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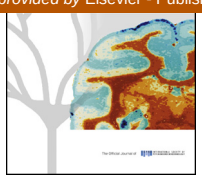

The longitudinal association of the diurnal cortisol rhythm with internalizing and externalizing problems in pre-schoolers. The Generation R Study

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Summary
Background: Studies investigating the association between diurnal cortisol rhythm and behavioural problems in young children have yielded inconsistent results. We tested the hypothesis that variations in diurnal cortisol rhythm in pre-schoolers are already related to problem behaviour early in life with a cross-sectional and longitudinal design.
Methods: This study was embedded in Generation R, a population-based cohort from foetal life onwards. Parents collected saliva samples from their infant at 5 moments during 1 day. In 322 infants aged 12—20 months, we determined the diurnal cortisol rhythm by calculating the area under the curve (AUC), the cortisol awakening response (CAR), and the diurnal slope. Problem behaviour was assessed at ages 1.5 and 3 years with the Child Behavior Checklist/1.5—5 years.
Results: No cross-sectional associations between the cortisol composite measures and problem behaviour were found at 1.5 years. However, cortisol predicted change in internalizing problems as assessed from 1.5 to 3 years, but not change in externalizing problems. Children with higher AUC levels, flatter slopes and a more positive CAR at baseline were more likely to score higher

Abbreviations: AUC, area under the curve; CAR, cortisol awakening response; CBCL, Child Behavior Checklist.

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on the Internalizing Problems scale (β per nmol/L AUC: 0.08, 95% CI: 0.00; 0.17, $p = 0.04$; β per nmol/L/h slope: 0.57, 95% CI: 0.17; 0.98, $p = 0.006$; β per nmol/L CAR: 0.04, 95% CI: 0.01; 0.08, $p = 0.02$) at follow-up.

Conclusions: Variations in diurnal cortisol rhythm are associated with change in internalizing problems in pre-schoolers. The results suggest that variations in diurnal cortisol patterns early in life precede internalizing problems.

1. Introduction

The relationship between regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis and behavioural problems in children has been studied repeatedly, as discussed by Granger and Kivlighan (2003) and Jessop and Turner-Cobb (2008). Cortisol, the hormonal end-product of the HPA-axis, is important for a wide variety of adaptive functions and is released in response to stressors. This hormone is also involved in numerous essential bodily functioning involving energy metabolism and immunology (de Kloet, 2003). Cortisol shows a diurnal pattern characterized by post-waking peak cortisol levels (cortisol awakening response) and subsequent declining cortisol levels throughout the day in healthy adults (Edwards et al., 2001).

It is important to know if the developing HPA axis in young children is already related to internalizing and externalizing problems early in life. In adults and adolescents variations in the HPA axis co-occur with many psychiatric disorders such as major depression, bipolar disorder and PTSD (Yehuda and Seckl, 2011; Spijker and van Rossum, 2012). Thus studying the relationship between HPA axis and problem behaviour in young children can unravel the corresponding temporal sequence. Moreover, many of these severe psychiatric disorders find their origin in childhood (see for example review by Wilkinson and Goodyer, 2011). Also, the concept of the allostatic load model (McEwen and Stellar, 1993) suggests that individuals raised in a more stressful environment accumulate biological consequences, such as alterations in the HPA axis, resulting in higher risk for maladaptive behaviour.

Internalizing and externalizing problems in young children were found moderately stable (Smith et al., 2004; Bufferd et al., 2012) and also predictive of later maladaptive behavioural outcomes (Keenan et al., 1998; Bayer et al., 2010). Early childhood or infancy is a developmental period of rapid growth in neurological, physical, and emotional systems. Further according to the allostatic load model, adverse environmental effects are thought to have a lasting impact exceeding the infancy period. These adverse effects affect the infant's frontal brain activity (Dawson et al., 2003), which is involved in emotion regulation and inhibition of inappropriate responses, and also on HPA axis activity (Francis et al., 1996), which is involved in cortisol regulation.

The diurnal cortisol rhythm is not present at birth, but emerges during the first 18 months of life. Watamura et al. (2004) even posited that there is an on-going maturation of the HPA axis up to the third year of life. Although diurnal cortisol levels have been associated with internalizing and externalizing problems or a higher problem score in pre-schoolers, the results of different studies show some inconsistencies. de Haan et al. (1998) found in 2-year-old children that midmorning cortisol levels were positively correlated with internalizing problem scores and that changes in these cortisol levels after starting preschool were positively associated with externalizing problem scores. The study of Scher et al. (2010) showed that in 12—36 month old children elevated awakening cortisol levels were positively associated with internalizing problem scores but not with externalizing problem scores. The study of Ouellet-Morin et al. (2010) showed with a cross-sectional and longitudinal design that (after 1 year) there was no association between basal cortisol levels and internalizing and externalizing problem scores in 3-year olds.

There are several explanations for these discrepant results. First, the assessments used to characterize the diurnal cortisol rhythm differed in the studies. For example, the study of de Haan et al. (1998) collected one saliva sample midmorning, whereas Essex et al. (2002) collected one saliva sample between 1500 h and 1900 h on at least two of three consecutive days to characterize the diurnal cortisol rhythm. In the study of Dougherty et al. (2009) parents were instructed to collect their child's saliva 30 min after awakening and 30 min before bedtime. The latter instruction was also used in the studies of Ouellet-Morin et al. (2010) and Scher et al. (2010). As timing of sampling and number of samples during the day differed the results of these and other studies are difficult to compare. To obtain a good representation of the diurnal cortisol rhythm it is important to sample at least on three or four different time points during the day (Adam and Kumari, 2009), as was done in the study by Watamura et al. (2004). But not only differences in timing of sampling can be found, differences in the way summary measures are defined also make results hard to compare. Second, most studies in pre-schoolers relating diurnal cortisol levels to internalizing and externalizing problems are cross-sectional in design (e.g. Watamura et al., 2004; Scher et al., 2010). To assess the directionality of the relationship between diurnal cortisol levels and problem behaviour it is important for studies to have a longitudinal design. Ideally internalizing and externalizing problems at baseline are accounted for in such longitudinal studies to reduce the possibility of reversed causality. Third, some studies only focused on externalizing problems (see meta-analysis of Alink et al., 2008) and did not assess internalizing problems as well. It is important to study diurnal cortisol levels in relation to internalizing and externalizing problems to understand the specificity of any observed relation.

Although there are methodological problems in research on cortisol levels and child development, there is good evidence linking variations in diurnal patterns to problem behaviour. Both the studies of the de Haan et al. (1998)

and Scher et al. (2010) showed that higher cortisol levels were positively correlated to internalizing problems. In school-aged children, Cicchetti et al. (2010) found that children with more internalizing symptoms exhibited an attenuated diurnal decrease in cortisol. In adolescents, Shirtcliff and Essex (2008) showed that high cortisol levels predicted internalizing problems 2 years later. Also, Adam et al. (2010) demonstrated that in adolescents a higher cortisol awakening response predicted major depressive disorder. Thus, diurnal cortisol patterns indicative of higher HPA axis activity have been prospectively related to internalizing symptoms during different developmental periods. The relationship of diurnal cortisol patterns with externalizing problems seems less clear. HPA axis hypoactivity has been related to higher levels of callous-unemotional traits and externalizing problems as antisocial behaviour; however Hawes et al. (2009) pointed out that this association may be characteristic for a particular severe subgroup only. The meta-analysis of Alink et al. (2008) suggests that the relationship between basal cortisol levels and externalizing problems is moderated by age during development. Lower basal cortisol levels (hyporeactivity) were associated with externalizing problems in elementary school-aged children, while this was the case for higher basal cortisol levels (hyperreactivity) in pre-schoolers.

To study the association between diurnal cortisol patterns and behavioural problems early in life we used a population-based sample. This offers the opportunity to study developmental patterns in normally developing children. We focused on the diurnal rhythm and collected several saliva samples for cortisol assessment throughout the day. Several pre-defined summary measures were used to assess different aspects of the diurnal cortisol rhythm. Measuring diurnal cortisol levels in the home environment allows less control than in the laboratory, but defining composite cortisol measures reduces the situational effects and reflects the functioning of the HPA axis in everyday life better (Saxbe, 2008). Internalizing and externalizing problems were measured repeatedly, that is at baseline and follow-up. Our hypothesis is that variations in diurnal patterns signalling hyperactivity of the HPA axis precede higher scores on problem behaviour. More specifically we postulate that diurnal cortisol patterns indicative of higher HPA axis activity during the day precede more internalizing and externalizing problems.

2. Methods

2.1. Setting

This study was conducted in a subsample of the Generation R Study, a cohort study investigating growth, development and health from foetal life onwards in Rotterdam, the Netherlands. The cohort has been described in detail elsewhere (Jaddoe et al., 2012; Tiemeier et al., 2012). The Generation R Focus Study, a subcohort within the Generation R Study, is conducted to obtain detailed measurements of the child's development in an ethnically homogeneous subgroup to exclude confounding or effect modification by ethnicity. Only children of Dutch national origin were included in this group, i.e. the children, their parents and their grandparents were all born in the Netherlands. The participating children were born between February 2003 and August 2005. The children visited the research centre regularly for various somatic and behavioural assessments. Written informed consent was obtained from all participants. The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam.

2.2. Study population

For the current study, children who visited the research centre for the Focus Study around 14 months were eligible for assessment of the diurnal cortisol profile. Parents of 602 children who attended the Focus Cohort examination returned one or more saliva samples. Of these, 236 children had to be excluded, because in these children less than two morning samples or less than three samples during the day were obtained, which is insufficient to compute a cortisol composite measure. The area under the curve was calculated in 277 children, the diurnal cortisol slope in 297 children and the cortisol awakening response in 314 children. For 366 children at least one of these cortisol composite measures could be computed.

Information on problem behaviour at age 18 months was available in 345 children (94% of 366). At age 36 months, information on problem behaviour was available in 332 children (91% of 366). This resulted in a total of 322 children (88% of 366) that were included in one or more analyses of the relation between cortisol and problem behaviour.

2.3. Salivary cortisol measurements

An extensive description of the cortisol measurement and analysis was presented previously (Saridjan et al., 2010). Prior to the Focus Study visit at 14 months, parents were instructed to collect five saliva samples at home using Salivette sampling devices (Sarstedt, Rommelsdorf, Germany). Parents received detailed written instructions with pictures concerning the saliva sampling. These saliva samples were collected during one single weekday: immediately after awakening, 30 min later, around noon, between 1500 h and 1600 h, and at bedtime. Supplement Table 4 gives an overview over this schedule. Parents were asked not to let their infant eat or drink 30 min before saliva sampling to avoid disturbances of the cortisol levels. Besides these restrictions, the infants were free to follow their normal daily routines on the sampling day. Parents were asked to record information about sampling times on the Salivette tubes as well as on an enclosed schematic form (see Supplement Table 4 for mean sampling times). On these forms parents were asked to add information about napping time and food intake. Between 97.9% and 99.3% of the parents reported not feeding their child 30 min before sampling. This high adherence to the protocol makes it unlikely that feeding practice influenced the results. The Salivettes were gathered at the laboratory of the Department of Epidemiology at the Erasmus MC, where the samples were centrifuged and frozen at −80 °C. After completion of the data collection, all frozen samples were sent on dry ice in one batch by courier to the laboratory of the Department of Biological Psychology laboratory at the Technical University of Dresden for

analysis. Salivary cortisol concentrations were measured using a commercial immunoassay with chemiluminescence detection (CLIA; IBL Hamburg, Germany). Intra- and interassay coefficients of variation were below 7% and 9%, respectively.

Supplementary Table 4 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2014.08.008.

For each time point, cortisol values that were above the 99th percentile (>200 nmol/L) were excluded ($n = 18$, outliers from 12 children) from the analysis to reduce impact of outliers. Although values >44 nmol/L or 3—4 SD above the mean at each sampling time have been excluded in previous studies (Gunnar and White, 2001; Gunnar and Talge, 2007), because of positively skewed distributions we used the 99th percentile, equaling 2.5 SD after *Z*-standardizing the cortisol values, per time point. Our procedure for exclusion of outliers was also used by Dekker et al. (2008). Excluding values >44 nmol/L ($n = 12$) did not change our results.

We calculated three composite variables of the separate cortisol measurements within a day: the area under the curve (AUC), the diurnal cortisol slope and the cortisol awakening response (CAR). These independent variables characterize different aspects of the HPA axis activity. The AUC was used as a measure of total cortisol secretion during the day (from awakening in the morning until bedtime in the evening). It was determined by the total area under the curve given by the cortisol measurements in nmol/L on the y-axis and the time between the cortisol measurements on the x-axis, in the same way as previously described by Pruessner et al. (2003) using the formula for calculating the area under the curve with respect to the ground. To correct for differences in length of total sampling interval time, the AUC was divided by number of hours between the first cortisol measurement at awakening and the last cortisol measurement before going to bed. The AUC was computed only for those who collected at least three saliva samples. Sleeping hours during the day were not associated with the AUC.

The diurnal cortisol slope was used as a measure of the diurnal cortisol decline. It was calculated by fitting a linear regression line for each child, which predicted the cortisol values from time since awakening. The slope was computed by using the first saliva sample and at least two other cortisol time point measures. To avoid any effect of the CAR (Adam et al., 2006), the second cortisol sample (30 min after awakening) was not included in this measure of the slope. Flatter slopes, as indexed by less negative betas, imply a slower cortisol decline during the day. This can be due to relatively lower morning cortisol levels or relatively higher levels in the afternoon or evening. To determine the influence of the first and last cortisol levels on the slope, the correlation between these cortisol levels and the slope was analysed.

The CAR was also used as an index of the HPA axis activity. It was calculated as the difference between the cortisol value at awakening and the value 30 min after awakening (Kunz-Ebrecht et al., 2004). The CAR was only calculated if the cortisol value 30 min after awakening was taken between 15 min and 60 min after awakening. 95% of the parents reported to have sampled the first saliva sample immediately or within 15 min after awakening. In a previous study, we also found that on average the CAR of children aged 14 months was negative. As older children in this sample were more likely to have a positive CAR, this probably reflects maturation of the HPA-axis (Saridjan et al., 2010).

2.4. Child internalizing and externalizing problems

At age 18 and 30 months parents received postal questionnaires to assess their child's problem behaviour. The Child Behavior Checklist (Achenbach and Rescorla, 2000) for toddlers (ages 18 months to 5 years) was used to obtain standardized parental reports of children's internalizing and externalizing problems. This questionnaire contains 99 problem items, which are scored with regard to 7 empirically based syndromes that were derived by factor analyses: Emotionally Reactive, Anxious/Depressed. Somatic Complaints, Withdrawn, Sleep Problems, Attention Problems, and Aggressive Behavior. The summary Internalizing scale is a summary score for items on the first 4 syndrome scales, and the Externalizing scale is a summary score for Attention Problems and Aggressive Behavior. Each item is scored 0, 1, or 2 (0 = not true, 1 = somewhat or sometimes true, 2 = very true or often true) on the basis of the child's behaviour during the preceding 2 months. Higher scores indicated more problems. Good reliability and validity have been reported for the CBCL (Achenbach and Rescorla, 2000). The internal consistency of the Internalizing scale at 18 months in this sample was $\alpha = 0.72$, and $\alpha = 0.88$ for the Externalizing scale. At 36 months this was $\alpha = 0.75$ and 0.89, respectively. Outliers were defined as scores below the 0.1 or higher than the 99.9 percentile and were excluded ($n = 9$ children) to meet model assumptions. In our study population 3.3% of the children fell in the borderline range and no child was in the clinical range of internalizing problems at age 18 months. For externalizing problems the percentages were respectively 10.7% and 2.6% at 18 months. At 36 months, 3.7% of the children fell in the borderline range and 0.3% in the clinical range of internalizing problems, while 5.3% fell in the borderline range and 1.3% in the clinical range for externalizing problems. The cut-off scores were derived according to Achenbach's manual and based on Dutch norms according to Tick et al. (2007).

2.5. Assessment of covariates

The choice of potential confounders was determined a priori and based on earlier literature. Socio-economic status and lifestyle related variables (maternal educational level, maternal smoking during pregnancy), obstetric and neonatal variables (parity, gestational age at birth, birth weight, Apgar score 5 min after birth) and other known determinants of (mother' reported) infant behavioural problems (maternal age, maternal distress during pregnancy, infant gender, infant age, maternal parenting stress at 18 months) were considered as possible confounders (Gutteling et al., 2005; Saridjan et al., 2010).

Maternal age, maternal educational level, and parity were determined at enrolment using self-report. Educational level was categorized in three levels: low (no or primary education, and lower vocational training), middle (intermediate and higher vocational training) and high education (university or higher). Information about maternal smoking was obtained by postal questionnaires

during pregnancy. Mothers were classified as smokers or non-smokers during pregnancy.

Date of birth, gestational age at birth, birth weight, Apgar score 5 min after birth, and gender of the infant were obtained from community midwife and hospital registries at birth.

Maternal psychiatric symptoms during pregnancy were assessed using the Brief Symptom Inventory (BSI), a validated 53-item (5-point scale) self-report symptom inventory outlined to ascertain the psychological state of individuals (Derogatis and Melisaratos, 1983). Of the 322 mothers in our study population, 305 completed the BSI. The mean total score of the BSI, the Global Severity Index (GSI) and indicator of current psychological distress levels, was obtained by dividing the sum score by the numbers of completed items. The internal consistency of the GSI in this sample was $\alpha = 0.92$.

Maternal parenting stress was measured by the Nijmeegse Ouderlijke Stress Index-Kort (NOSIK; de Brock et al., 1992), the Dutch version of the Parenting Stress Index-Short Form. The NOSIK comprises 25 questions on two domains: parenting stress due to parental factors and parenting stress due to child factors. Only the 11 items of the parental domain were used in the present analyses. In our sample 312 mothers competed these items from the NOSIK. Items were assessed on a four-point Likert scale. Following the manual (de Brock et al., 1992), scores were summed and divided by the number of completed items. Higher scores indicate greater levels of stress. The NOSIK has good reliability (Cronbach's alpha = .95) and validity (de Brock et al., 1992). Internal reliability for the 11 items in the current study, measured by Cronbach's alpha, was 0.69.

2.6. Statistical analyses

In the non-response analysis we compared the maternal and child characteristics of our study population with the characteristics of the mothers and infants with no information on the cortisol composite measures and problem behaviour. The following statistical tests were used in the non-response analyses to compare the children included in the analyses to the children who were excluded because of missing data: independent *t*-tests for continuous variables approaching a normal distribution, Mann—Whitney *U* tests for continuous non-normally distributed variables, and chi-square statistics for categorical variables. Analyses of missing data showed that children without information on the cortisol composite measures and without information on their problem behaviour were more often girls (52.9% vs. 42.5%, chi-square = 6.39, df = 1, $p = 0.01$) and had lower Apgar scores 5 min after birth (Apgar score below 8: 9.0% vs. 4.8%, chi-square = 4.21, df = 1, $p = 0.04$). The non-responding children were more likely to have less educated mothers as well (% low educational level: 11.6% vs. 6.3%, chi-square = 5.26, df = 1, $p = 0.02$). However, these children did not differ in any other characteristics from the children in our study population.

The computed variables AUC, slope and CAR, and the CBCL scores showed a slightly skewed distribution. We did not transform these variables since regression residuals were normally distributed and this makes interpretation of the results more straight-forward. The correlation between CBCL scores at 18 months and 36 months was tested for both the Internalizing and Externalizing scale scores using Spearman's correlation coefficient.

We used linear regression models to test the associations between the composite variables of cortisol and the CBCL Internalizing and Externalizing scale scores. First, we tested the associations adjusting for age at cortisol sampling, gender and for age at CBCL measurement (age and gender adjusted analyses). In the final models we additionally adjusted for maternal age, maternal educational level, maternal psychological problems during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months. We did not include birth weight and Apgar score in our models, since these covariates did not change the effect estimates meaningfully (<5%). Percentages of missing values on covariates ranged from 0% to 5.3%. For missing values on continuous variables the median value was imputed and for missing values on categorical variables the median category was used for imputation. In the longitudinal analyses with the Internalizing and Externalizing Problem scores at 36 months as an outcome, CBCL score at 18 months were also added to the models to correct for initial values at 18 months and thereby testing the associations between the composite variables of cortisol and the change in reported problem behaviour. In an alternative approach to test stability of results, we tested the relation of cortisol measures delta-scores (difference scores between 36 and 18 months) of Internalizing and Externalizing scale scores with linear regression models. Delta-scores, however, do not account for the level of problem scores (the same absolute change occurs at low and high levels of problems).

Additionally adjusting for time of waking did not change results; therefore we did not include time of waking in our final models. Also, we performed a sensitivity analysis by calculating the AUC more conservatively by including only children with at least four saliva samples and again by including only children with all five samples. Next we reran the analyses of the CAR by including only those who provided information that the first sample was taken within 10 min after awakening and the second sample was obtained between 30 and 45 min after the first sample, which led to a reduction of the *n* to 177 because of non-compliance and missing data. This exclusion reduced the power by more than 20% (data not shown).

Also, the interaction between the cortisol composite measures and gender was tested. In post hoc analyses we tested the associations between the composite cortisol variables and the subscales of the CBCL Internalizing scale scores (emotionally-reactive, anxious-depressed, somatic complaints, withdrawn). All statistical analyses were performed with the Statistical Package for the Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

3. Results

Table 1 presents the characteristics of the participating mothers and children. 57.5% of this sample was male. Maternal distress during pregnancy did not differ between girls and boys (Mann—Whitney $Z = -1.18$, df = 1, $p = 0.24$), but

Table 1 Subject characteristics.

	Total $N = 322$	Mean ± SD (range) or %
Maternal characteristics		
Age (years)		32.0 ± 3.6 (21.0–43.3)
Educational level		
Low	20	6.3
Middle	170	53.3
High	129	40.4
Smoking during pregnancy (% yes)	34	10.6
Psychiatric symptoms (GSI-score)		0.16 ± 0.18 (0.00–1.67)
Parity (% nulliparous)	193	59.9
Parenting stress at 18 months (Nosik-score)		0.23 ± 0.26 (0.00–1.82)
Child characteristics		
Gender (% boy)	185	57.5
Gestational age at birth (weeks)		40.1 ± 1.56 (34.4–42.9)
Birth weight (grams)		3530 ± 505 (1960–4795)
Apgar-score 5 min after birth		9.6 ± 0.6 (5–10)
Age of cortisol sampling (months)		14.4 ± 1.0 (11.7–19.3)
Cortisol values		
AUC (nmol/L)	247	8.27 ± 4.5 (0.21; 27.83)
Slope (nmol/L/h)	265	−1.03 ± 8.3 (−3.82; 2.91)
CAR (nmol/L)	267	−1.73 ± 9.3 (−19.7; 37.6)
Problem behaviour at 18 months		
Internalizing problems scale	307	3.86 ± 3.3 (0.00–16.0)
Externalizing problems scale	309	9.95 ± 6.4 (0.00–31.00)
Problem behaviour at 36 months		
Internalizing problems scale	321	4.02 ± 3.5 (0.00–17.0)
Externalizing problems scale	320	8.16 ± 5.9 (0.00–30.0)

Values are means ± standard deviations (range) for continuous variables, and percentages for categorical variables.
GSI = Global Severity Index of the Brief Symptom Inventory, measured during pregnancy; Nosik = Nijmegen Parenting Stress Index; AUC = Area under the curve; CAR = Cortisol awakening response.

perceived parenting stress at 18 months was lower in girls than in boys (median = 0.10 in girls vs. median = 0.18 in boys, Mann–Whitney $Z = -2.11$, df = 1, $p = 0.035$). The following median cortisol values were observed at the different time points during the day: at awakening 15.21 nmol/L (range: 0.08–51.03), 30 min after awakening 13.05 nmol/L (range: 0.07–55.56), at noon 5.45 nmol/L (range: 0.05–47.30), around 1600 h 4.94 nmol/L (range: 0.21–40.48) and at bedtime 2.05 nmol/L (range: 0.09–58.50). These cortisol values and cortisol composite measures did not differ between girls and boys. On average, the children in our study did not show a rise of cortisol after awakening (mean CAR −2.64 nmol/L, range: −19.7; 37.6). There was a correlation between the Internalizing Problem scale scores at 18 and 36 months (Spearman's rho: 0.48, $p < 0.001$), and between the Externalizing Problem scores at 18 and 36 months (Spearman's rho: 0.56, $p < 0.001$). Also, the Internalizing Problem scale scores at 18 months were correlated to the Externalizing Problem scale scores (Spearman's rho: 0.61, $p < 0.0001$); this was also observed at 36 months (Spearman's rho: 0.64, $p < 0.0001$). The scores on the Externalizing Problems scale at 18 months were significantly different between girls and boys (median = 8.17 in girls vs. median = 10.0 in boys, Mann–Whitney $Z = -2.13$, df = 1, $p = 0.03$).

Table 2 shows the cross-sectional associations between the diurnal cortisol rhythm and problem behaviour reported at 18 months. As none of the interactions between gender and the different cortisol composite measures were significant, the results are shown for girls and boys together. The AUC, the slope and the CAR at 14 months were not associated with Internalizing Problem scores at 18 months (see Table 2 for details). Likewise, there were no associations between the cortisol composite measures and the Externalizing Problem scores (see Table 2 for details).

Table 3 presents the associations between the diurnal cortisol rhythm at baseline and problem behaviour reported at 36 months. In the age and gender adjusted analyses, the associations just fell short of reaching the level of significance (β per nmol/L AUC: 0.09, 95% CI: −0.00; 0.19, $p = 0.06$; β per nmol/L/h slope: 0.42, 95% CI: −0.07; 0.98, $p = 0.09$; β per nmol/L CAR: 0.04, 95% CI: −0.00; 0.09, $p = 0.07$; see also Supplement Table 1). Supplement Table 3 shows the association of several important confounders with internalizing and externalizing problems. After additionally adjusting for CBCL scores at 18 months as well as potential confounding factors, associations between the diurnal cortisol rhythm and the Internalizing Problem scores reported at 36 months were found. Children with higher AUC levels, flatter slopes and a more positive CAR at 14 months were more likely to score higher on the Internalizing Problems scale at 36 months (β per nmol/L AUC: 0.08, 95% CI: 0.00; 0.17, $p = 0.04$; β per nmol/L/h slope: 0.57, 95% CI: 0.17;

Table 2 Cross-sectional associations between cortisol composite measures and behavioural problems at 18 months.

Cortisol measures	CBCL broadband scales					
	Internalizing problems[a]			Externalizing problems[a]		
	N	Beta (95% CI)	*p*	*N*	Beta (95% CI)	*p*
AUC (nmol/L)	260	−0.01 (−0.10; 0.09)	0.91	261	0.02 (−0.15; 0.19)	0.81
Slope (nmol/L/h)	280	−0.15 (−0.65; 0.34)	0.54	282	−0.03 (−0.92; 0.87)	0.96
CAR (nmol/L)	295	−0.03 (−0.07; 0.01)	0.20	296	−0.01 (−0.08; 0.07)	0.84

[a] Models adjusted for age at cortisol sampling, age of CBCL measurement at 18 months, gender, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months.

0.98, $p = 0.006$; β per nmol/L CAR: 0.04, 95% CI: 0.01; 0.08, $p = 0.02$). The associations between the cortisol composite measures and Internalizing Problem scores adjusted for confounders but not for baseline Internalizing Problem scores at 18 months, however, were very similar (see Supplement Table 1).

Supplementary Tables 1 and 3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2014.08.008.

The sensitivity analyses testing the associations between the cortisol composite measures and delta-scores of the Internalizing and Externalizing Problem scores, showed very similar results (see Supplement Table 2 and Fig. 1). Both the slope and the CAR predict the difference in Internalizing score from baseline to follow-up, in particular the slope showed a very clear dose-response pattern. In the sensitivity analyses calculating the AUC only in children who provided at least four saliva samples and again in those without any missing samples, the results for Internalizing Problem scores remained essentially unchanged. However, they no longer reached the same level of significance in the latter model because of a smaller sample size (4 samples: β per nmol/L AUC: 0.07, 95% CI: −0.02; 0.15, $p = 0.12$; 5 samples: β per nmol/L AUC: 0.07, 95% CI: −0.03; 0.16, $p = 0.19$). The association between AUC and Externalizing Problem scores became significant in those who provided all samples (5 samples: β per nmol/L AUC: 0.15, 95% CI: 0.00; 0.31, $p = 0.05$; 4 samples: β per nmol/L AUC: 0.08, 95% CI: −0.05; 0.21, $p = 0.23$).

Supplementary Table 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2014.08.008.

By calculating the CAR only in those with documented compliance, the sample size was reduced to 177 and the association with Internalizing Problem Scores was no longer significant (β per nmol/L CAR: 0.02, 95% CI: −0.02; 0.07, $p = 0.31$). None of the interactions between gender and the different cortisol measures were significant with one exception. Gender moderated the association between the slope and internalizing problems. After stratifying for gender, the slope was positively associated with the Internalizing Problem scores reported at 36 months in girls (β per nmol/L/h slope: 0.90, 95% CI: 0.32; 1.49, $p = 0.003$) but not in boys (data not shown).

In contrast, we found no association between the cortisol composite measures, i.e. AUC, slopes and CAR with Externalizing Problem scores (see Table 3 for details).

Post hoc analyses showed that the CAR was positively associated with the anxious-depressed subscale of the Internalizing Problems scale (β per nmol/L CAR: 0.02, 95% CI: 0.01; 0.03, $p = 0.005$) and that the AUC was positively associated with the somatic complaints subscale of the Internalizing Problems scale (β per nmol/L AUC: 0.06, 95% CI: 0.02; 0.09, $p = 0.003$). However, when corrected

Table 3 Longitudinal associations between cortisol composite measures and behavioural problems at 36 months.

Cortisol measures	Child problem behaviour									
	Internalizing problems[a]					Externalizing problems[a]				
	N	Beta (95% CI)	*p*	R^2	F^2	*N*	Beta (95% CI)	*p*	R^2	F^2
AUC (nmol/L)	246	0.08 (0.00; 0.17)	0.04*	0.351	0.541	245	0.10 (−0.03; 0.23)	0.14	0.400	0.667
Slope (nmol/L/h)	264	0.57 (0.17; 0.98)	0.006**	0.372	0.592	263	0.42 (−0.26; 1.10)	0.23	0.416	0.712
CAR (nmol/L)	275	0.04 (0.01; 0.08)	0.02*	0.307	0.443	274	0.03 (−0.03; 0.09)	0.33	0.366	0.577

[a] Models adjusted for age at cortisol sampling, CBCL score at 18 months (continuously), gender, age of CBCL measurement at 36 months, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months.
R^2 = adjusted *R* Square for fully adjusted models; F^2 = Cohen's *F*-Square effect size.
* Significant at the 0.05 level.
** Significant at the 0.01 level.

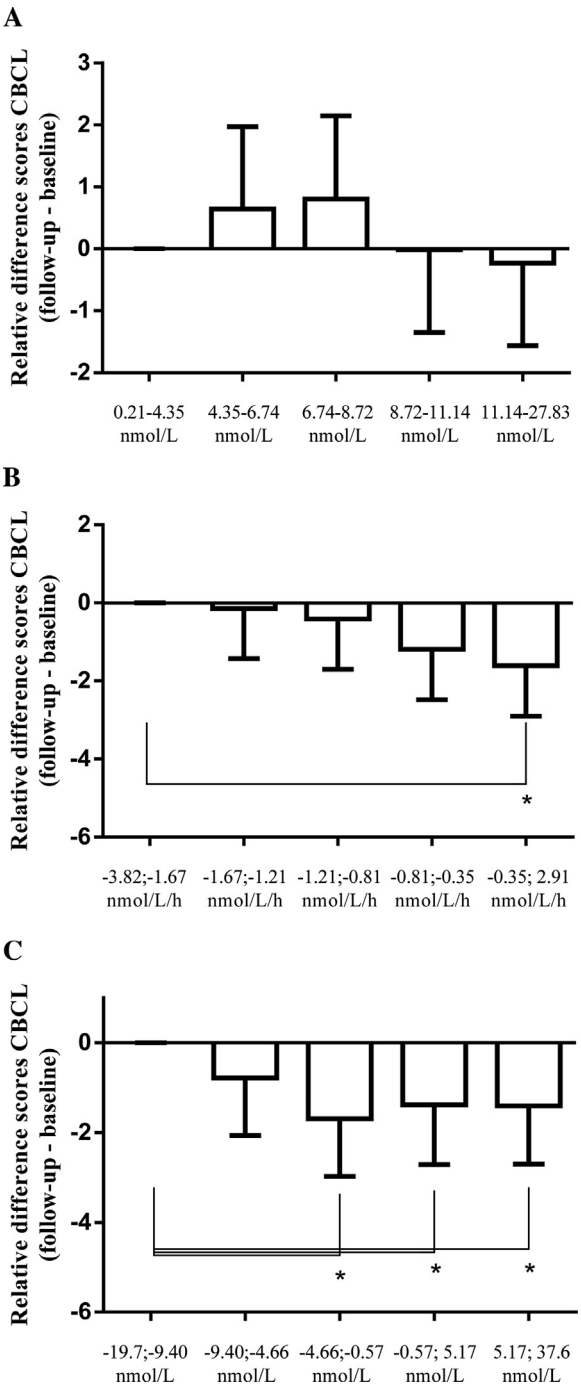

Figure 1 (A) Association between AUC at baseline and difference scores in child problem behaviour from baseline to follow-up. Per quintile of AUC at baseline, the mean change in internalizing problems is calculated and compared with the change of problems in the lowest quintile of AUC (reference) using ANCOVA adjusted for age at cortisol sampling, gender, age of CBCL measurement at 36 months, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months. The mean change in internalizing problems in the reference group over time was 0.25. (B) Association between slope at baseline and difference scores in child problem behaviour from baseline to follow-up.

for multiple testing, only the latter association remained significant ($p = 0.003 < p = 0.05/12$).

4. Discussion

This prospective study showed that higher HPA axis activity during the day in infants predicted higher internalizing problem scores in the pre-school age. Children with higher AUC levels, flatter slopes or a more positive CAR were more likely to have higher scores on internalizing problem behaviour at 36 months. Findings were largely similar in boys and girls.

To our knowledge this is one of the few population-based studies to assess diurnal HPA axis activity at an early age in relation to internalizing or externalizing problems. Importantly, this design enabled us to study temporal sequence of the relation between diurnal cortisol patterns and problem behaviour and reduced the possibility of reversed causality. Also, our results contribute to the few studies in young children with a longitudinal design (e.g. Smider et al., 2002), and to those with repeated measurement of internalizing and externalizing problems over a 6-month period (Gunnar et al., 2011).

Although we observed no cross-sectional association between the diurnal cortisol rhythm and internalizing problems at 18 months, we found that higher HPA axis activity during the day at 14 months was related to a significant change of internalizing problems with higher levels at 36 months. Our results are in line with the prospective study of Smider et al. (2002), who found that in boys higher afternoon cortisol levels at age 4.5 years predicted more internalizing symptoms 1.5 years later. Interestingly in 4-year-old children, high basal cortisol levels co-occurring with higher internalizing behaviour scores has been reported in boys also in a cross-sectional study (Pérez-Edgar et al., 2008). First we will discuss possible explanations for the longitudinal associations between infants' diurnal cortisol rhythm and internalizing problems, in the absence of significant cross-sectional associations. At 18 months internalizing problem scores are much less specific and less stable than at 36

Per quintile of slope at baseline, the mean change in internalizing problems is calculated and compared with the change of problems in the lowest quintile of slope (reference) using ANCOVA adjusted for age at cortisol sampling, gender, age of CBCL measurement at 36 months, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months. The mean change in internalizing problems in the reference group over time was −0.66. * Relative difference to the reference group was significant ($p < 0.05$). (C) Association between CAR at baseline and difference scores in child problem behaviour from baseline to follow-up. Per quintile of CAR at baseline, the mean change in internalizing problems is calculated and compared with the change of problems in the lowest quintile of CAR (reference) using ANCOVA adjusted for age at cortisol sampling, gender, age of CBCL measurement at 36 months, maternal age, maternal educational level, maternal distress during pregnancy, maternal smoking during pregnancy, parity, gestational age at birth, and maternal parenting stress at 18 months. The mean change in internalizing problems in the reference group over time was −0.88. * Relative difference to the reference group was significant ($p < 0.05$).

months (Rose et al., 1989). Since 18 months is at the very bottom of the standardization range for the CBCL, it is conceivable that persistent behavioural patterns, although present, are not yet clearly recognized by the parent or caregiver. The moderate to high correlation between the internalizing scores at 18 and at 36 months, however, suggests that other factors may account for these results. Alternatively, a bi-directional relation between cortisol and internalizing problems could explain our observations. If the direction of the association between internalizing problems and cortisol levels is in opposite direction (i.e. high internalizing problem scores lead to reduced cortisol levels in some infants) the overall observed association in the cross-sectional models at 18 months would be diluted (but not the baseline adjusted longitudinal association). Given the lack of research in the age group of our participants strong bi-directional effects remain speculative, but this illustrates the importance of adjustment for internalizing problems at baseline. It can never be ruled out that the results of our study are chance findings but longitudinal analyses with baseline assessment are certainly a powerful design to detect associations because individual variations at baseline, which can also reflect confounding, are adjusted for.

There are also several biological mechanisms, which make our observed longitudinal associations plausible, and constitute possible pathways that may underlie our findings. First, higher cortisol levels have been linked directly to anxiety via activation in the central nucleus of the amygdala (MacMillan et al., 2003), which is involved in both behavioural inhibition and anxiety (Fox et al., 2005). However, higher cortisol levels can also lead to physiological changes in the body, which can be experienced as stress and therefore reinforce anxiety or internalizing problems. Our results showed that the diurnal cortisol slope had a particularly strong relationship with a change in internalizing problem scores. As flatter cortisol slopes have been associated with chronic stress in healthy children (Wolf et al., 2008), this may explain our findings. Second, prenatal and postnatal stress can negatively influence brain development and thereby also HPA activity. Several brain regions, such as the frontal cortex and fusiform gyrus (see review by Nosarti, 2013) have been implicated in internalizing behaviour and neurodevelopmental changes due to stress are a possible explanation for our findings. This concept has been referred to as the allostatic load model (McEwen and Stellar, 1993). Kagan integrated this concept with the well-established psychobiological model of internalizing problems (Kagan et al., 1984) when he demonstrated increased morning cortisol levels in behaviourally inhibited children (Kagan et al., 1987). More recently Buss et al. (2011) showed that increased allostatic load, measured by cumulative indices (in which flatter diurnal cortisol slopes were included), was related to more internalizing problems in young children. Also the study of Ruttle et al. (2011) showed different relationships between morning cortisol levels and internalizing problems analysed concurrently and longitudinally in adolescents. In concurrent analyses internalizing problems were associated with hyperactivity of the HPA axis, whereas in longitudinal analyses internalizing problems predicted hypoactivity. These findings provide a strong case for testing the temporal relation between HPA axis activity and internalizing problems in both directions at different ages. Third, a genetic predisposition could underlie the observed association. It is conceivable that variants of genes involved in brain or endocrine development, such as the serotonin transporter gene with its well documented role in internalizing problems and HPA axis activity in humans (see for example Goodyer et al., 2010), give rise to individual differences in cortisol levels and directly or indirectly also lead to more internalizing problems. This remains speculative, cortisol levels and internalizing problems are moderately heritable but molecular genetic variances influencing cortisol levels or internalizing problems have not been reliably demonstrated. A fourth explanation for our findings is the possible effect of unmeasured confounders in our models. For example socio-economic status or concurrent daily stressors such as family stress can influence internalizing problems and cortisol levels and affect the relation between HPA activity and internalizing problems. Although we cannot rule out the influence of residual confounding, we tried to minimize the effect of confounding by adjusting our models with several possible indicators of socio-economic background and child development.

In our study we did not find differences between boys and girls in the associations of the cortisol composite measures with internalizing problem scores. There was one exception, girls, but not boys, with a more positive cortisol slope at 14 months (i.e. less decline during the day) had higher internalizing problem scores at 36 months. Our findings are in line with the study of Kryski et al. (2013), who found elevated cortisol reactivity only in girls, and suggested that cortisol reactivity to stress in early childhood has a sex-specific association with girls' internalizing symptoms.

In this study we found no relationship between diurnal cortisol rhythm and externalizing problem behaviour in pre-schoolers. Although the effect of cortisol patterns on externalizing and internalizing problem scores did not differ greatly, our hypothesis that diurnal patterns demonstrating higher HPA axis activity predict more externalizing problems was not supported by our findings. Our results are in line with the findings of the prospective study of Ouellet-Morin et al. (2010), who also found no association between cortisol levels and externalizing problem scores. However, the meta-analysis of Alink et al. (2008) suggests that higher basal cortisol levels are associated with more externalizing problems in pre-schoolers. Possibly our study did not have enough power to find a relationship between diurnal cortisol levels and externalizing problem scores. If not a chance finding, the observed association of HPA axis activity and internalizing problems may reflect that internalizing problems are more reliable at young ages. Internalizing problems in toddlers have been found to be more stable as compared with externalizing problems (Achenbach and Rescorla, 2000). Another explanation could be that externalizing problems are influenced more by environmental risk factors such as low socio-economic status and poverty as compared with internalizing problems in young children (Costello et al., 2003).

The strengths of our study are the large population-based sample and the prospective design. Yet, some limitations of the current study need to be considered. First, the sampling of saliva occurred only on one single day, so day-to-day variability could not be taken into account (Hellhammer et al.,

2007). However, to ask parents participating in a large cohort with multiple other assessments to sample on several days increases the risk of drop-out or non-response. Second, the compliance of the saliva sampling was not assessed by an objective measurement such as a timing device, for this we relied on parental report. As can be seen from the sensitivity analyses, our approach to include children from families who did not adhere well to the protocol increased the power but can also introduce noise to the calculation of the CAR and the AUC. Importantly, the associations largely remained very similar. Furthermore, we relied on parental report for our problem behaviour outcome measures. However, good reliability and validity have been reported for the questionnaire we used to determine problem behaviour (Achenbach and Rescorla, 2000). Another aspect, relevant for clinicians who diagnose child psychiatric disorders, is that we did not use clinical cut-off scores for problem behaviour in our study. This maximizes power and avoids the relying on cut-offs defined arbitrarily or by convention. Our analyses of missing data showed that attrition was not at random. There was a selective dropout of girls, children with lower Apgar scores and children of lower educated mothers. Due to possible selection effects, our results may be less representative of the general population.

In conclusion, our study shows that variations in diurnal cortisol rhythm are longitudinally associated with the change in internalizing problems in pre-schoolers. The study highlights the importance of longitudinal studies that can unravel temporal sequence. Our results suggest that variations in diurnal cortisol patterns are a cause rather than a consequence of internalizing problems in young children.

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Conflict of interest

All authors declare that they have no conflict of interest.

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