; Glass et al., 2010; Jodczyk et al., 2014; Kiecolt-Glaser et al., 2011; Mason et al., 2015; Robertson et al., 2012; Robles et al., 2016; Savolainen et al., 2014; Schaakxs et al., 2016; van Ockenburg et al., 2015). Methodological differences, including method of telomere assessment, measures of childhood adversity, and tissue source are likely responsible for these divergent findings (Ridout et al., 2018a). Adversity is difficult to measure precisely and comprehensively, and numerous exposures (e.g., adversity, toxins) and other factors (e.g., illnesses) can influence telomere length. Developmental timing of exposures and telomere measurement is also important as telomere length dynamics dramatically change during early life, with substantial telomere attrition observed from birth to age 3 compared to the gradual attrition observed later in life (Frenck et al., 1998; Zeichner et al., 1999), and a plateau from age 4 through young adulthood (Frenck et al., 1998; Wojcicki et al., 2016).

Several studies have examined the impact of early social environment on telomere length in children. These have studied telomere length in DNA from saliva or buccal swabs in modest-sized samples of children from disadvantaged social backgrounds as a broad indicator of early adversity. Longer childhood buccal telomere length has been reported with higher percent time institutionalized in Romanian orphanages (Drury et al., 2012), a designation of "high risk" for maltreatment according to child welfare services (Asok et al., 2013), and exposure to family violence and family disruption, particularly in girls, in a sample of predominantly African American youth (Drury et al., 2014). Similarly, saliva DNA telomere length has been negatively related to community level disorder, poverty, and disadvantage in African American children and youth from urban neighborhoods (Theall et al., 2013), disadvantaged social environments in African American boys (Mitchell et al., 2014), and lower parental education, but was not significantly associated with family income in a mixed-race sample of 7–13 year-olds (Needham et al., 2012). Longitudinal studies have documented accelerated telomere shortening in buccal cell DNA across middle childhood and adolescence among children with early institutional care (Humphreys et al., 2016), and from age 5–10 in relation to witnessing violence, bullying, and physical abuse (Shalev et al., 2013).

Given their importance to the stress response system, there is interest in telomeres as a biomarker of stress-related illness exposure and, potentially, as an underlying mechanism increasing risk for such disorders (Entringer et al., 2016; Ridout et al., 2015). Telomere length has been associated with psychiatric disorders (Darrow et al., 2016), including MDD (Lin et al., 2016; Ridout et al., 2016b), PTSD (Li et al., 2017a), and anxiety disorders (Malouff and Schutte, 2017). While there is a growing body of literature examining psychiatric symptoms and disorders in relationship to telomere length in adults, there is a paucity of such studies in children. Internalizing symptoms have been negatively associated with telomere length in a cohort of kindergarteners (Kroenke et al., 2011). In adolescents aged 13–18, decreased telomere length was associated with MDD (Henje Blom et al., 2015). Finally, decreased telomere length was associated with oppositional defiant disorder and anxiety at 5 years old, while there was a trend towards longer telomeres in children aged 3 years with affective symptoms (Wojcicki et al., 2015).

Mitochondria are intracellular organelles that are the main source of cellular energy and play a central role in multiple cellular processes (Streck et al., 2014), including those governing responding and adapting to stress (Juster et al., 2016; Picard et al., 2018; Ridout et al., 2016a,b). Similar to telomeres, mitochondrial function has been hypothesized to be integral to cellular aging, and mitochondrial DNA copy number (mtDNAcn) has been proposed as a marker of mitochondrial dysfunction and cellular aging (Kong et al., 2014; Sun et al., 2016). In line with this theory, restoration of mtDNAcn preserves mitochondrial function and delayed tissue aging in murine models (Foote et al., 2018). Variations in mtDNAcn in saliva or peripheral blood cells may reflect changes in mitochondrial energy production, demands, or compensation (Juster et al., 2016; Picard et al., 2018; Ridout et al., 2016a,b).

Recent evidence suggests mitochondria are affected by childhood trauma and may be involved in the effects of trauma on psychiatric disorder risk (Picard et al., 2018; Ridout et al., 2018b). Increased mitochondrial DNA copy number (mtDNAcn) has been reported from whole blood samples in adults with a history of childhood maltreatment (Tyrka et al., 2016) and saliva samples of adults with childhood sexual abuse (Cai et al., 2015). High mtDNAcn in blood or saliva samples has also been reported in adults with MDD (Cai et al., 2015; Edwards et al., 2016; Tyrka et al., 2016) and suicide (Lindqvist et al., 2016; Otsuka et al., 2017), as well as anxiety disorders and substance use disorders (Tyrka et al., 2016). Other investigations of mtDNAcn from whole blood samples have reported an association with cell-free mtDNAcn from plasma but not plasma (Lindqvist et al., 2018), no association with depressive symptoms (Verhoeven et al., 2017), or a decrease of mtDNAcn in peripheral blood mononuclear cells (PBMCs) of older adults with MDD (Kim et al., 2011) and in granulocytes of male combat veterans with PTSD compared to those without PTSD (Bersani et al., 2016). Variable findings may be due to effects of the course, severity, and chronicity of disorders, exposures, and other effects on mtDNAcn, such as tissue source, chronic or acute illnesses (Edwards et al., 2016; Nicod et al., 2016). No studies have examined mtDNAcn as related to maltreatment or behavior in children.

In the present study, we aimed to examine these biomarkers of stress exposure and aging as related to recent maltreatment and behavioral problems in a sample of children aged 3–5-years. This study aimed to 1) examine cross-sectional and longitudinal relationships of telomere length and mtDNAcn with recent maltreatment and other adverse exposures at two time points in early development, and 2) examine the relation of telomere length and mtDNAcn with behavioral adjustment over time in early development.

2. Methods

2.1. Participants

Two hundred and fifty-six children were included in this study. Of these, 133 had child welfare documentation of moderate-severe maltreatment in the past six months at baseline. Children ranged in age from 3 to 5 years (M = 51.1 months; SD = 8.9 months), were racially and ethnically diverse (140 non-Hispanic, 115 Hispanic, 1 unknown ethnicity; 100 white, 41 black, 57 biracial, 50 other and 7 unknown), and 122 were male. Fifty caregivers had less than a high school degree, 104 completed high school, 77 had some post-secondary education, 24 had a bachelor's degree, and 1 did not provide education information. One hundred and thirty-seven caregivers were single parents and 141

were unemployed. Two hundred thirty-one of the families qualified for public assistance. No more than one child per family was included.

2.2. Procedure

This study was approved by the Lifespan – Rhode Island Hospital Institutional Review Board. Children with a history of maltreatment were identified from the local child welfare agency or an emergency maltreatment assessment service via record review. Families without a history of maltreatment within the past six months were recruited at a pediatric medical clinic during a well-child visit or at childcare centers. Children with a chronic medical or neurological condition, regular medication use, obesity, and failure-to-thrive as determined by medical record review and parent report, were excluded. In the case of acute illness or medication use, sample collection was delayed at least two weeks following resolution of illness and medication use.

Families completed two home visits and questionnaires between the visits at baseline, and two home visits for follow-up. Visits were spread out throughout the year over the course of five years for the entire study. The first home visit at each assessment wave, during which caregivers completed interviews on child stress exposure and a saliva sample for DNA isolation was collected from the children, is the focus of the current report.

2.3. Measures

2.3.1. Child maltreatment status

All families consented to examination of child welfare records to determine maltreatment status. Trained research staff coded the records using the System for Coding Subtype and Severity of Maltreatment in Child Protective Records (Barnett et al., 1993). Five maltreatment subtypes and severity scores were derived, ranging from 1 (least severe) to 5 (most severe). Children with moderate to severe levels of maltreatment (score of 3–5) within the last six months were considered as part of the maltreated group ($n = 133$). Twenty-five children had substantiated cases involving physical abuse, 32 sexual abuse, 16 physical neglect/failure to provide, 36 physical neglect/lack of supervision, and 83 emotional maltreatment. The comparison group ($n = 123$) included children who had never had a substantiated case of maltreatment regardless of severity type.

2.3.2. Contextual stress

Caregivers completed a semi-structured interview developed in our laboratory to assess the child's experience of contextual stressors in the child's lifetime. Categories were: death of a caregiver, separation from a caregiver, frequent change of residence or homelessness, inadequate food or clothing, and other events including witnessing neighborhood violence or parental arrest. Each domain was scored positive if at least one episode occurred, and domains were summed for lifetime. Possible scores ranged from 0 (no stressors) to 5 (stressors in all five domains) for each summary scale.

2.3.3. Traumatic life events

The Diagnostic Infant and Preschool Assessment (Scheeringa and Haslett, 2010) interview was conducted with caregivers to assess child traumatic life events. Interviews were conducted by trained clinical social workers and PhD-level psychologists, reviewed in a group supervision format, and scored based upon group consensus. The number of types of traumas experienced in the child's lifetime was summed. Categories were: experiencing an accident, animal attack, man-made disaster, natural disaster, witnessing violence, accidental burning, medical emergency/hospitalization/invasive medical procedure, kidnapping, and other events, such as a near drowning. Physical and sexual abuse were not included in this variable because they were assessed as maltreatment (above). Possible scores ranged from 0 to 9.

2.3.4. Adversity composite

The number of types of maltreatment experienced, the number of lifetime contextual stressors, and the number of other traumatic life events were summed to create an adversity composite to examine the cumulative effect of adversity exposures, consistent with the literature suggesting a cumulative effect of multiple exposures (Ridout et al., 2018a,b) and in line with our previous publications (Tyrka et al., 2015a,b). Possible scores ranged from 0 to 18.

2.3.5. Socioeconomic adversity

Indicators of socioeconomic adversity (parental education ≤ high school degree, parental unemployment, and single parenthood) were summed. Possible scores ranged from 0 to 3.

2.3.6. Behavior problems

Caregivers completed the Child Behavior Checklist for Ages 1.5–5 (CBCL; (Achenbach and Ruffle, 2000)) to assess internalizing and externalizing behavior problems. For each of the 100 behaviors, parents assessed their children on a 3-point scale from 0 (not true) to 2 (very true). T scores were used for data analysis. The CBCL is a reliable and valid measure with strong test-retest reliability ($r = .90$ and 0.87 for internalizing and externalizing scales, respectively), as well as discriminant validity between children who were and were not referred for behavioral health services (Achenbach & Rescorla, 2000). Possible scores ranged from 0 to 100.

2.4. DNA isolation and real-time quantitative PCR (qPCR)

Saliva samples were obtained using the Oragene DISCOVER kits (OGR-575) for Assisted Collections (DNA Genotek, Kanata, Ontario, Canada) at baseline and follow-up visits. DNA was isolated using the QIAamp DNA isolation kit (Qiagen, Hilden, Germany); reagents were added to the saliva and samples centrifuged as specified in the kit. Per the manufacturer, the kit isolates mitochondrial DNA and nuclear DNA with minimal to no degradation.

Three parallel quantitative polymerase chain reactions (qPCR) were performed to quantitate copy numbers for telomeres, mitochondrial genomes, and the beta-hemoglobin gene as a single-copy standard as previously described (O'Callaghan and Fenech, 2011). Data were acquired using the ABI Prism HT79000 DNA Sequence Detection System (Applied Biosystems, Grand Island, New York). qPCR was performed using 384-well plates in a reaction volume of 10 mL containing approximately 25 ng genomic DNA, 300 nmol/L of each pair of primers, and 1 X Sybr Select Master Mix (Life Technologies Corporation, Grand Island, New York). For telomere length the reaction also included 2% dimethyl sulfoxide. Each reaction plate contained wells with serial dilutions of cloned telomere amplicon, mitochondrial amplicon, and beta-hemoglobin amplicon to permit quantitation of mtDNA and beta-hemoglobin copy number. A 12-point standard curve (log 0 ng–5.25 ng) was used to directly determine copy number for telomere, mitochondria, or the single-copy gene in each PCR run. All analyses were performed in triplicate. PCR efficiency criteria for all measures were 99–104%. Coefficients of variation (CVs) were calculated within each triplicate; samples with CVs > 5% were repeated.

2.4.1. Beta-hemoglobin

The sequences of the forward and reverse primers for the beta-hemoglobin gene were GCT TCT GAC ACA ACT GTG TTC ACT AGC and CAC CAA CTT CAT CCA CGT. An initial heating step of 95 °C for 10 min was followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.4.2. mtDNA copy number

The sequences of the forward and reverse mitochondrial primers (directed toward the D-loop region) were CAT CTG GTT CCT ACT TCA GGG and TGA GTG GTT AAT AGG GTG ATA GA (Bai and Wong, 2005). An initial heating step of 95 °C for 10 min was followed by 40 cycles of

95 °C for 15 s and 60 °C for 1 min. mtDNA copy number relative to beta-hemoglobin gene copy number was calculated; data used in data analysis reported as mtDNAcn per diploid genome.

2.4.3. Telomere length

Modified telomere primer sequences were provided by Richard Cawthon, M.D., Ph.D. (Eccles Institute of Human Genetics, University of Utah, Salt Lake City, Utah): CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT (Tel1b) and GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT (Tel2b). An initial heating step of 95 °C for 30 min was followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Telomere copy number relative to beta-hemoglobin copy number was obtained.

2.5. Statistical analysis

Analyses were conducted with SPSS version 24 (IBM, Armonk, NY, USA). All analyses were two-tailed with alpha = .05. Demographic characteristics were examined and controlled for where appropriate. Telomere length and mtDNAcn were Winsorized to 3 standard deviations. Missing data were imputed using SPSS 24. Less than 4% of data was missing overall. Little's Missing Completely at Random test (Little, 1988) demonstrated that the data were missing completely at random. Ten imputed datasets were derived from the original dataset, analyses were run on each individual dataset, and parameter estimates from all ten datasets were pooled to derive the final results. Associations of demographic characteristics, telomere length, mtDNAcn, adversity variables and child behavior problems were examined using Pearson correlations and *t*-tests. Repeated measures general linear models (GLMs), controlling for covariates, were used to examine the effects of the adversity variables on telomere length or mtDNAcn over time. Child age was included as an a-priori covariate given prior findings on age and telomere length. Other demographic variables were examined for association with the outcome variables and included in subsequent analyses if significant (described below). The primary goal of the repeated measures GLM was to simultaneously examine between (overall mean) and within (change over time) effects. Additional GLMs tested associations of behavioral problems with telomere length and mtDNAcn, controlling for covariates. Partial correlations, controlling for covariates, were used to examine associations of adversity variables and behavioral problems with telomere length and mtDNAcn at each individual time point. Sensitivity analyses to assess whether effects differed in males and females were conducted using GLMs with sex included as an interaction term.

3. Results

3.1. Preliminary analyses

Table 1 presents demographic, adversity, and child behavior data by maltreatment status. There were no differences between the groups in child age, sex, or qualifying for public assistance. Significant differences between the groups were observed based on child race and ethnicity. Children who were maltreated were less likely to be Hispanic and more likely to be Non-Hispanic White. Children who were maltreated also had greater socioeconomic adversity, higher scores on the adversity composite, more traumatic life events, and greater lifetime stress. Children who were maltreated had more internalizing, but not externalizing, behavior problems. Child age, sex, and race were not associated with telomere length or mtDNAcn at baseline or follow-up, but given well-established effects of age on telomere length, child age was included in subsequent analyses as a covariate. Child ethnicity was significantly associated with mtDNAcn at baseline, with Hispanic children having lower mtDNAcn (p = .022). Hispanic children also had longer telomere length than non-Hispanic children at follow-up (p = .009). Thus, ethnicity was included with age as covariates in all subsequent analyses. Variation in length of time between the baseline and follow-up assessments was not associated with telomere length or mtDNAcn at baseline or follow-up, nor did it predict change in telomere length or mtDNAcn over time, and was therefore considered no further. Socioeconomic adversity within this sample was not associated with telomere length or mtDNAcn at baseline or follow-up, nor did it predict change in telomere length or mtDNAcn over time, therefore it was considered no further. Baseline telomere length and mtDNAcn were significantly positively correlated (r = .38, p < .001), as were follow-up telomere length and mtDNAcn (r = .37, p < .001).

Table 1
Descriptive Statistics.

	No Maltreatment *n = 123*	Maltreated *n = 133*	*p*
Age (months), *M (SD)*	50.1 (9.0)	51.9 (8.8)	.11
Sex, *N (%)* Female	63 (51.2)	71 (53.4)	.73
Race/Ethnicity			.00
Hispanic, *N (%)*	65 (52.8)	50 (37.6)	
Non-Hispanic White, *N (%)*	21 (17.1)	52 (39.1)	
Non-Hispanic Black, *N (%)*	19 (15.4)	15 (11.3)	
Non-Hispanic, Other/Biracial, *N (%)*	18 (14.6)	16 (12.0)	
Qualified for Public Assistance, *N (%)*	109 (89.3)	122 (91.7)	.52
Socioeconomic Adversity, *M (SD)*	1.5 (0.9)	1.8 (0.9)	.01
Adversity Composite, *M (SD)*	1.6 (1.6)	4.9 (2.0)	.00
Traumatic Life Events, *M (SD)*	0.7 (0.9)	1.5 (1.0)	.00
Lifetime Stress, *M (SD)*	1.0 (1.0)	1.9 (1.2)	.00
Internalizing Behavior Problems, *M (SD)*	50.9 (8.6)	53.8 (7.7)	.00
Externalizing Behavior Problems, *M (SD)*	46.5 (10.3)	48.0 (12.4)	.26

3.2. Association of telomere length with maltreatment status and other adversities

In repeated measures GLMs controlling for child age and ethnicity, there was a significant between-subjects effect of adversity composite (F(1,252) = 25.08, p < .001), child maltreatment status (F(1, 252) = 13.85, p < .001), number of traumatic life events (F (1,252) = 12.27, p < .001), and lifetime stress (F(1,252) = 15.30, p < .001) on telomere length. As illustrated in Table 2, all four adversity variables were positively associated with telomere length at both the baseline and follow-up assessments. In contrast, within-subjects effects of the adversity composite (F(1,252) = .24, p = .625), child maltreatment status (F(1,252) = .10, p = .752), number of traumatic life events (F(1,252) = .16, p = .689), and lifetime stress (F (1,252) = .10, p = .752) were not significant, and therefore these adversity measures did not predict change in telomere length over time.

3.3. Association of mtDNAcn with maltreatment status and other adversities

There was a significant between-subjects effect of adversity composite (F(1,252) = 7.55, p = .006), child maltreatment status (F(1, 252) = 5.85, p = .016) and number of traumatic life events (F (1,252) = 10.60, p = .001) on mtDNAcn in repeated measures GLMs controlling for child age and ethnicity. As illustrated in Table 2, the adversity composite, child maltreatment status, and number of traumatic life events were positively associated with mtDNAcn at the follow-up assessment, but not at the baseline assessment. There was no significant between-subjects effect of lifetime stress in the repeated measures GLM (F(1, 252) = 2.12, p = .147), but examination of the pattern of findings revealed that consistent with the other adversity variables, lifetime stress was positively associated with mtDNAcn at the follow-up assessment, but not at the baseline assessment (Table 2).

Table 2
Associations of adversity variables with telomere length and mtDNAcn.

	Telomere Length Baseline	Telomere Length Follow-Up	mtDNAcn Baseline	mtDNAcn Follow-Up
Adversity Composite	r = 0.21**	r = 0.26**	r = 0.08	r = 0.20**
Maltreatment Status	r = 0.18**	r = 0.17**	r = 0.12	r = 0.13*
Traumatic Life Events	r = 0.15*	r = 0.19**	r = 0.07	r = 0.26**
Lifetime Stress	r = 0.18**	r = 0.20**	r = 0.02	r = 0.13*

Notes: n = 256. * p < .05. ** p < .01. r indicates partial correlations with child age and ethnicity included as covariates. Maltreatment Status: 1 = Maltreated, 0 = No Maltreatment. Children who were maltreated had longer telomeres at baseline (M = 3.03, SE = .03) and follow-up (M = 3.12, SE = .03) than children who were not maltreated at baseline (M = 2.89, SE = .03) and follow-up (M = 2.99, SE = .03). Children who were maltreated had greater mtDNAcn at follow-up (M = 2.18, SE = .02) than children who were not maltreated (M = 2.12, SE = .03), but there was no difference at baseline between the maltreated groups (M = 2.09, SE = .02) and the no maltreatment group (M = 2.03, SE = .02).

Within-subjects effects of adversity composite (F(1, 252) = 2.90, p = .090), child maltreatment status (F(1, 252) = .19, p = .663, and lifetime stress (F(1, 252) = 2.37, p = .125) were not significant, and therefore did not predict change in mtDNAcn over time. In contrast, there was a significant within subjects effect of traumatic life events on change in mtDNAcn over time (F(1,252) = 6.48, p = .012). Children with two or more traumatic life events at baseline had the sharpest increases in mtDNAcn over time compared to children with zero or one traumatic life event.

3.4. Association of telomere length with child behavior problems

There were significant between-subjects effect of internalizing (F(1,252) = 39.71, p < .001) and externalizing (F(1, 252) = 11.84, p < .001) behavior problems on telomere length in repeated measures GLMs controlling for child age and ethnicity. As illustrated in Table 2, internalizing and externalizing behavior problems were positively associated with telomere length at the baseline assessment. Internalizing, but not externalizing, behavior problems were positively associated with telomere length at the follow-up assessment. There was a significant within subjects effect of internalizing behavior problems on change in telomere length over time (F(1,252) = 5.78, p = .017). Children at or above the median score for internalizing problems (Median = 51) had sharper increases in telomere length over time compared to children below the median score for internalizing problems. In contrast, the within-subjects effect of externalizing behavior problems was not significant (F(1,252) = 3.22, p = .074), and therefore did not predict change in telomere length over time.

3.5. Association of mtDNAcn with child behavior problems

There was a significant between-subjects effect of internalizing behavior problems (F(1,252) = 16.86, p < .001) on mtDNAcn controlling for child age and ethnicity. As shown in Table 3, internalizing behavior problems were positively associated with mtDNAcn at both the baseline and follow-up assessments. There was no significant between-subjects effect of externalizing behaviors (F(1,252) = 0.41, p = .523); externalizing behavior problems were not associated with mtDNAcn at the baseline or follow-up assessments. Within-subjects effects of internalizing (F(1,252) = .92, p = .338), and externalizing (F(1,252) = .19, p = .663) behavior problems were not significant, and therefore behavior problems did not predict change in mtDNAcn over time.

3.6. Sensitivity analyses

In order to determine whether the above effects differed by sex, we repeated each of the GLMs and included a sex × adversity or sex × behavior interaction term. There were no significant interactions of sex with any of the adversity or behavior variables in the prediction of telomere length or mtDNAcn (p's > .05).

All reported effects remained significant without multiple imputation except for the between-subjects effect for telomere length and externalizing behaviors and the within-subjects effect for telomere length and internalizing behaviors, which dropped to a trend level. Scatterplots of the significant effects using raw non-imputed data are shown in the supplemental material.

4. Discussion

These results indicate that exposure to stress and maltreatment during the preschool age (3–5 years old) is detectable through markers of cellular aging, telomere length and mtDNAcn. In addition to being altered with exposure to adversity, these biological indicators of cellular aging and stress exposure were associated with behavioral problems. To our knowledge, this the first study to examine mitochondrial involvement in exposure to stress or trauma in children, and also the first to examine the acute effects of recent (in the preceding 6 months) maltreatment exposure and after a 6-month follow-up period. These results provide compelling evidence that both markers are associated with maltreatment exposure and behavioral symptoms, supporting the idea that such exposures may impact biological aging (Kiecolt-Glaser and Wilson, 2016; Ridout et al., 2015, 2018a; Ridout et al., 2018b) even in young children.

These findings contribute to the mounting evidence that maltreatment and other stressful exposures may deeply impact children's health, by influencing cellular aging. Telomere length is responsive to stress-related changes in intracellular signaling and can dynamically change with exposures such as psychosocial stress, illness, and inflammation (Ridout et al., 2016a, 2015; Svenson et al., 2011). Shortened telomeres are associated with a number of chronic diseases, including asthma, diabetes, and cardiovascular disease (Ridout et al., 2015). Mitochondria are cellular organelles critical to metabolism, energy production, calcium signaling, cell growth and differentiation, cell cycle control, and cell death (Ridout et al., 2016a). Alterations in mitochondrial function are linked to increased risk of diseases associated with aging, such as diabetes and heart disease (Ridout et al., 2016a). Together, the results

Table 3
Associations of child behavior symptoms with telomere length and mtDNAcn.

	Telomere Length Baseline	Telomere Length Follow-Up	mtDNAcn Baseline	mtDNAcn Follow-Up
Internalizing Behavior	r = 0.37**	r = 0.20**	r = 0.17**	r = 0.24**
Externalizing Behavior	r = 0.23**	r = 0.10	r = 0.01	r = 0.01

Notes: n = 256. * p < .05. ** p < .01. r indicates partial correlations with child age and ethnicity included as covariates.

of this study suggest that maltreatment may impact these biological markers of stress and aging and increase an individual's risk of age-related illness later in life.

Additionally, these results provide preliminary evidence that maltreatment exposure may contribute to psychopathology risk by impacting biological markers of stress and aging as early as ages 3–5 years. These findings underscore the importance of early screening, detection, and intervention for childhood maltreatment. Although studies of the impact of early intervention after maltreatment exposure are limited, preliminary results suggest that trauma-informed intervention may help reverse the biological impact of childhood maltreatment (Asok et al., 2013; Dozier et al., 2018; Slopen et al., 2014). Such intervention may be vital to moderating the impact of maltreatment on mental and physical well-being in childhood and into adulthood.

In this study, we observed longer telomeres in the maltreated cohort compared to controls, in contrast to previous literature that showed shorter TL with stress and trauma in childhood (Hanssen et al., 2017; Li et al., 2017b; Ridout et al., 2018). Our study is unique in that it was designed to examine the acute effects of recent (in the preceding 6 months) maltreatment exposure and included a 6-month follow-up. Most previous studies did not report the timing of maltreatment exposure (Asok et al., 2013; Drury et al., 2014; Mitchell et al., 2014; Needham et al., 2012; Robles et al., 2016; Shalev et al., 2013; Theall et al., 2013), or they examined telomere length more remotely after the documented exposure (Drury et al., 2012; Humphreys et al., 2016). Moreover, this is the only study to include children whose maltreatment was verified via coding of child welfare records, and we included measures of parental report of other stressors and traumas to provide a comprehensive assessment of major adversities. That our telomere findings were consistent 1) for several measures of adversity, 2) between measures of adversity and measures of behavior, 3) with findings related to mtDNAcn in addition to telomere length, and 4) at baseline and 6-month follow-up, lends confidence to their validity.

As discussed above, telomere attrition occurs at a fast pace through age 3 (Frenck et al., 1998; Zeichner et al., 1999) and plateaus from age 4 through young adulthood (Frenck et al., 1998). In a cohort of n = 77 Latino children studied longitudinally over a year from age 4 and 5, attrition was rare, and telomeres showed predominantly lengthening or maintenance (Wojcicki et al., 2016). It is not clear how telomere dynamics in this developmental phase are influenced by adversity, and it is notable that we did not find an association of age and telomere length in this sample of children aged 3–5, but it is possible that the positive association between adversity and telomere length was influenced by a normative process of lengthening or maintenance that may occur in the later part of this phase. Telomere lengthening has previously been reported in children followed from age 5 to 10 (Shalev et al., 2013), and some studies that find overall shorter telomeres with adversity have reported that some adverse conditions or stress measures were associated with longer telomeres (Drury et al., 2017; Theall et al., 2013). In a longitudinal study of Dutch military personnel before and 6 months following deployment to Afghanistan, Boks and colleagues (2015) found that the development of PTSD symptoms was associated with telomere lengthening as well as *decreased* DNA methylation aging.

Our observation of relatively longer telomeres with recent stress exposure could reflect the increased activity of telomerase, which adds base pairs to the ends of telomeres, thereby lengthening them. The data in this study were derived from saliva. Saliva is made up of multiple tissue types, however has a large proportion of leukocytes, (Nature, 2011; Thiede et al., 2000; Thomas et al., 1994), in which telomerase is active (Counter et al., 1995). Telomerase has been reported to be active in saliva (Zhong et al., 2005), which likely reflects the telomerase activity in leukocytes. While chronic stress is associated with decreases in telomerase activity (Deng et al., 2016), acute stress is associated with telomerase activity activation (Deng et al., 2016; Epel et al., 2010). Additionally, the catalytic subunit of telomerase, telomerase reverse transcriptase (TERT), localizes to mtDNA in post-mitotic cells, where it serves to protect the mitochondrial genome from oxidative damage (Ale-Agha et al., 2014) and decreases mitochondrial production of reactive oxygen species (Haendeler et al., 2009). Increased oxidative stress is a known mechanism for telomere shortening (von Zglinicki, 2002). Thus, our findings may reflect an upregulation of telomerase in children with recent maltreatment exposure, which provides a relative protection against oxidative stress and telomere shortening compared to controls. Telomere length was positively related to measures of lifetime trauma exposure as well as recent maltreatment; however, maltreatment and other lifetime traumas frequently co-occurred, so it is difficult to disentangle these effects.

In this cohort, we did not find an association of sex or race with telomere length or mtDNAcn. A meta-analysis on sex and telomere length found that females have longer telomeres than males, though only when Southern blot was used to measure telomeres (Gardner et al., 2014). Several studies have found that Caucasian adults have shorter telomeres than African Americans or Hispanics (Sanders and Newman, 2013) though one study found that black infants have longer placenta telomeres than white infants (Drury et al., 2015). We found that telomere length was longer among Hispanics at the follow-up assessment, and we controlled for this in the analyses; telomere length did not differ by race in the present sample. It should be acknowledged that our study is limited by the use of saliva DNA and we had no means to assess or control for differences in distribution of cell type. However, we did not collect saliva samples from children with acute or chronic illnesses, or those taking medications.

It is possible that engagement in social or psychological services had an impact on telomere length or mtDNAcn. To address this, we examined the number of unique episodes of service utilization (including outpatient mental health treatment, home based services, and services provided by the local school department). Although telomere length and mtDNAcn were positively associated with service utilization, after controlling for the composite adversity variable, these associations were no longer significant ($F(1,251) = .95$, $p = .33$ for telomere length, and $F(1,251) = 2.06$, $p = .15$ for mtDNAcn). In contrast, the between subjects effect of the adversity composite remained significant even when controlling for service utilization ($F(1,251 = 21.7$, $p < .001$ for telomere length, and $F(1, 251) = 5.61$, $p = .02$ for mtDNAcn).

This is the first study to examine mtDNAcn in children in relation to adversity. Our findings of higher mtDNAcn in relation to maltreatment and other adversities are consistent with prior evidence showing mtDNAcn increases in adults with a history of childhood maltreatment in the form of abuse (Tyrka et al., 2016), neglect (Tyrka et al., 2016), or sexual abuse (Cai et al., 2015). That the significant effects for mtDNAcn were seen at 6 months of follow-up for the adversity measures, and that those with more traumas showed the greatest increases over the 6-month follow-up period, suggests that mtDNAcn might show dynamic changes in response to adverse exposures. We did not observe a relation between baseline mtDNAcn and the measures of adversity, which may reflect the dynamic nature of mtDNAcn in response to stress as children in the maltreatment group ranged from zero to six months since maltreatment exposure at baseline, although all within the six-month inclusion criteria, rather than a delay in mtDNAcn compared to telomere length. It may be that all children in the maltreated group at follow-up were further away from the maltreatment exposure, allowing the chronic changes in mtDNAcn to be statistically observed. Indeed, both mtDNAcn and telomere length can dynamically change in the period of weeks to months (Epel and Prather, 2018; Nicod et al., 2016). Together, these findings support the concept of mtDNAcn as a dynamic and possibly cumulative marker of stress exposure and add to growing evidence of the potential role of mitochondria in psychiatric and other stress-related disorders. Increases or decreases in intracellular mtDNA reflect the systemic energy demands on the cell (Juster et al., 2016; Ridout et al., 2016a, 2018b). Acutely, physiologic stress responses can lead to upregulation of mtDNAcn through intracellular pathways including oxidative stress, glucocorticoid signaling, and peroxisome

proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) signaling, while chronic stress may show different patterns of mtDNAcn and reflect cellular stress and senescence (Juster et al., 2016; Ridout et al., 2016a,b). Thus, our results suggest that adversity exposure in preschool-aged children is related to dynamic changes in cellular energy and aging pathways.

Furthermore, the results of this study show that mtDNAcn increases are related to internalizing behaviors (such as depressive and anxiety symptoms) in children, which is consistent with prior findings in adults showing mtDNAcn increases with depression (Cai et al., 2015; Lindqvist et al., 2018; Tyrka et al., 2016) and changes with depressive severity (Edwards et al., 2016; Nicod et al., 2016). While some studies have found increased mtDNAcn with MDD, some have reported no change among adults with depressive symptoms (Verhoeven et al., 2017) or decreased mtDNAcn among elders with elevated depressive symptoms or who were taking antidepressants (Kim et al., 2011). The apparent discrepancies between these findings may be explained by differences in the clinical course and severity of depression (Edwards et al., 2016; Nicod et al., 2016), treatment effects, or exposure to stress or trauma in these samples. MDD episode duration has been associated with positive increases in mtDNAcn (Edwards et al., 2016). Together, these results highlight the potential importance of mitochondria in the etiology of depression.

Saliva samples were used in this study due to the young age of the children and ease of access to saliva compared to blood. Saliva is one of the most commonly sampled tissues (Theda et al., 2018) and consists of multiple tissue sources, including a majority of leukocytes, some monocytes and lymphocytes, and epithelial cells (Theda et al., 2018). A limitation to saliva as a sample source is the tissue heterogeneity. In a small study of post-mortem samples, there was variability of relative telomere length between tissues, including skin epithelial cells and PBMCs. MtDNAcn is regulated in a tissue-specific way, although mtDNAcn per tissue mass appears similar between tissues (Herbers et al., 2019). Thus, given that different cell types have different telomere lengths and mtDNAcn, and given that saliva consists of multiple cell types, the results presented in this manuscript could be confounded by the relative contribution of each cell type per child. However, current literature suggests that cellular composition in saliva is relatively consistent between subjects in the absence of oral infections (Theda et al., 2018). Issues around cellular heterogeneity are applicable to all studies utilizing saliva to quantify telomere length and mtDNAcn, and as a majority of studies to date in children are from saliva or buccal samples, which has similar cellular heterogeneity issues (Theda et al., 2018), this is a factor to consider in the interpretation of this literature.

Overall, these results contribute to the evidence that maltreatment in early childhood may increase an individual's risk for psychiatric conditions and both are impact stress-related biological mechanisms. Based on the present findings, these changes can be seen as early as ages 3–5 years. The results of this study suggest that telomeres and mitochondria may be important biomarkers to track in relation to maltreatment exposures, and as indicators of the biological impact of preventive interventions and treatments. Future work could examine these measures as related to specific dimensional symptoms to examine the role of such biological mechanisms across psychiatric disorders.

Declarations of interest

None.

CRediT authorship contribution statement

Kathryn K. Ridout: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Stephanie H. Parade:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Hung-Teh Kao:** Methodology, Validation, Investigation, Resources, Writing - review & editing, Supervision. **Stevie Magnan:** Validation, Investigation, Writing - review & editing. **Ronald Seifer:** Methodology, Investigation, Resources, Writing - review & editing, Supervision, Project administration. **Barbara Porton:** Methodology, Validation, Investigation, Resources, Writing - review & editing. **Lawrence H. Price:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision. **Audrey R. Tyrka:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Acknowledgements

We are grateful to the children and families who participated in this study, and we thank Hasbro Children's Hospital, Rhode Island Head Start, and the Rhode Island Department of Children, Youth, and Families for assisting in recruitment of study participants. We thank Asi Polly Gobin for her work on data management, and Brittney Josefson and other research assistants who contributed to this project. Isolation of DNA was done in the laboratory of Joel Gelernter, M.D., and we are grateful to Dr. Gelernter and his staff for their contribution.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.05.015.

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