Associations among Diurnal Salivary Cortisol Patterns, Medication Use, and Behavioral Phenotype Features in a Community Sample of Rett Syndrome

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Abstract

Rett syndrome (RTT) is a severe neurodevelopmental disorder resulting from mutations of the MECP2 gene. Hyperactivity of the HPA axis and abnormal stress responses have been observed in animal models of RTT, but little is known about HPA axis function among individuals with RTT. Diurnal salivary cortisol patterns from 30 females with RTT were examined in relation to mutation type, medication use, and features of the RTT behavioral phenotype. Cortisol patterns were significantly related to mutation severity, anticonvulsant medication status, and bruxism (tooth grinding). This study provides preliminary support for the hypothesis that RTT may be at risk for outcomes associated with aberrant HPA axis function, and that this risk may be mediated by mutation type.

Associations among Diurnal Salivary Cortisol Patterns, Medication Use, and Behavioral Phenotype Features in a Community Sample of Rett Syndrome

Rett syndrome (RTT) is a severe neurodevelopmental disorder associated with mutations of the X-linked Methyl-CpG-binding protein 2 (MECP2; (Amir et al., 1999). Individuals with classic or typical RTT demonstrate apparently normal post-natal development until around 6 to 18 months of age, at which point they experience a severe developmental regression that results in loss of any acquired language and functional hand use. Subsequently, individuals with RTT experience profound, life-long deficits in communication and motor function. Several associated health conditions, including autonomic dysfunction, seizures, and gastrointestinal problems are common (e.g., (Motil et al., 2012; Jeffrey L. Neul et al., 2015; Weese-Mayer et al., 2006)).

Although the pathogenesis of RTT symptoms is not fully explicated, deficits in MeCP2 are known to affect transcription and repression of numerous other genes (e.g., (Brown et al., 2016; Guy et al., 2018). Preclinical mouse models show that one consequence is up-regulation of genes related to glucocorticoids production and signaling, including the Corticotrophin Releasing Hormone (CRH) gene, among others (Goffin et al., 2012; McGill et al., 2006; Nuber et al., 2005). In mouse models, this leads to hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis, as evidenced by larger increases in corticosterone in response to stress compared to wild type mice, and associated elevated behavioral and physiological stress responses (McGill et al., 2006). Pharmacological interventions targeting the glucocorticoid system have been shown to impact lifespan, motor function, and behavior in RTT mouse models (Braun, Kottwitz, & Nuber, 2012; De Filippis, Ricceri, Fuso, & Laviola, 2013), suggesting that this system may be an appropriate pharmacological target for treatment of some core symptoms of the disorder.

Cortisol is the human equivalent of corticosterone and the end product of the HPA axis. The HPA axis works with the sympathetic nervous system during times of stress to stimulate a series of adaptive behavioral and physiological responses (Carpenter & Gruen, 1982; Chrousos, 1995). Cortisol assists in the regulation of a wide range of biological activities, including respiration, blood pressure, and heart rate, as well as emotional regulation. The HPA axis, autonomic nervous system, and immune system are all part of an interactive network in which activity of each component can affect activity in the others (reviewed in (Boyce & Ellis, 2005). Under normal conditions, feedback loops ensure that the body returns to normal baseline conditions within a short time. Inadequate control of glucocorticoid responses, however, can have extensive downstream consequences, and lead to the development of myriad physiological and psychological disorders. Alterations to HPA-axis functioning have been implicated in systemic diseases such as obesity and cardiovascular disorders (e.g., (Kumari, Shipley, Stafford, & Kivimaki, 2011; Matthews, Schwartz, Cohen, & Seeman, 2006), affective disorders, including depression and anxiety disorders (e.g., (Shirtcliff & Essex, 2008; Van den Bergh, Van Calster, Pinna Puissant, & Van Huffel, 2008), and neurodegenerative disease (e.g., (Dong & Csernansky, 2009), among others.

Despite strong preclinical evidence that HPA axis alterations may be a direct consequence of MECP2 mutations, there is relatively little evidence specific to HPA axis function among individuals with RTT. Several single-time point measures of serum or urinary cortisol levels have been reported, with some documenting normal concentrations, and others documenting elevated levels (e.g., (Assadi, Crowe, & Rouhi, 2006; Echenne, Bressot, Bassir, Daures, & Rabinowitz, 1991; Motil, Schultz, Abrams, Ellis, & Glaze, 2006). The HPA axis is a dynamic system, however, limiting the value of single time-point evaluations. In contrast,

patterns of diurnal salivary cortisol have been demonstrated to be a robust marker of HPA axis functioning in a variety of populations (e.g., (Lupien, McEwen, Gunnar, & Heim, 2009). Under normal circumstances, concentrations show a peak shortly after morning awakening, followed by steady declines throughout the day (Van Cauter, Leproult, & Kupfer, 1996). In most studies, pronounced decreases of up to 90% if initial values are observed by the evening. In the only study investigating morning/evening change in cortisol in RTT to date, half of the sample failed to show the expected decrease (Huppke, Roth, Christen, Brockmann, & Hanefeld, 2001). The results of this study were limited, however, by small sample size (N = 14) and limited additional clinical information.

Based on the available evidence, there is reason to believe that individuals with RTT may be at increased risk for physical and psychiatric disorders due to aberrant HPA axis function. Consistent with this hypothesis, mood disorders, including anxiety and depressed mood are common, and parents frequently report that their children with RTT show excessive behavioral reactions, including apparent fear and panic, in novel situations (e.g., (Mount, Charman, Hastings, Reilly, & Cass, 2002; Sansom, Krishnan, Corbett, & Kerr, 1993). Aberrant cardiovascular and respiratory responses when awake (Julu et al., 2001; Katz, Dutschmann, Ramirez, & Hilaire, 2009; Weese-Mayer et al., 2006), and endocrine disorders, including glucose intolerance, thyroid disorders, and bone mineral loss have also been reported (e.g., (Jefferson et al., 2011; Stagi et al., 2015). Because of the many downstream consequences of MECP2 mutations, however, it is unclear whether any of these conditions are directly attributable to HPA axis dysfunction. Evidence linking mood and health disorders with abnormal cortisol production would provide support for the potential of the HPA axis as a clinical treatment target.

A significant challenge to assessing diurnal cortisol among individuals with RTT and similar conditions is the unknown effects of most medications on cortisol production. As noted by (Granger,

Hibel, Fortunato, & Kapelewski, 2009), medications may influence measurement of cortisol in at least five ways: (a) direct agonistic or antagonistic effects on the HPA axis, (b) indirect effects on physiological systems that interact with HPA axis, (c) pharmacologically induced change in subjective experience (e.g., reduced anxiety or stress responses), (d) changes to the availability or composition of saliva, and (e) cross-reactivities with antibodies used to detect cortisol by immunoassay. Although the effects (or non-effects) of some medications have been documented, many others have not been evaluated. Because of the many comorbid health conditions that occur in RTT, it is expected that, in any community sample of individuals with RTT, a majority will be taking one or more daily medication. For this reason, it is important to also include information about the medications commonly used in this population to test, at least in part, the possible effect on cortisol concentrations and diurnal patterns.

Given the profound health and behavior disturbances associated with the disorder and the fact that there are no therapies yet that treat the disorder, further research documenting patterns of diurnal cortisol among individuals with RTT is needed to further understand whether HPA axis dysfunction may be a reasonable future treatment target. The purpose of this exploratory study was to examine diurnal salivary cortisol patterns in a sample of individuals with RTT. The study had three primary goals: 1) determine whether the patterns of elevated morning levels followed by large decreases throughout the day observed in healthy, typically-developing populations would be replicated in this sample; 2) evaluate whether relationships between frequently used medications and diurnal salivary cortisol patterns would be detected, and 3) determine whether relationships between functional status, health, and behavior variables and diurnal salivary patterns could be detected after controlling for significant medication effects.

Methods

Participants

Participants were recruited through RTT parent support organizations. All study procedures were approved by the university's Institutional Review Board, and informed consent was obtained from all parents. Samples from 33 individuals were collected. Three participants were excluded due to missing information regarding time of sample collections (N =1), or because of medications known to affect cortisol values (i.e., steroid-based medications; N = 2). The final sample consisted of 30 females with clinical diagnoses of RTT, ranging in age from 2 to 35 years (mean = 13.2). Specific MECP2 mutations were reported for 25 participants (83%). One parent reported that genetic testing had confirmed a MECP2 mutation, but did not have further information. Three of the participants (10%) had not had genetic testing, and one had a clinical diagnosis of RTT with no MECP2 mutations identified during genetic testing (see Table 1 for participant characteristics).

[INSERT TABLE 1 HERE]

Saliva Collection and Assay

Parents were instructed to collect eight saliva samples over two days at four time points (pre-breakfast, pre-lunch, mid-afternoon, pre-bedtime). Specifically, parents were instructed to collect samples within 30-60 minutest of the participant awakening in the morning (prior to breakfast), between mid-morning and lunch time (typically between 10am and 12pm), sometime in the mid-afternoon (typically between 3 and 5pm), and approximately 30 minutes prior to going to bed at night. Parents were instructed to select typical days (i.e., days during which the participant was healthy, and without unusually exciting or stressful events) for collection, and to avoid collecting the samples within 30 minutes of eating or tooth-brushing. Samples were collected using salivary swabs (SalivaBio Children's Swabs) and were refrigerated until the collection was complete, after which they mailed the samples in unrefrigerated packages. Parents

recorded the timing of all sample collections, as well as the timing of meals, bedtime, wake-up, and naps using a daily paper diary. Once returned, samples were frozen at -20°C for up to 9 months, and then analyzed using the salivary cortisol ELISA assay kit from Alpco following the manufacturer's instructions by a trained laboratory technician.

Questionnaires

Parents were asked to complete questionnaires that included information on the child's date of birth, prescription and over-the-counter medications and supplements, genetic testing, seizure status, ambulation status, and frequency of hyperventilation and bruxism (tooth-grinding). Information and medications and mutation type were reported in open-ended questions. Information on ambulation status was collected in a yes/no format (i.e., "Does your child currently walk?"). The question regarding seizure status included three response options (i.e., "No history of seizures", "History of seizures, but currently under control", and "History of seizures, not currently controlled by medication"). Questions regarding hyperventilation and bruxism also included three response options (i.e., "No", "Sometimes", "Often"). Information on sleep problems and emotional lability was collected using a modified version of the Diagnostic Assessment for the Severely Handicapped - revised (DASH-II; Matson, 1995), which was altered to include only the items from the sleep, mood lability, self-injurious behavior, and eating subscales.

Data management and statistical analysis

Cortisol concentrations were scaled in nmol/L to be in line with previously reported values. Due to the skewed nature of the distribution, all cortisol values were log-transformed for analyses, but back-transformed to the original metric for ease of interpretation. Data management decisions were based on guidelines described in (Miller et al., 2016). Five samples had

insufficient saliva volume to complete the assay. One sample was excluded based on an implausibly high cortisol concentration (i.e., > 60 nmol/L). A total of 235 samples (7 or 8 per participant) were included in the subsequent analyses.

Preliminary analyses of cortisol values and diurnal patterns. A recent, large-scale study found that, on average, cortisol concentration decreases of over 90% were observed between peak morning samples and those collected 12 hours later (Miller et al., 2016) among typically-developing individuals of all ages. Therefore, a conservative cut-point of a minimum 50% decrease from morning to evening levels was used to identify abnormal patterns, as has been done in previous studies (e.g., Huppke et al., 2001). It was noted that the time elapsed between the morning and evening samples was less than has been reported in previous studies of typically developing individuals due to increased time in bed in the current sample. As a result, it is possible that the samples obtained missed the nadir of salivary cortisol among those individuals whose samples were collected after only 8 to 10 hours of waking time, potentially accounting for any observed abnormal patterns. To explore this possibility, a regression model analysis investigating the relationship between the time elapsed between the morning and evening samples and the percentage decrease, controlling for participant age, was conducted to determine whether a decrease in time spent awake was associated with a smaller morning to evening cortisol decrease.

Linear mixed models. Three-level mixed-effects growth curve regression models were implemented to evaluate the relationships between medication, health and behavior variables, and diurnal cortisol patterns. The first level of the model represents individual saliva sample values, which are nested within the second level representing collection days, which are nested within the third level representing participants. A preliminary set of analyses was conducted to

determine which random effects to include in the model (e.g., individual- and day-level random slopes and intercepts). This analysis indicated that participant- and day-level slopes, but not intercepts, contributed to model fit. In addition to these random effects, fixed effects in the base model included time of sample collection (centered at time of awakening) and a dummy variable for samples collected between 15 and 60 minutes of wake to account for the cortisol awakening response (CAR; e.g., (Stalder et al., 2016) for level 1, and time of awakening (centered at 7am) for level 2. Total sleep time and a quadratic form of time were also evaluated, but were excluded from subsequent models as they were not found to contribute to model fit.

Preliminary assessments of data patterns showed that age did not appear to be linearly associated with cortisol patterns. For this reason, a categorical variable representing four age groups (i.e., 4 years and younger, 5-10 years, 11-20 years, 20 years and older) was included in the model, with all comparisons referencing the youngest group. Due to its theoretical relevance, this age variable was included in all models regardless of statistical significance. All linear mixed models were implemented with the lme4 (Bates et al., 2015), and lmerTest (Kuznetsova, Brockhoff, & Christensen, 2015) packages in R.3.3.2 statistical software (Core Team, 2013). Predicted values were extracted using the ggeffects package (Lüdecke, 2018). All covariates were evaluated for collinearity, and residuals plots were reviewed for potential problems. *P*-values were generated using the Satterthwaite's approximate degrees of freedom. Due to the exploratory nature of the study, *p*-values were not corrected for multiple comparisons.

After fitting specifying the base model, a saturated model, including all medication variables, was tested. To minimize the risk of overfitting the models, a top-down strategy was used to reduce the saturated models as described in (West, Welch, Galecki, Welch, & Galecki, 2014), using a criterion of a log-likelihood ratio test p-value <.05 as evidence in favor of

retaining individual model terms. Once the reduced medication model was specified, variables related to health and behavior were added to create a second saturated model, and the model reduction process was repeated for these variables. All medication variables that were retained in the reduced medication-only model were retained in the health and behavior model regardless of effect size/significance.

Independent Variables for Linear Mixed Models. In addition to the core predictors from the base models, two sets of variables were tested in statistical models: a set of medication variables, and a set of health and behavior variables. The medication variables included 11 dummy variables representing different medications taken by at least three participants on data collection days, and one continuous variable representing the total number of anti-epileptic drugs (AEDs) taken by each participant. The dummy variables included five classes of anti-epileptic drugs (AEDs): benzodiazepines (N =3), lamotrigine (N = 4), levetiracetam (N = 6), valproic acid (N= 4), and zonisamide (N= 6). Additionally, three AEDs were being taken by one or two participants, and could not be included in the model: topiramate (N = 2), carbamazepine (N = 1), and oxcarbazepine (N = 1). In addition to AEDs, the medications included in the saturated model included anxiolytics (i.e., buspirone, trazodone, and sertraline; N = 3), glycopyrrolate (a medication prescribed to reduce drooling; N = 3), melatonin (N = 5), levocarnitine supplements (N = 4), polyethylene glycol (laxative; N = 13), and acid reducers (N = 11). Other medications being taken by participants but not included in any models were cholesterol medications (N =1), beta agonists (N = 1), oral baclofen (N = 2), trihexyphenidyl (N = 2), atypical antipsychotics (N = 2), non-steroidal anti-inflammatories (N = 1), and simethicone (N = 2).

The health and behavior variables included dummy variables for ambulation status, uncontrolled seizures, and mutation status, and ordinal variables for sleep problems, emotional

lability, hyperventilation, and bruxism. Mutation status was reflected by a dummy variable, with "1" representing a "mild" mutation group based on previous studies documenting differences in clinical severity across mutation types (e.g., (Cuddapah et al., 2014; J. L. Neul et al., 2008; Urbanowicz, Downs, Girdler, Ciccone, & Leonard, 2015). This "mild" group included p.Arg133Cys, p.Arg294X, and p.Arg306Cyc, and contained 8 participants. All other mutations were coded as "0". Emotional lability and sleep problem scores were calculated by summing the 0-2 scores for each item as described previously (i.e.,(Johnny L. Matson & Malone, 2006; Sturmey, Matson, & Lott, 2004).

Results

The average time of collection for the first morning sample was 8:19 (min = 6:20, max = 10:20), 35 min post wake (min = 15, max = 60). For the second sample, the average collection time was 11:25 (min = 10:00, max = 15:15), 3 hr 29 min post wake (min = 1 hr 40, max = 6 hr 0). For the afternoon sample, average time of collection was 18:10 (min = 14:10; max = 20:30), 8 hr 30 min post wake (min = 5 hr 0 min; max = 12 hr 50). Average time of collection for the evening sample was 20:06 (min = 16:00, max = 24:00), 12 hr 22 min post wake (min = 8 hr 30 min, max = 15 hr 15 min), and 39 minutes prior to bedtime (mix = 0 min, max = 1 hr 30 min). Raw cortisol concentrations for each collection day by age are shown in Figure 1. A total of 53 individual days from 30 participants were examined for the percentage decrease analysis (data for seven days excluded due to no collection within 1 hour of wake). The average time elapsed between sample collections was 11.8 hours (min =8.0, max = 13.9). The average percentage decrease was 46%, ranging from an increase of 276% to a decrease of 96%. In total, 16 (53%) participants showed a decrease of less than 50% between the morning and evening sample. Notably, however, only one participant showed this profile on both days, suggesting significant

day-to-day variability in this sample. When days with morning to evening increases in cortisol were excluded from the analysis as outliers, there was a small relationship between time elapsed between the morning and evening samples and the percentage decrease in cortisol concentrations, which remained after controlling for age (see Table 2 and Figure 2).

[INSERT FIGURES 1 & 2 HERE]

The results of the saturated and reduced medication models are presented in Table 3. Time of collection was strongly associated with cortisol concentrations, which is consistent with typical diurnal patterns of cortisol. Similarly, the awakening sample was associated with moderate increases in cortisol concentrations, which is consistent with the cortisol awakening response observed in typically-developing populations (although it should be noted that the data in the current study do not provide an accurate representation of the cortisol awakening response as only a single morning sample was collected). A later awakening time was associated with decreased morning cortisol concentrations and shallower diurnal slopes. There were no associations between age and morning concentrations or slopes in these models.

Five medication variables were retained in the reduced model. Three of these (polyethylene glycol, lamotrigine, and glycopyrrolate) were associated with small to moderate increases in morning cortisol levels, but were not related to differences in the slope across the day. Benzodiazepines were associated with a moderate decrease in morning cortisol level, but not with differences in slopes across the day. Levetiracetam was associated with shallower diurnal slopes (see Figure 3).

[INSERT TABLE 2 HERE]

The results of the saturated and reduced health and behavior models are reported in Table 4. A total of three health and behavior variables were retained in the reduced model. Milder

mutation types were associated with steeper diurnal slopes, whereas bruxism was associated with shallower diurnal slopes. Hyperventilation contributed significantly to model fit, suggesting that it should be retained in the model, although the association with morning levels was associated with a p-value greater than 0.05. Nevertheless, this suggests that hyperventilation may be associated with higher awakening cortisol levels (see Figure 4).

Discussion

The purpose of this study was to document the diurnal patterns of cortisol among individuals with RTT, and explore relationships between diurnal patterns and medication, health, and clinically-relevant behavior issues. Descriptively, over half of the participants in the sample showed blunted diurnal patterns of cortisol on at least one collection day, as defined by a decrease of less than 50% from morning to evening concentrations. As decreases of more than 90% are frequently observed in healthy children, adolescents, and adults (Miller et al., 2016), this suggests that diurnal cortisol patterns may be altered among individuals with RTT. The preliminary results suggest that individuals who spend more time awake during the day (and therefore have more time elapsed between their morning and evening samples) show larger morning to evening decreases in cortisol concentrations. Nevertheless, there remains significant variability in the size of the decrease, even among those who spend the same amount of time awake.

On the other hand, the results of the linear mixed models showed that cortisol concentrations were strongly associated with sample time. Samples collected within an hour of wake were significantly higher than those collected at other times during the day, suggesting that, on average, individuals with RTT do show awakening responses and diurnal patterns of cortisol production. These results point to the need for additional studies in which RTT samples

are compared directly to a matched control group to determine the degree to which diurnal patterns and absolute values differ from those observed in healthy females.

Several medications were associated with differences in cortisol concentrations and diurnal patterns in the current sample. Due to the observational nature of the study, it is impossible to draw causal inferences regarding the effects of the medications on cortisol production. It is therefore plausible that the observed effects are due to the underlying health conditions being treated, rather than the medications themselves. For some of the observed effects, there are theoretical reasons that may explain the observed effects. For example, in the case of glycopyrrolate, the medication is designed to reduce drooling by reducing saliva production. As a result, it is reasonable to expect that this medication would alter the composition of saliva, and would be reasonably expected to affect the concentrations of many different proteins. Similarly, benzodiazepines have anxiolytic and sedative effects, and therefore might result in decreased reactivity to environmental stressors, resulting in alterations to HPA activity, thereby resulting in the reduced cortisol concentrations observed in participants taking these medications in the current sample. In contrast, the reasons why individuals taking lamotrigine and polyethylene glycol would show overall higher levels of salivary cortisol are less clear. It is possible that these medications have direct or indirect effects of HPA axis activity, that they interfere with the cortisol assay, that the underlying health reasons for which they are prescribed are affecting cortisol concentrations, or that the effects in the current study are spurious due to the small sample. Additional work is needed to determine whether these effects are replicable and, if so, ascertain the source of the effects.

The largest effect in the reduced medication model was associated with levetiracetam, a commonly prescribed AED. In the current sample, taking levetiracetam was associated with

strongly blunted diurnal cortisol patterns, with lower morning concentrations and little change throughout the day. There is preliminary evidence that levetiracetam affects corticosterone release in animal models (Grimee et al., 2003), and levetiracetam appears to have higher risks of behavioral side-effects, such as aggression, hostility, and nervousness, in children, compared to other AEDs (Halma et al., 2014), but it is currently unclear whether changes in HPA axis activity are implicated in these side-effects. Longitudinal studies tracking diurnal cortisol prior to and following the introduction of this medication would help elucidate these relationships.

Among the health and behavioral variables tested, the strongest effects were associated with mutation subgroups and sleep problems, which somewhat smaller effects associated with and emotional lability scores. MECP2 mutations associated with less severe phenotypic presentations were associated with steep decreases in cortisol concentrations throughout the day, which is similar to the patterns typically observed among healthy individuals. This finding is intriguing, as it provides additional evidence supporting the hypothesis put forward by Goffin et al. (2012) that altered HPA axis activity may play a causal role in the development of some phenotypic characteristics of RTT. As three separate functional regions comprise the MeCP2 gene, and each of these regions has different interactions with other proteins and genes, it is possible that mutations that occur at different locations on the gene have effects on different downstream systems. It is, therefore, possible that the more typical diurnal cortisol patterns observed among the participants with the "mild" mutations are due to the relative preservation of the interaction between MeCP2 and the CRH gene. We cannot rule out, however, that the differences simply reflect differences in overall health between the two groups. No formal measure of clinical severity was available in the current study, making it unclear whether there would be a more general association between clinical severity and salivary cortisol patterns, or

whether this finding is specific to the types of missense mutations that were included in the 'mild' group. Nevertheless, the study is in line with the results of Peters, Byiers, and Symons (2016), which documented that blunting of diurnal cortisol patterns was associated with regression status and clinical severity in MECP2 duplication syndrome, a genetic syndrome caused by gain-of-function mutations in MECP2 (as opposed to the loss-of-function mutations associated with RTT). Together, although preliminary, these studies provide evidence that changes in MECP2 concentrations may impact diurnal cortisol patterns, and that these changes may be associated with differential clinical severity or disease progression.

Bruxism was associated with blunted diurnal patterns of cortisol in the current sample. This is somewhat consistent with previous work documenting blunted cortisol awakening responses among otherwise typically-developing children with sleep bruxism (e.g., (Castelo, Barbosa, Pereira, Fonseca, & Gavião, 2012), but bruxism in RTT is qualitatively different from sleep bruxism, as it occurs almost exclusively during wake (e.g., (Ribeiro, Romano, Birman, & Mayer, 1997). As the etiology of bruxism is RTT is unknown, and there is no work investigating the relationship between daytime bruxism and salivary cortisol in any population, to our knowledge, this is an area in need of further investigation.

Several health and behavior variables that have been linked to diurnal cortisol patterns in other populations were not retained in the final model in the current study. Specifically, neither emotional lability nor sleep problems contributed significantly to model fit in the current sample, although both showed relationships with awakening levels in the saturated model. This points to potential issues with statistical power, which is not surprising given the small sample and complex statistical model. Another limitation potentially contributing to this issue is the reliance

on parental report of symptoms using scales that have not been validated for specific use with RTT, which may have reduced the sensitivity of these measures.

Typically, diurnal cortisol concentrations follow a developmental pattern, with gradual increases during adolescence, followed by a stabilization during early and middle adulthood (e.g., Miller et al., 2016). In contrast, there were no age-related effects observed in the current sample. It is possible that some of the variability may be attributable to differences in the composition of the age groups that were not accounted for in the model. We did not, for example, collect information on pubertal status, which has been shown to impact salivary cortisol (e.g., Matchock, Dorn, & Susman, 2007). Further, it is possible that individuals with an earlier and more severe presentation would be more likely to be identified earlier, potentially introducing a bias into the under 5-year-old group. Similarly, although individuals with RTT frequently live well into adulthood, early mortality is not uncommon (e.g., (Kirby et al., 2010). Therefore, older individuals in the sample may represent a different population than those in the younger age groups. This issue may also be addressed in future studies via the inclusion of more comprehensive measures of clinical severity.

As previously noted, this study is limited by a relatively small, heterogeneous sample, making it difficult to fully investigate the relationships between the variables, which are likely much more complex than represented by the statistical model. Additional limitations include a reliance on parent caregiver report for all clinical/behavioral information and sample collection times. The lack of a comparison sample also limits the conclusions that can be drawn. Because the study was exploratory in nature, all results should be viewed as preliminary, and should be replicated in future studies.

Despite these limitations, this study provides preliminary evidence that, although individuals with RTT show evidence of cortisol awakening responses and diurnal patterns, the diurnal decline may be less steep than would be expected among healthy, typically-developing individuals. The results also suggest that some medications commonly prescribed in this population may affect cortisol concentrations, and that these effects need to be controlled in statistical models. This also raises the issue of selecting an appropriate comparison group for investigating salivary cortisol in RTT, as any comparison between individuals with RTT and healthy, typically-developing females will be inevitably confounded by medication differences. Future research could incorporate comparison groups with similar health problems, but who do not have the same potential genetic susceptibility to altered HPA axis function, such as individuals with cerebral palsy, or those with significant intellectual and developmental disabilities of unknown or mixed etiology.

The findings of the current study point towards several potentially intriguing avenues for future research, including: further elucidating links between cortisol patterns and specific medication classes; and investigating the directionality of relationships between cortisol patterns and mood and sleep problems among individuals with RTT (and other neurodevelopmental disorders). Additional research is needed to clarify whether individuals with RTT show age-related changes in diurnal cortisol patterns. Taking these steps may help further our scientific understanding of the HPA axis in RTT and whether its regulation would be a suitable treatment target to improve health and behavior outcomes in this vulnerable population.

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Table 1. *Participant clinical characteristics by age group*

Participant ID	Mutation	Age	Epilepsy	Ambulation	Medications											
					a	b	c	d	e	f	g	h	i	j	k	l
Mild																
1	R106W	4	-	-	-	-	-	-	-	+	+	-	-	-	+	-
2	R306C	4	+	+	-	-	-	-	-	-	-	-	-	+	-	+
3	R133C	6	-	+												
4	R106W	7	+	-	-	-	-	-	-	-	-	-	-	-	+	+
5	R294X	13	-	+	-	-	-	-	-	-	-	-	-	-	-	-
6	R294X	16	-	-	-	-	-	-	-	+	-	-	-	-	-	+
7	R294X	16	+	-	-	-	-	-	-	+	+	+	-	-	+	+
8	R294X	35	++	-	+	-	-	-	-	-	-	-	-	-	+	+
Other mutations																
9	R168X	2	-	-	-	-	-	-	-	-	-	-	+	+	-	-
10	R255X	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	R168X	4	+	-	-	-	+	-	+	-	-	-	-	-	+	-
12	R168X	4	-	+	-	-	-	-	-	-	-	-	-	-	-	-
13	Deletion	4	-	+	-	-	-	-	-	-	-	-	-	-	-	-
14	S49X	5	+	-	+	+	-	-	-	+	-	-	-	-	-	-
15	R270X	7	-	+	-	-	-	-	-	+	-	-	-	-	+	-
16	Deletion	7	++	-	-	-	-	-	+	-	-	-	+	+	-	-
17	R168X	8	+	-	-	-	+	-	-	+	-	-	-	-	-	-
18	Deletion	9	+	-	-	-	-	+	-	+	-	-	-	-	-	+
19	MECP2*	9	+	+	+	+	-	-	-	+	-	-	-	-	+	-
20	272RfsX1	10	++	-	-	-	+	+	-	+	-	-	+	-	+	+
21	Q262X	13	++	-	-	-	+	-	+	+	-	+	-	-	+	-
22	None	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	K144X	14	+	+	-	-	+	-	-	-	-	-	-	-	+	-
24	R255X	18	-	+	-	-	-	-	-	+	-	-	-	-	+	+
25	T158M	21	++	-	-	-	+	-	+	-	-	+	+	+	-	-
26	Deletion	24	+	-	-	+	-	-	-	+	+	-	-	+	-	+
27	Not tested	25	+	+	-	-	-	-	+	-	-	-	-	-	-	+
28	Not tested	27	+	+	-	+	-	-	-	-	-	-	-	-	-	-
29	Not tested	33	+	-	-	-	-	-	-	-	-	-	-	-	+	+
30	R168X	35	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Note: Epilepsy: - No history of seizures, + History of seizures, but controlled, ++ History of seizures, not controlled; Ambulation: - Does not walk (with or without assistance), + Walks (with or without assistance). Medications: for lettered columns, + represents participant taking each medication class at time of saliva collection: a = benzodiazepines, b = lamotrigine, c = levetiracetam, d = valproic acid, e = zonisamide, f = acid reducers, g = anxiolytic, h = glycopyrrolate, i = levocarnitine, j = melatonin, k = polyethylene glycol, l = other medication(s) not included in models. *This participant had a diagnosed MECP2 mutation, but the parents did not know the specific mutation type.

Table 2. Results of regression model predicting percent change in morning to evening cortisol concentrations by time elapsed between sample collections and participant age.

Model	Variable	Unstandardized coefficient	Coefficient SE	Standardized coefficient	t	p
1	Constant	0.28	0.21		1.36	0.18
	Time elapsed	0.03	0.02	0.25	1.86	0.07
2	Constant	0.28	0.22		1.28	0.21
	Time elapsed	0.03	0.02	0.25	1.77	0.08
	Age	0.00	0.00	-0.02	-0.16	0.88

Table 3. Results of the saturated and reduced medication models

Model terms	Saturated medication model					Reduced medication model				
	Est	SE	df	t	p	Est	SE	df	t	P
Time of collection	-0.10	0.02	190	-5.14	<0.001	-0.09	0.02	204	-5.62	<0.001
Awakening sample	0.25	0.10	193	2.46	0.015	0.24	0.10	205	2.39	0.018
Time of wake										
Intercept	-0.13	0.06	159	-2.08	0.039	-0.12	0.05	152	-2.25	0.026
Slope	0.02	0.01	191	2.55	0.012	0.02	0.01	209	2.46	0.015
Age 5-10										
Intercept	0.11	0.24	56	0.48	0.635	0.03	0.17	79	0.19	0.854
Slope	0.02	0.03	193	0.70	0.486	0.02	0.02	203	1.09	0.275
Age 11-20										
Intercept	-0.07	0.21	50	-0.33	0.746	-0.18	0.18	68	-0.98	0.329
Slope	0.00	0.02	190	-0.18	0.855	0.00	0.02	201	-0.02	0.987
Age 20+										
Intercept	-0.04	0.20	56	-0.21	0.838	0.00	0.18	76	-0.01	0.995
Slope	0.00	0.02	190	0.20	0.840	0.00	0.02	202	0.03	0.975
Benzodiazepine										
Intercept	-0.07	0.40	64	-0.17	0.866	-0.44	0.17	23	-2.54	0.018
Slope	-0.09	0.05	193	-1.92	0.057	-	-	-	-	-
Lamotrigine										
Intercept	0.23	0.23	50	0.98	0.332	0.50	0.13	22	3.72	0.001
Slope	0.04	0.03	190	1.55	0.123	-	-	-	-	-
Levetiracetam										
Intercept	-0.37	0.21	60	-1.74	0.087	-0.24	0.16	60	-1.49	0.141
Slope	0.09	0.02	193	3.56	<0.001	0.07	0.02	203	3.85	<0.001
Valproic acid										
Intercept	0.17	0.20	54	0.84	0.404	-	-	-	-	-
Slope	-0.03	0.02	193	-1.13	0.259	-	-	-	-	-
Zonisamide										
Intercept	0.05	0.23	58	0.22	0.825	-	-	-	-	-
Slope	0.01	0.02	190	0.24	0.814	-	-	-	-	-
Acid reducers										
Intercept	-0.01	0.21	61	-0.03	0.979	-	-	-	-	-
Slope	0.00	0.02	192	-0.16	0.873	-	-	-	-	-
Anxiolytic										
Intercept	-0.14	0.30	50	-0.46	0.651	-	-	-	-	-
Slope	0.04	0.03	191	1.27	0.207	-	-	-	-	-
Glycopyrrolate										
Intercept	0.46	0.31	50	1.48	0.146	0.44	0.16	20	2.76	0.012
Slope	-0.02	0.03	190	-0.58	0.562	-	-	-	-	-
Levocarnitine										
Intercept	-0.20	0.28	59	-0.72	0.472	-	-	-	-	-
Slope	0.05	0.03	193	1.67	0.096	-	-	-	-	-
Melatonin										
Intercept	0.35	0.22	48	1.60	0.117	-	-	-	-	-
Slope	-0.04	0.03	190	-1.46	0.147	-	-	-	-	-
Polyethylene glycol										
Intercept	0.18	0.16	53	1.15	0.257	0.21	0.09	22	2.36	0.028
Slope	0.01	0.02	192	0.41	0.679	-	-	-	-	-

Note: Est = Estimate; SE = Standard error; df = degrees of freedom

Table 4. Results of the saturated and reduced health and behavior models.

Model terms	Saturated model					Reduced model				
	B	SE	df	t	p	B	SE	df	t	p
Time of collection	-0.11	0.02	195	-4.56	0.000	-0.11	0.02	201	-4.79	<0.001
Awakening sample	0.23	0.10	198	2.31	0.022	0.24	0.10	203	2.50	0.013
Time of wake										
Intercept	-0.07	0.06	137	-1.15	0.251	-0.10	0.05	136	-1.84	0.069
Slope	0.01	0.01	199	1.18	0.241	0.01	0.01	205	2.02	0.045
Age 5-10										
Intercept	-0.08	0.21	52	-0.37	0.711	0.03	0.17	74	0.18	0.860
Slope	0.02	0.02	195	0.66	0.511	0.01	0.02	201	0.54	0.593
Age 11-20										
Intercept	-0.62	0.25	66	-2.47	0.016	-0.29	0.18	67	-1.58	0.118
Slope	0.04	0.03	194	1.46	0.145	0.02	0.02	199	0.82	0.413
Age 20+										
Intercept	-0.17	0.21	68	-0.82	0.416	-0.12	0.19	76	-0.66	0.513
Slope	0.02	0.03	196	0.76	0.450	0.01	0.02	200	0.56	0.576
Medications										
Benzodiazepines										
Intercept	-0.35	0.19	14	-1.79	0.095	-0.33	0.18	20	-1.86	0.078
Lamotrigine										
Intercept	0.34	0.18	14	1.85	0.086	0.32	0.15	18	2.11	0.049
Levetiracetam										
Intercept	-0.07	0.20	54	-0.36	0.723	-0.33	0.16	52	-2.03	0.047
Slope	0.03	0.02	195	1.34	0.181	0.05	0.02	201	3.05	0.003
Glycopyrrolate										
Intercept	0.31	0.20	14	1.54	0.147	0.47	0.15	18	3.10	0.006
Polyethylene glycol										
Intercept	0.13	0.11	15	1.24	0.234	0.19	0.09	19	2.22	0.039
Health and behavior										
Ambulation										
Intercept	-0.10	0.14	53	-0.73	0.470	-	-	-	-	-
Slope	0.00	0.02	192	-0.07	0.944	-	-	-	-	-
Mild mutations										
Intercept	0.09	0.16	55	0.55	0.584	0.09	0.15	73	0.62	0.540
Slope	-0.05	0.02	193	-2.91	0.004	-0.05	0.02	198	-3.09	0.002
Uncontrolled seizures										
Intercept	0.18	0.25	34	0.72	0.477	-	-	-	-	-
Slope	0.00	0.03	196	-0.04	0.968	-	-	-	-	-
Bruxism										
Intercept	-0.12	0.11	46	-1.04	0.304	-0.17	0.10	74	-1.61	0.111
Slope	0.02	0.01	194	1.76	0.080	0.02	0.01	199	2.11	0.036
Mood lability										
Intercept	0.06	0.03	47	2.22	0.031	-	-	-	-	-
Slope	0.00	0.00	193	-1.05	0.295	-	-	-	-	-
Hyperventilation										
Intercept	0.16	0.11	35	1.43	0.163	0.11	0.06	18	1.72	0.103
Slope	0.00	0.01	194	0.14	0.891					
Sleep problems										
Intercept	-0.11	0.06	45	-2.06	0.045	-	-	-	-	-
Slope	0.01	0.01	194	1.33	0.184	-	-	-	-	-

Note: Est = Estimate; SE = Standard error; df = degrees of freedom

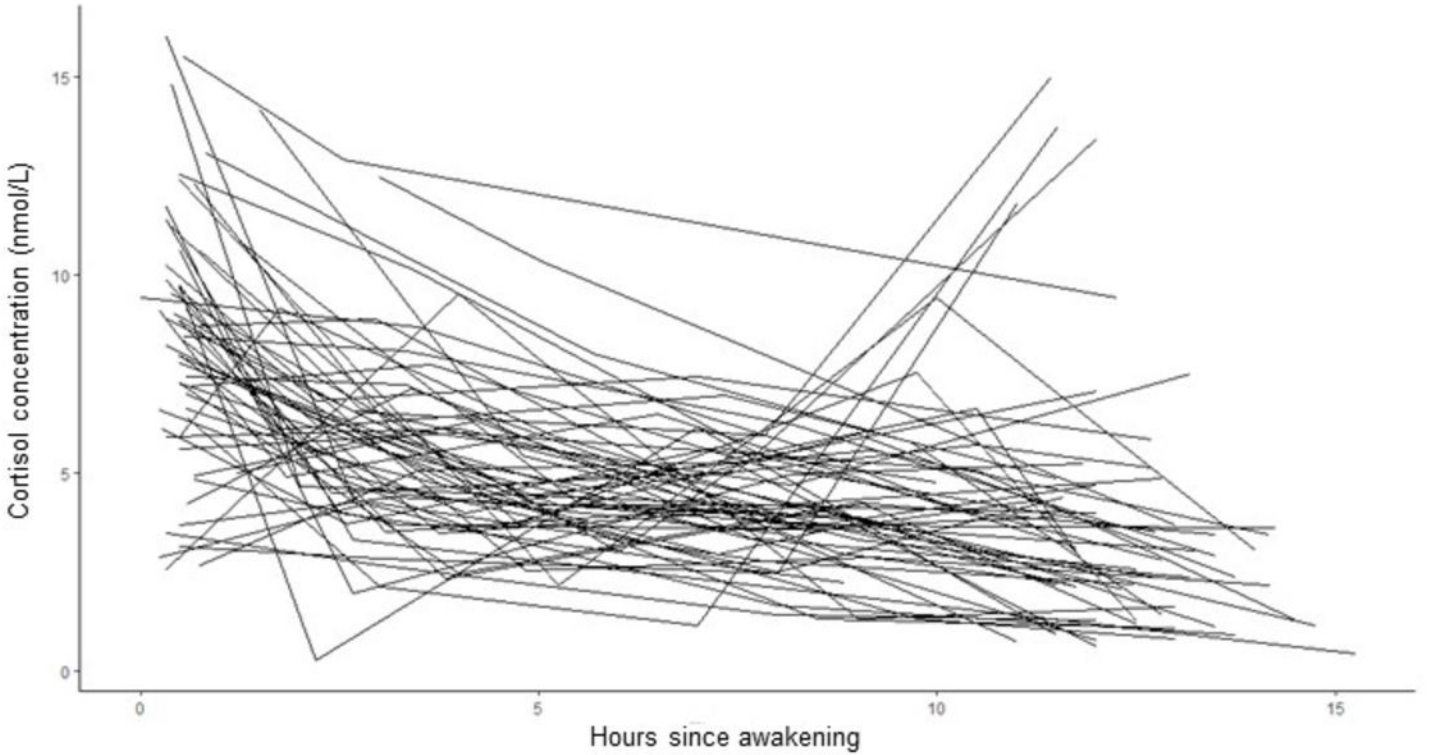
Figure captions.

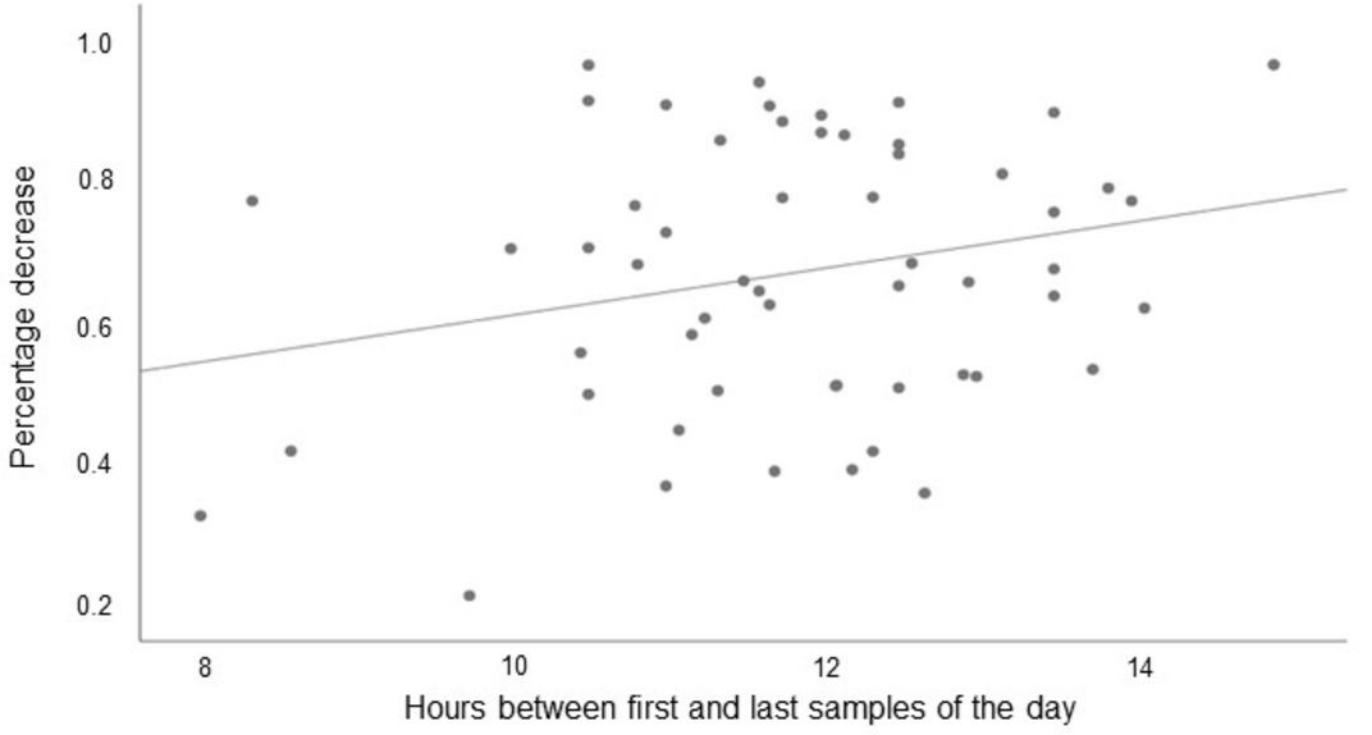
Figure 1. Raw cortisol concentrations by time of day by participant/day.

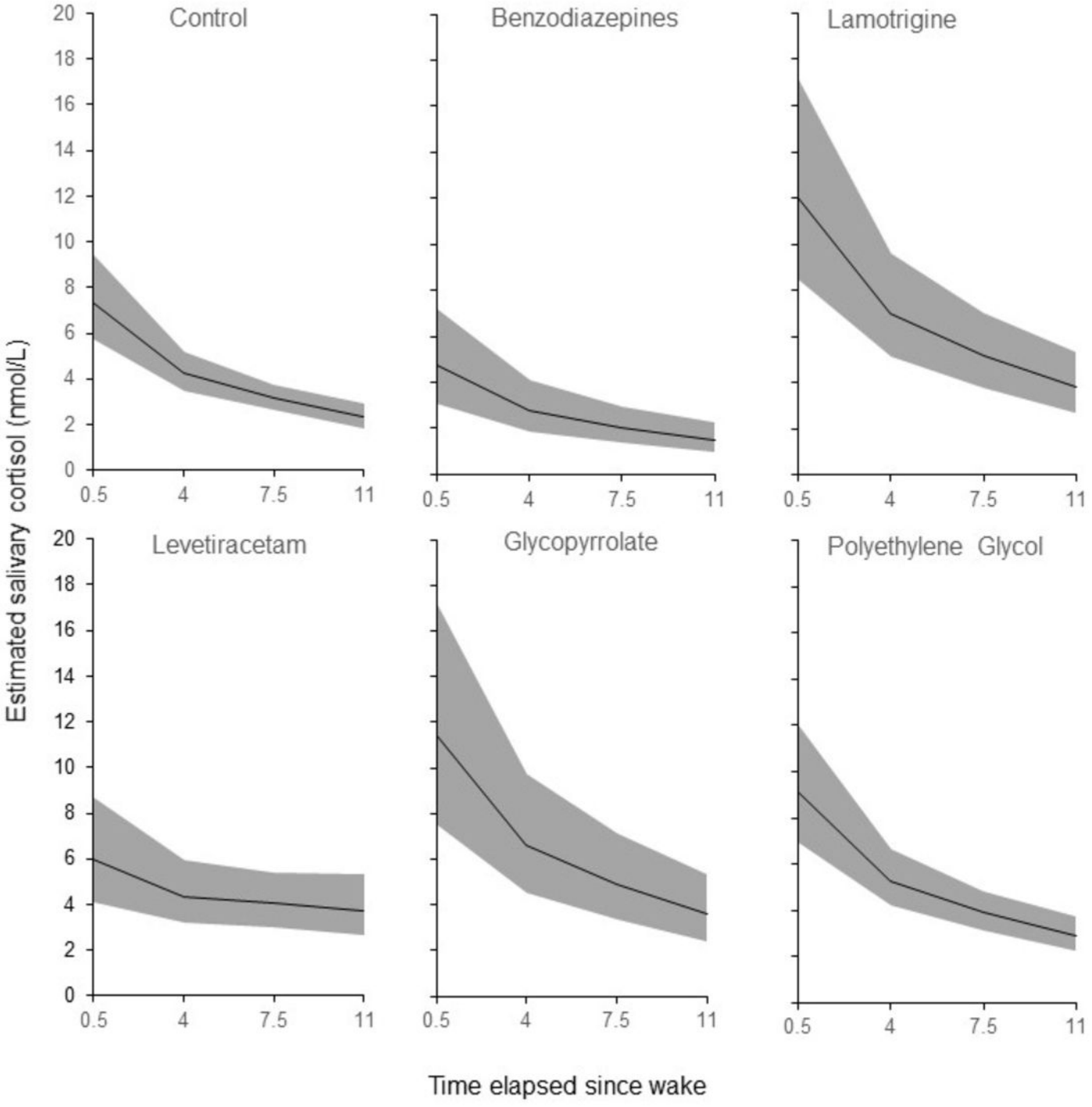
Figure 2. Relationship between time elapsed between first and last samples of each day and percentage decrease in cortisol concentrations. Percent change values smaller than 0 (i.e., increases from morning to evening) were replaced with 0 to minimize the effects of potential outliers.

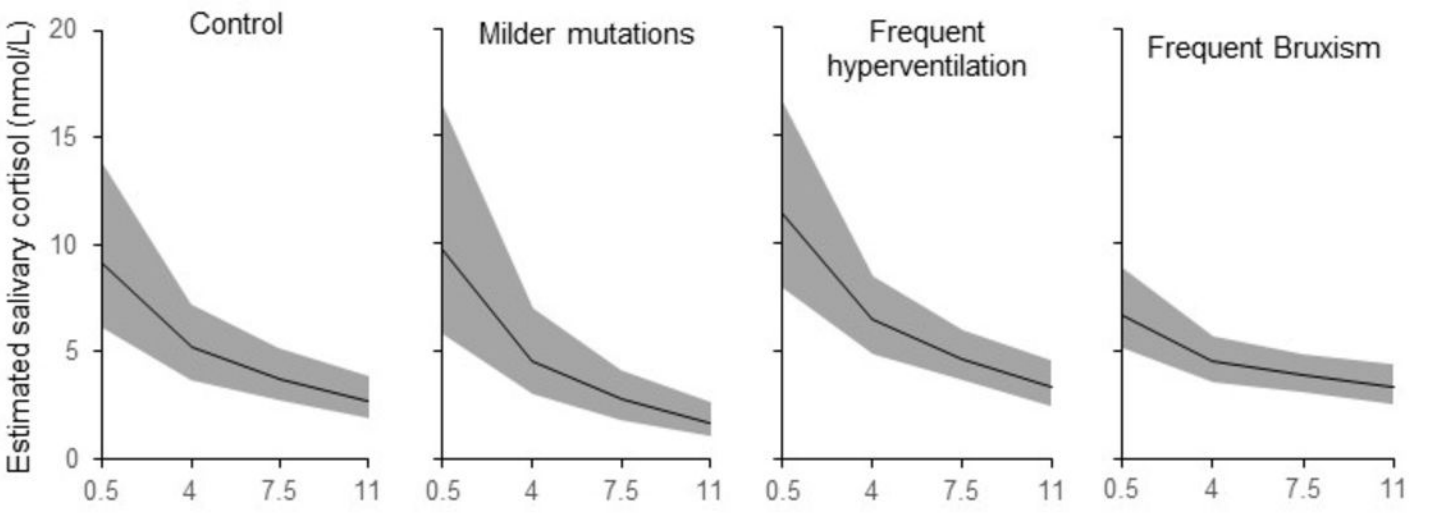
Figure 3. Predicted diurnal salivary cortisol patterns estimated by the reduced medication model for all medication effects associated with significantly improved model fit compared to individuals not taking any of the identified medications (control).

Figure 4. Predicted diurnal salivary cortisol patterns estimated by the reduced health and behavior model for all health and behavior effects associated with significant improvements in model fit, compared to individuals without mild mutations, hyperventilation, or bruxism (control).


Cortisol concentration (nmol/L)
15
10
5
0
0
5
10
15
Hours since awakening


1.0
0.8
0.6
0.4
0.2
Percentage decrease
8
10
12
14
Hours between first and last samples of the day


Control
Benzodiazepines
Lamotrigine
Levetiracetam
Glycopyrrolate
Polyethylene Glycol
Estimated salivary cortisol (nmol/L)
Time elapsed since wake
0
2
4
6
8
10
12
14
16
18
20
0.5
4
7.5
11


Control
Milder mutations
Frequent hyperventilation
Frequent Bruxism
Estimated salivary cortisol (nmol/L)
20
15
10
5
0
0.5
4
7.5
11
Time elapsed since wake