Examining physiological stress (re)activity as an endophenotype for adolescent substance use

Evans, B.E.

2013

document version
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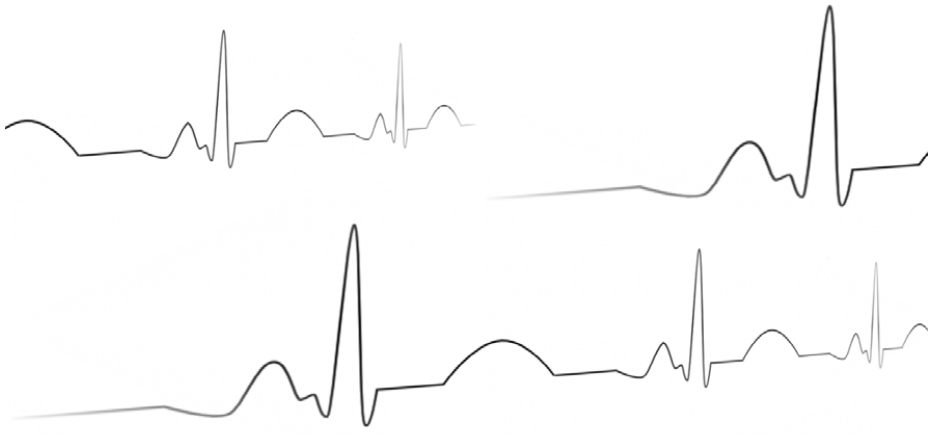
Chapter 6

Cortisol levels in children of parents with a substance use disorder

Brittany E. Evans, Kirstin Greaves-Lord, Anja S. Euserm, Ingmar H.A. Franken &

Anja C. Huizink



Accepted for publication in Psychoneuroendocrinology, March 2013

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ABSTRACT

Background

Children of parents with a substance use disorder (CPSUDs) are at increased risk for the development of substance use disorders later in life, and therefore may manifest vulnerability markers for these disorders at a higher level than children from the general population. Our aim was to examine hypothalamic-pituitary-adrenal (HPA) axis activity as a potential vulnerability marker in CPSUDs as compared to healthy controls. We further examined whether having experienced more adverse life events (ALEs) accounted for differences in cortisol levels between CPSUDs and controls.

Method

Eighty-three CPSUDs were matched to 83 controls on the basis of age, sex and socioeconomic status. Salivary cortisol was assessed at four time points during a normal day and at six time points during a psychosocial stress procedure, during which perceived stress was also measured. We implemented piecewise multilevel growth curve modeling to examine group differences in diurnal and stress-evoked cortisol levels.

Results

Diurnal cortisol levels of CPSUDs did not differ from those of controls. Only stress-evoked cortisol levels at onset of the experiment were explained by group status, such that CPSUDs exhibited lower cortisol levels at onset of the stress procedure. CPSUDs reported experiencing significantly more ALEs, yet number of ALEs was not related to cortisol levels. CPSUDs furthermore reported less perceived stress than controls at onset of the procedure.

Conclusion

HPA axis dysregulation may be a vulnerability marker for substance use disorders, as CPSUDs show blunted activation in anticipation of stress. These blunted cortisol levels were not the result of having experienced more stressful experiences during their lifetimes, thus might reflect an inborn vulnerability to substance use disorders.

INTRODUCTION

An estimated 9 million young people in the European Union face everyday challenges with the added burden of having a parent who is struggling with a substance use disorder (SUD; Anderson & Baumberg, 2006). These children of parents with a SUD (CPSUDs) are at heightened risk for developing psychopathology later in life, including SUDs (e.g. Chassin, et al., 1991), as well as for experiencing more stressful events during their lifetimes as compared to children who do not have a parent with a SUD (Hussong, et al., 2008).

Because CPSUDs are at increased risk for developing SUDs later in life (e.g. Chassin, et al., 1991), they are expected to be more likely to manifest vulnerability markers for SUDs than individuals without a parent with a SUD. Stress is closely tied to the development and maintenance of SUDs (Sinha, 2008), therefore the physiological response to stress is a viable vulnerability marker.

One of the main physiological stress systems is the hypothalamic-pituitary-adrenal (HPA) axis. The system has a diurnal rhythm, with cortisol levels reaching a peak approximately 30 minutes after awakening (i.e. cortisol awakening response), and subsequently declining over the day towards an evening nadir. In healthy individuals, when faced with stress, the hypothalamus induces a cascade of events which ends in the secretion of cortisol from the adrenal glands. This activity in the hypothalamus is observable in salivary cortisol approximately 20 minutes after stressor (Sapolsky, et al., 2000). Cortisol levels then decline following the cessation of the stressor (e.g. Seyle, 1950).

HPA axis hyper- and hypo-activation have been theoretically linked to vulnerability to SUDs. Researchers have proposed alcohol use by some individuals to function as a way of dampening an inherent hyper-activation of the stress system, or of self-medicating (Khantzian, 1985). Another view draws upon the observation that individuals vulnerable to substance use exhibit an inherent physiological hypo-activation (e.g. Huizink, et al., 2006). These individuals may be characterized by high sensation-seeking tendencies (e.g. Creemers, et al., 2009) or by the lack of a physiological 'brake' when confronted with dangerous or prohibited activities, thus increasing their chance of engaging in delinquent, externalizing or substance use behaviors (Raine, 1993).

In SUD patients and heavy drinkers, resting cortisol may be increased or not differ from controls (e.g. Boschloo, et al., 2011; Lovallo, et al., 2000). Evoked by a psychosocial

stressor, cortisol levels have been reported to be blunted (e.g. Lovallo, et al., 2000; Richards, et al., 2011, although see Munro, et al., 2005). From these studies it remains unclear whether differences in HPA axis (re)activity are inherent, or whether they are the consequence of chronic and/or heavy substance use.

In order to investigate the possibility that stress is a vulnerability factor for SUD, research has focused on cortisol levels in individuals who may be at high risk for developing SUDs. Some studies have examined resting and stress-evoked cortisol levels in children exposed prenatally to maternal substance use, with differing results. Children prenatally exposed to substances exhibited lower cortisol levels in the morning and decreasing cortisol levels during a psychosocial stressor compared to controls (Bauer, et al., 2011; Fisher, Kim, Bruce, & Pears, 2012; Lester, et al., 2010). Other studies reported cortisol hyper-reactivity in infants and adolescents exposed to intrauterine substances (Chaplin, Freiburger, Mayes, & Sinha, 2010; Hunter, Minnis, & Wilson, 2011). Based on these studies, it is still not possible to exclude direct teratogenic effects of substances on the HPA axis.

Other research has investigated HPA axis activity in adolescent or adult CPSUDs who have not yet used substances heavily or for long periods, and who were not exposed to intrauterine substances. Several studies examined acute cortisol responses to alcohol or other substances versus placebo in CPSUDs as compared to controls (e.g.Hernandez-Avila, et al., 2002; Zimmermann, et al., 2004). While the cortisol response to alcohol is not relevant to the present study, the placebo conditions provide an indication of resting cortisol levels. The majority of studies reported no differences between adult CPSUDs and controls in resting cortisol levels (e.g. Gianoulakis, et al., 2003; Hernandez-Avila, et al., 2002), or in diurnal cortisol patterns (e.g. Sorocco, et al., 2006). However, differences have been reported in stress-evoked cortisol levels. In adolescents and adults, most studies observed lower cortisol levels in CPSUDs as compared to controls in anticipation of a novel situation (e.g. Hardie, et al., 2002; Moss, et al., 1999), and in response to psychosocial stress (e.g. Sorocco, et al., 2006). In two studies HPA axis hyper-activity was observed in adult CPSUDs in response to psychosocial stress, although only in European-Americans (Uhart, et al., 2006), and only if the placebo session was administered first (as opposed to the alcohol administration session; Zimmermann, et al., 2004). The participants in these studies were either adults (mostly 18-25 years) or young adolescents (10-12 years), thus, no study so far has included adolescents from a wide age range, and furthermore, only one study included girls (i.e. Hardie, et al., 2002).

Aside from the observed aberrations in the stress response, it is widely known that CPSUDs experience greater lifetime adversity than their peers who do not have a parent with a SUD, especially in the family domain (Hussong, et al., 2008). CPSUDs are met with greater family-related environmental adversity (Johnson & Leff, 1999), and are more likely to be physically and sexually abused (Miller, et al., 1997). A commonly cited finding in HPA axis literature is that life stressors lead to subsequent blunted cortisol responses in adults (e.g. Lovallo, et al., 2012) and in children (e.g. Gunnar & Vazquez, 2001). Therefore, it seems plausible that blunted cortisol responses in CPSUDs could also be (partially) accounted for by having experienced more lifetime environmental adversity. Some first empirical evidence in CPSUDs provided marginal support for this view. Two studies found additive effects of exposure to intrauterine substances and environmental adversity, such that children who reported both risks were most likely to portray decreasing cortisol levels during a psychosocial stressor (Fisher, et al., 2012; Lester, et al., 2010). One study examined the role of family disruption in CPSUDs, but found no relation between family disruption and cortisol levels, although CPSUDs exhibited blunted cortisol levels compared to controls (Hardie, et al., 2002). Other than this study, no earlier research has examined simultaneously stress-evoked cortisol levels and adverse life events in adolescent CPSUDs.

The first aim of this study was to examine whether diurnal and stress-evoked cortisol levels differed between CPSUDs and controls. We hypothesized that both diurnal and stress-evoked cortisol levels would be lower in CPSUDs. Since experiencing chronic stressors can lead to blunted cortisol levels and blunted cortisol responses to stress (e.g. Gunnar & Vazquez, 2001) and CPSUDs have reported experiencing more adverse life events (ALEs; Hussong, et al., 2008), we examined whether they did indeed report more adverse life events than controls, and whether this accounted for any observed differences in cortisol levels. As a secondary analysis, we examined whether CPSUDs and controls differed on perceived stress (PS) levels during the stressor, and whether ALEs affected the relation between group status and PS levels.

Chapter 6

METHOD

Participants

Children of parents with a substance use disorder

The sample of CPSUDs consisted of 83 adolescents (11-20 years) who had at least one parent who had been diagnosed with a SUD (lifetime DSM-IV diagnosis of substance abuse and/or dependence other than nicotine). The majority of these adolescents were recruited from Bouman GGZ outpatient clinics, the major addiction care provider in the province of South Holland (the Netherlands), where their parents were in treatment for a SUD. Diagnosis of SUD in parents was based on clinical consensus obtained by staff at Bouman GGZ. A few adolescents (*N*=6) were recruited through the Bouman GGZ Youth Clinic, being in treatment themselves, and their parents were known to have a diagnosis of SUD. A number of participants (*N*=6) had parents who were diagnosed with a SUD but were not currently in treatment. These participants were recruited by word of mouth, and diagnosis of SUD in parents was obtained via a structured interview of all DSM-IV axis 1 disorders (Composite International Diagnostic Interview; CIDI; Robins, et al., 1989), performed by a trained interviewer.

Clinical staff at Bouman GGZ informed patients with children in the targeted age range of the study and gave them an information brochure. If the patient consented to being contacted, a researcher telephoned the patient to explain the study, confirm eligibility, and make an appointment for the test session if both parent and adolescent agreed to participation. See Table 6.1 for descriptive characteristics of the SUD patients.

Table 6.1. Characteristics of SUD patients

Characteristic[1]	Percent
Parent (mothers/fathers)	47/53
Diagnosis (abuse/dependence)	8/92
Treatment status (in treatment/not)	94/6
Substance used[2]	
Alcohol	65
Polydrug	26
Cannabis	6
Cocaine	2
Sedatives	2
Substance use during pregnancy (use/no use)[3]	
Alcohol	6/94
Cannabis	2/98
Other drugs	2/98
Diagnoses other axis 1 disorders[4]	
Attention deficit hyperactivity disorder	9
Anxiety disorders	4
Mood disorders	11
Psychotic disorders	2
No disorder	73

[1]All characteristics (unless otherwise specified) based on *n*=66 parents (of which for two subjects both parents had been diagnosed)

[2]Substances used based on *n*=65 parents, as this was unknown for one parent

[3]Substances used during pregnancy based on *n*=65 (alcohol use) and *n*=67 (cannabis and other drug use) CPSUDs for whom information was available

[4]Other Axis 1 diagnoses based on *n*=45 patients for whom all Axis 1 disorders had been diagnosed

Controls

Controls were part of a larger sample of children and adolescents that participated in a longitudinal Dutch general population study. For this larger study, participants were randomly drawn from registers of 35 representative municipalities in South Holland including urban and rural areas. As part of the second wave of measurements, 711 participants (aged 7-20 years) participated in a psychosocial stress procedure. After excluding observations during which cortisol levels were greater than three standard deviations above the mean, 682 participants had cortisol observations for at least one of the stress tasks and one of the rest periods on the test day. We excluded those whose parents had been diagnosed with a SUD (other than nicotine dependence), leading to 632 eligible controls. For the purpose of this study, in order to obtain a highly comparable control group, a sample of 83 individuals was randomly selected, matched as closely as possible on age, sex and socioeconomic status (SES) to CPSUDs. For a detailed description of the study sample and design, please see Huizink, et al. (2012).

Measures and procedure

Written informed consent was obtained from all participants and their parents and participants received a gift certificate. The study was approved by the Ethics Committee of the Erasmus University Medical Center (MEC 230.37/2003/123).

Diurnal cortisol levels (Cort1-4)

All salivary cortisol samples were taken by passively drooling into a test tube. Four tubes were sent to the participants prior to the stress experiment for diurnal cortisol assessment. Detailed written and verbal instructions were given on the time and manner of sample collections, and to preserve the tubes in the freezer until the experiment. Participants were instructed to provide the first sample directly upon awakening (Cort1), the second 30 minutes afterwards (Cort2), the third at 12 p.m. (Cort3) and the fourth at 8 p.m. (Cort4).

Stress-evoked cortisol levels (Cort5-10)

The psychosocial stress procedure was modeled after the Trier Social Stress Test (Kirschbaum, et al., 1993), characterized by uncontrollability and social-evaluative threat, which are key elements to eliciting a stress response (Dickerson & Kemeny, 2004). This procedure has been shown to elicit a physiological response in children, adolescents and

adults (Dickerson & Kemeny, 2004; Gunnar, et al., 2009; Kudielka, Hellhammer, & Kirschbaum, 2007). The sessions commenced with an explanation of the procedure, two questionnaires and a ten minute rest period. Subsequently, the social stress tasks began, entailing a mental arithmetic task (serial mental subtraction), a public speaking task (the participant was told to imagine he/she had been accused of stealing and was asked to prepare and give a speech in front of a camera to explain why he/she could not have done it) and a computer mathematics task (numerical ordering as quickly and accurately as possible). Participants were told their performance on all tasks would be compared to performances of their peers. An experiment leader oversaw the tasks, gave scripted prompts to ensure the tasks were completed, and did not offer encouragement or praise. The session ended with a five minute recovery period and a relaxing nature documentary (25 minutes), after which the participants were fully debriefed. After each period/task, at the middle of the movie and at the end of it, the participant was asked to provide saliva samples (see Figure 6.1; Cort5-10). These samples reflect activity in the hypothalamus approximately 20 minutes earlier due to the delay in observable cortisol response (Sapolsky, et al., 2000; see arrows in Figure 6.1). This procedure was implemented in previously published studies (i.e. Dieleman, et al., 2010; Evans, et al., 2012; Evans, et al., 2012).

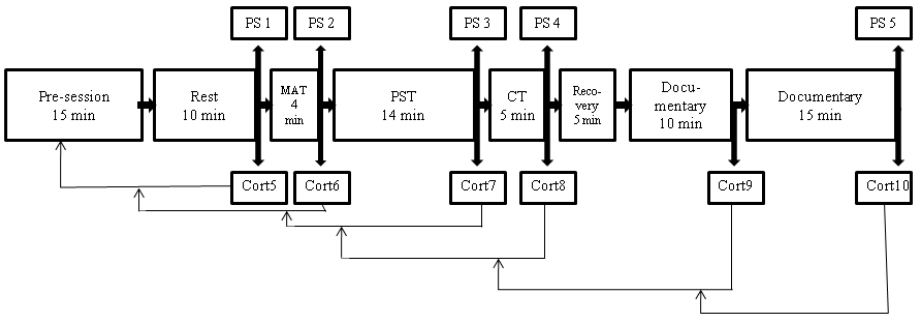

Figure 6.1. Depiction of the psychosocial stress procedure.

Note. MAT=mental arithmetic task; PST=public speaking task; CT=computer task; Cort5-Cort10=cortisol tubes 5 through 10; PS=perceived stress. Arrows extending from Cort5-10 point to moments during the procedure to which cortisol levels correspond, due to the delay in observable cortisol increase after the onset of the stressor (Sapolsky, et al., 2000).

Saliva samples were kept in a freezer and collectively sent to the laboratory for analysis. A time-resolved fluorescence immunoassay was implemented to determine the cortisol concentration in the samples. Outliers greater than three standard deviations above the mean were removed from the analysis due to possible contamination (e.g. blood, medicine). All cortisol values were square root transformed in order to approximate normal distributions.

Adverse life events

Adverse life events (ALE) were selected from an extensive Life Events Questionnaire (LEQ; Amone-P'Olak, et al., 2009) which included both severely and mildly adverse events as well as positive events, and from the post traumatic stress disorder section of the NIMH Diagnostic Interview Schedule Composite (DISC). Eighteen severely adverse events were chosen from these sources, modelled after Lovallo, et al. (2012). Events included multiple forms of adversity, most importantly physical and sexual adversity and emotional adversity and furthermore economic hardship, illness/death and natural disaster. Both the DISC interview and the LEQ were completed by the adolescent and his/her parent. An event was considered an ALE if either the parent or adolescent confirmed that the event was experienced by the adolescent. For the LEQ, events were only considered an ALE if the informant coded the event as 'unpleasant' (for the adolescent). In accordance with similar studies (e.g. Elzinga, et al., 2008; Lovallo, et al., 2012), ALEs were summed and subsequently split on the median in order to form two groups (0-3 ALEs: x =0, 4-12 ALEs: x=1).

Perceived stress

Self-reported perceived stress (PS; Dieleman, et al., 2010) was assessed after the rest period and after each of the tasks, prior to collection of Cort5-8 and -10 (see Figure 6.1). Seven questions (e.g. *Can you feel your heart beating? Are you nervous?*) were answered using a visual thermometer ranging from 0 (not at all) to 8 (very much). The scores were summed into a total score of PS for each period/task. Cronbach's alphas for the scale at each period in this sample ranged between .60 and .77.

Potential covariates

In previous studies examining cortisol levels, age (Gunnar, et al., 2009), sex (Kudielka & Wüst, 2010), ethnicity (DeSantis, et al., 2007), body mass index (BMI), oral contraceptive

(OC) use (Kirschbaum, et al., 1999), menstrual phase (Bouma, et al., 2009), SES (Sorocco, et al., 2006), urbanicity (Lederbogen, et al., 2011), and test-day factors such as season (i.e. time of year that the session took place; Rosmalen, et al., 2005), time (of day) of the test session (Bouma, et al., 2009), time of awakening, caffeine intake (Pincomb, Lovallo, Passey, Brackett, & Wilson, 1987), physical exercise (Budde, Windisch, Kudielka, & Voelcker-Rehage, 2010) and medication use (Kudielka, et al., 2009) have been taken into account as covariates. Because siblings were included in the CPSUD sample (N=33 participants had at least one sibling in the study), and due to the heritability of cortisol levels (Wust, et al., 2004), case dependency (sibling in study: x=0; no sibling: x=1) was also examined as a potential covariate. SES was based on the higher occupational level of either parent (Statistics, 2010) and coded into low (x=0), average (x=1) or high (x=2). Age, sex (boy: x=0; girl: x=1), ethnicity (of Dutch origin, x=0; of non-Dutch origin, x=1) and OC use (no use: x=0; use: x=1) were assessed using a demographics self-report questionnaire. Height and weight were measured prior to the test session and used to calculate BMI. Urbanicity was based on the population rate of the home city/town of the participant at the time of the test session, coded as rural (x=0), town (>10,000 inhabitants; x=1) or urban (>100,000 inhabitants; x=2; Lederbogen, et al., 2011). Population statistics were based on online national archives (Statistics, 2011). Season was coded as spring (March-May, x=0), summer (June-August, x=1), autumn (September-November, x=2) or winter (December-February, x=3). Time of test session was coded as morning (x=0), noon (x=1), late afternoon (x=2) or evening (x=3). Time awake/test difference was calculated as the difference in minutes between waking on the day of the test session and the start of the test session. Other test-day factors were self-reported immediately prior to the test session (coded as no coffee/coca cola intake/physical exercise/medication use: x=0, use: x=1).

Statistical analysis

In order to confirm that the stressful tasks were perceived as stressful, first a manipulation check was performed by way of repeated measures analysis of variance (ANOVA) in the entire sample, examining differences in PS after each of the stressful tasks compared to the pre-task rest period. For the main analysis, two two-level piecewise mixed effects models were fit for diurnal and stress-evoked cortisol levels in statistical package R (RDCT, 2012) using the 'lmer' function from the lme4 package (Bates, Maechler, & Bolker, 2011). Piecewise models were estimated due to the nonlinear nature of cortisol levels across the time

periods. Visual inspection of the individual plots of the cortisol response curve across the periods determined at which time point the slope would be split (according to Llabre, Spitzer, Saab, & Schneiderman, 2001). The intercept was set at the first measurement of each day in order to examine differences upon awakening on a normal day, and at onset of the stress procedure on the test day. At level 1, within-individual differences in cortisol measures across the day were controlled. At level 2, we added between-individual predictors of cortisol levels. Between models, fit was compared using log likelihood ratio tests.

In the first step, we fitted models (one for diurnal and one for stress-evoked cortisol) containing the intercept, slope and (if necessary) quadratic slope regressed on cortisol measurements. Secondly, we univariately tested the influence of each potential covariate on these models. We then added all significant covariates into each model and subsequently backwards step-wise excluded them until only significant covariates remained in the final model. Because *p* values are not estimated in lmer, estimates with *t* values greater than 2 were considered significant. Thirdly, group (CPSUDs, controls) was added to the model as an explanatory variable. Both the main effect of group (effect of group on the intercept) and interactions with period (effect of group on slopes) were added. Fourthly, ALE was added to the model, along with group by ALE and group by ALE by period interactions. Period was coded taking into account the time interval between measurements.

For the secondary analysis of group and ALE effects on PS, we followed the same procedure as described above, fitting a model for PS. All models were fit with Maximum Likelihood estimation, all continuous variables were centered and categorical variables were coded beginning with x=0 to facilitate interpretation.

RESULTS

Manipulation check

Repeated measures ANOVAs showed that two of the tasks were perceived as stressful compared to the pre-task rest period, as evidenced by significant main effects in the entire sample for both the mental arithmetic task ($F(1, 329)=16.49$, $p<.001$) and the public speaking task ($F(1,330)=39.78$, $p<.001$), but not the computer task ($F(1,329)=0.668$, $p=.414$).

Preliminary analyses

See Table 6.2 for descriptive statistics for all variables. Participants reported between 0 and 12 ALEs. Sixty two (74.7%) controls reported a low number of ALEs (0-3 ALEs), 21 (25.3%) a high number of ALEs. In CPSUDs, 42 (50.6%) reported a low and 41 (49.4%) a high number of ALEs, which was a significant difference (χ^2=10.30, p<.01).

Diurnal cortisol levels

Individual plots showed a strong increase in cortisol from the first measurement to the second (cortisol awakening response), and a subsequent decrease from the second measurement through the last. Therefore, the model included a fixed slope defined by the first two time points (Cort1-2; AwakeSlope) and a fixed slope defined by the last three time points (Cort2-4; DaySlope). Omission of the random effect for slope led to a significantly worse model, therefore we retained this term in the model, which supported the examination of between-individual predictors. See Table 6.3 for parameter estimates of the basic and final models.

We then examined potential covariates that could influence cortisol levels. Case dependency, age, sex and SES remained significant in the model. In order to examine potential effects of menstrual cycle phase and OC use, we reran the model again in girls only. Neither variable remained significant.

Next, we added group variables to the model. Group did not influence the intercept or either of the slopes significantly, and inclusion of these variables did not lead to a significantly improved model (χ^2(4)=4.14, p=.39). Thus, CPSUDs did not differ from controls on diurnal cortisol levels.

In the final step, ALE[1] variables were included. ALE did not influence the cortisol intercept or slopes, and the model was not significantly improved (χ^2(8)=8.33, p=.40). Interactions between group and ALE were also non-significant.

[1] An additional analysis on ALE was conducted which included ten additional less severe life events (e.g. Have you ever moved to a different city?) that were coded by the adolescent as at least slightly adverse. This did not change the results for either diurnal, stress-evoked cortisol or PS (data available upon request).

Table 6.2. Descriptive statistics for each familial risk status group

	Control		**CPSUD**		**Group differences**
	N	Mean (SD) or frequency (%)	N	Mean (SD) or frequency (%)	χ^2 or *t*
Cort1	73	3.71 (0.85)	67	3.52 (0.89)	1.28
Cort2	73	4.23 (0.99)	66	4.02 (1.07)	1.20
Cort3	72	2.66 (0.72)	61	2.46 (0.88)	1.40
Cort4	73	1.94 (0.75)	56	1.56 (0.58)	3.12**
Cort5	81	2.87 (0.71)	82	2.12 (0.60)	7.31***
Cort6	83	2.78 (0.70)	80	2.05 (0.63)	7.00***
Cort7	83	2.76 (0.66)	81	2.18 (0.81)	5.10***
Cort8	83	2.80 (0.70)	80	2.12 (0.77)	5.86***
Cort9	81	2.55 (0.64)	78	1.99 (0.73)	5.14***
Cort10	83	2.43 (0.63)	82	1.90 (0.63)	5.37***
Adverse life events median split	83		83		10.30**
Low (0-3)		74.7		50.6	
High (4 or more)		25.3		49.4	
Age	83	16.07 (2.49)	83	16.10 (2.46)	-0.06
Sex (boys/girls)	83	49/51	83	49/51	0.00
Ethnicity (Dutch/non-Dutch origin)	81	85/15	82	92/8	1.56
Body mass index	83	21.42 (3.93)	76	20.58 (3.41)	1.44
MC phase (follicular/luteal)	25	52/48	23	65/35	0.86
Oral contraceptive use (no use/use)	36	64/36	36	61/39	0.06
Case dependence (ind./dep.)	83	100/0	83	60/40	41.19***
SES (low/average/high)	81	4/71/25	74	3/73/24	0.13
Urbanicity (rural/town/urban)	83	18/52/30	83	15/55/30	0.43
Perceived stress response	83	6.89 (6.76)	83	5.57 (5.36)	1.40
Adverse life events sum	83	2.30 (1.74)	83	3.86 (2.36)	-4.83***

Table 6.2 continued.

	Control		**CPSUD**		**Group differences**
	N	Mean (SD) or frequency (%)	N	Mean (SD) or frequency (%)	χ^2 or *t*
Season (spring/summer/fall/winter)	83	23/31/18/28	83	22/20/28/30	3.68
TTS (morn./noon/afternoon/evening)	82	0/67/33/0	83	2/24/51/23	40.59***
Difference between waking on test day and start of test session (min)	82	328.60 (118.01)	83	425.71 (194.40)	-3.91***
Test day physical exercise (no/yes)	81	93/7	83	88/12	1.70
Test day dairy products (no use/use)	83	54/46	83	47/53	0.87
Test day coca-cola (no use/use)	83	93/7	83	85/15	2.24
Test day coffee (no use/use)	83	99/1	83	95/5	1.86
Medicine use past year (no use/use)	83	81/19	83	98/2	12.21***

*p<.05; **p<.01; ***p<.001

Note: all cortisol values are root transformed; MC=menstrual cycle; ind.=independent; dep.=dependent; SES=socio-economic status; TTS=time of test session; morn.=morning

Table 6.3. Fixed effects estimates for basic and final Diurnal cortisol models

	Diurnal cortisol basic		**Diurnal cortisol final**	
N level 1	541		521	
N level 2	143		137	
Fixed effects	Estimate (SE)	T	Estimate (SE)	T
Intercept	**3.61 (0.08)**	**46.65**	**3.64 (0.42)**	**8.57**
Case dependence			0.23 (0.13)	1.77
Age			**-0.06 (0.03)**	**-2.34**
Sex			0.16 (0.08)	1.9
SES (average)			-0.29 (0.40)	-0.73
SES (high)			-0.14 (0.42)	-0.34
Group			0.00 (0.20)	0.02
ALE			-0.03 (0.24)	-0.14
Group x ALE			-0.19 (0.32)	-0.60
AwakeSlope	**0.51 (0.09)**	**5.81**	-0.57 (0.44)	-1.29
SES (average)			**0.98 (0.45)**	**2.19**
SES (high)			**1.09 (0.47)**	**2.33**
Group			0.12 (0.22)	0.57
ALE			0.44 (0.27)	1.63
Group x ALE			-0.46 (0.37)	-1.26
DaySlope	**-0.22 (0.01)**	**-14.80**	-0.05 (0.07)	-0.75
Age			**0.02 (0.01)**	**3.27**
SES (average)			**-0.17 (0.07)**	**-2.35**
SES (high)			**-0.17 (0.08)**	**-2.14**
Group			0.01 (0.04)	-0.33
ALE			-0.04 (0.04)	-0.88
Group x ALE			0.09 (0.06)	1.43
$DaySlope^2$	**0.00 (0.00)**	**9.06**	-0.00 (0.00)	-0.02

Table 6.3 continued.

	Diurnal cortisol basic		**Diurnal cortisol final**	
N level 1	541		521	
N level 2	143		137	
Fixed effects	Estimate (SE)	T	Estimate (SE)	T
Age			**-0.00 (0.00)**	**-2.98**
SES (average)			**0.01 (0.00)**	**2.17**
SES (high)			0.01 (0.00)	1.77
Group			-0.00 (0.00)	-0.11
ALE			0.00 (0.00)	0.32
Group x ALE			-0.00 (0.00)	-1.06

Note. **Bold** estimates are considered significant (t>2)

Stress-evoked cortisol levels

Individual plots showed the general pattern of cortisol being slightly lower at the second measurement (Cort6) as compared to the first (Cort5). This was expected because the first most likely reflects anticipatory stress to a greater degree. We therefore excluded the first measurement and used Cort6-10 to assess stress-evoked cortisol levels. Cortisol levels remained elevated during the next two measurement points (Cort7-8; mental arithmetic and public speaking tasks). Cortisol levels then generally began to drop markedly, already during the last (computer) task, and further towards the last measurement. Due to this pattern, coupled with the finding that the computer task did not induce a significant increase in PS relative to the pre-task rest period (see above), we split the cortisol response curve at Cort8. Thus, we estimated a ReactivitySlope (Cort6-8) and a RecoverySlope (Cort8-10), which were both fixed effects, and included a random effect for the entire slope (TestSlope). Table 6.4 shows the parameter estimates for all Stress cortisol and Perceived stress models.

In the second step, covariates were added to the model. In this model, case dependency, age, sex, season, physical exercise on the testing day and the difference in minutes between awaking and the start of the test session were significant covariates. Again, we ran the model in girls only in order to examine the effects of menstrual cycle phase and

OC use. The interaction between OC use and the recovery slope was significant; cortisol levels of girls who used OCs decreased less strongly during the recovery phase.

Group variables were then added, which significantly improved the model ($\chi^2(3)=18.72$, $p<.01$). The effect of group on the intercept was significant, with CPSUDs exhibiting lower cortisol levels at onset of the experiment than controls. CPSUDs did not differ in cortisol slopes during the reactivity and recovery phases. In girls only, the effect of group was not altered. See Figure 6.2 for an illustration of cortisol values across the experimental procedure.

In the final step, ALE variables were included. None of these were significant, and the model was not significantly improved ($\chi^2(6)=5.73$, $p=.45$), indicating that ALE did not influence cortisol levels or the relation between group and cortisol. The group effect on the cortisol intercept remained significant in the model. In girls only, the results remained unaltered.

As a post hoc test, we examined how high cortisol levels at onset of the experiment were relative to diurnal cortisol levels. We subtracted Cort3 (assessed in the afternoon on a normal day) from Cort6 (first cortisol assessment used in the analyses). This difference score differed significantly between groups ($F(1, 127)=8.73$, $p<.01$) when controlling for time of test session. The difference was positive in controls and negative in CPSUDs (means were 0.13 versus -0.39), indicating that cortisol levels were slightly higher in controls at onset of the experiment as compared to on a normal day and lower in CPSUDs.

Perceived stress

PS generally increased from the first through third measurements (pre-task rest through public speak task), followed by a decrease from the third through fifth measurements (computer task and post-task rest). Therefore, we split the curve at PS3 to obtain a ReactivitySlopePS (PS1-PS3) and a RecoverySlopePS (PS3-PS5), which were both fixed effects, and included random effects for both the intercept and the entire slope. In the second step, the influence of covariates (case dependency, age, sex, SES, urbanicity) was examined, but none of them influenced PS significantly.

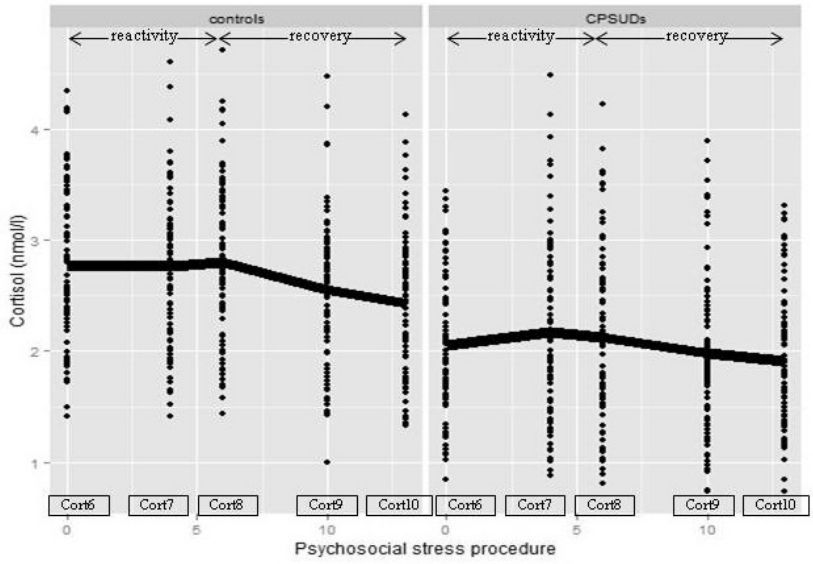

Figure 6.2. Cortisol across the stress procedure for controls and CPSUDs

Group variables were then added to the model, which led to a significantly improved model ($\chi^2(3)=9.04$, $p<.05$). The main effect of group on the intercept was significant, indicating that CPSUDs perceived the session as less stressful at onset of the experiment. Interaction effects between group and slopes were not significant. We then added ALE variables to the model. This did not significantly improve the model ($\chi^2(6)=7.49$, $p=.28$), nor were any of the ALE variables significant in the model.

DISCUSSION

The present study investigated whether HPA axis activity could be a vulnerability marker for SUDs. For this purpose we examined diurnal and stress-evoked cortisol levels in children of parents with a substance use disorder (CPSUDs) and matched controls. We found that diurnal cortisol levels did not differ between groups. In response to a psychosocial stressor, only cortisol levels at onset of the experiment were lower in CPSUDs compared to controls. Neither the reactivity slope, indicating the rise in cortisol in response to the first tasks, nor the

recovery slope, indicating the decrease in cortisol after cessation of the stressful tasks, differed between CPSUDs and controls. Although CPSUDs reported experiencing significantly more adverse life events (ALEs; 25.3% in controls reported a high number versus 49.4% in CPSUDs), this did not influence cortisol levels or the relation between group and cortisol levels.

Most importantly, we found that cortisol levels were lower in CPSUDs at onset of the psychosocial stress procedure in our study. This is in line with previous studies showing lower cortisol levels in CPSUDs in anticipation of a novel situation (e.g. Hardie, et al., 2002; Moss, et al., 1999). The current study was the first to examine diurnal and stress-evoked cortisol levels in adolescents from a broad age range, including boys and girls, and furthermore the first to include multiple cortisol measurements across both a normal day and an experimental procedure in an adolescent sample. We found no differences regarding reactivity and recovery patterns in adolescent CPSUDs versus matched controls. This is in contrast to previous research, albeit in adults, where blunted cortisol levels in response to psychosocial stress were found in CPSUDs compared to controls (e.g. Sorocco, et al., 2006).

Our finding of diurnal cortisol levels being similar in CPSUDs and controls confirms previous research in adults (e.g. Sorocco, et al., 2006). It seems to be a tenable conclusion that the diurnal rhythm of the HPA axis is not dysregulated in CPSUDs. As underlined by previous research as well as our own, cortisol levels evoked by anticipation of, or during, psychosocial stress may be a more important indicator of vulnerability to SUDs.

It seems reasonable that our observation of lower cortisol levels at onset of the experiment reflect *anticipation of stress* as opposed to being a true baseline measure. First of all, a baseline measurement of cortisol should reflect normal, resting cortisol levels. This is perhaps best examined on a normal day, in the usual environment and during the usual daily activities of the subject (measured by diurnal cortisol levels in this study). In order to obtain a reliable baseline measurement prior to an experimental procedure, it is important that there is sufficient time for the HPA axis to recover from anticipatory stress caused by the impending tasks, as anticipation may blunt subsequent cortisol responses (Nicolson, 2008). We excluded the first cortisol measurement assessed during the experiment in order to minimize anticipation effects (both the first and second cortisol measurements reflect levels when subjects were filling in questionnaires prior to the stressful tasks), however, as the pre-task period lasted only about 25 minutes, this was perhaps insufficient time for cortisol levels to

return to resting levels. To obtain more information on this possibility, we post hoc examined the relation between the second cortisol measurement (Cort6, first measurement used in the analysis, in almost all cases in the afternoon) and the cortisol measurement taken on a normal day in the afternoon (Cort3). Controlling for the time of day that the experiment took place, this difference score varied significantly by group, indicating that cortisol levels were slightly higher in controls at onset of the experiment as compared to on a normal day and lower in CPSUDs. Thus, possibly, an anticipation response was blunted in CPSUDs. Previous studies on cortisol responses to psychosocial stress have noted strongest between-group effects in anticipation of upcoming stress (e.g. Sumter, et al., 2010). Nevertheless, we did observe an increase in cortisol levels across groups in response to the tasks after inclusion of covariates (this allowed more power due to more explained variance in the model), which did not differ between CPSUDs and controls. All in all, our results revealed lower cortisol levels in CPSUDs compared to controls in anticipation of stress, which may reflect an underlying dysregulation of the HPA axis in these adolescents.

Though not yet clear what physiological activation in anticipation of stress signals, it may form the normative adolescent HPA axis response to stress, possibly indicating cognitive preparation for an upcoming stressor. This response could be related to the development of executive functions (i.e. planning, cognitive flexibility) which increases during adolescence (Davidson, et al., 2006). Dysregulation of this response may denote developmental delays in CPSUDs in cognitive and/or physiological functioning, and limited ability to prepare for an upcoming stressor.

Interestingly, blunted cortisol levels in anticipation to stress were mirrored in blunted PS levels in anticipation of the stressor in CPSUDs compared to controls. In the few studies that examined this previously, results were equivocal. Two studies (Uhart, et al., 2006; Zimmermann, et al., 2004) reported higher PS during the stressor in CPSUDs compared to controls, while another reported no difference (Sorocco, et al., 2006). Thus far, it is not entirely clear what the relation is between CPSUD status and PS during an experimental stressor, yet the current findings suggest that lower levels of objectively assessed stress (i.e. cortisol) are also reflected in lower levels of subjective stress (PS).

Table 6.4. Fixed effects estimates for basic and final Stress cortisol and Perceived stress models

	Stress cortisol basic		**Stress cortisol final**		**Perceived stress basic**		**Perceived stress final**	
N level 1	814		799		828		828	
N level 2	166		163		166		163	
Fixed effects	Estimate (SE)	T	Estimate (SE)	T	Estimate (SE)	T	Estimate (SE)	T
Intercept	**2.43 (0.06)**	**39.27**	**3.03 (0.22)**	**13.52**	**2.40 (0.10)**	**24.57**	**2.66 (0.16)**	**17.15**
Case dependence			0.16 (0.14)	1.13				
Age			**0.07 (0.02)**	**3.18**				
Sex			-0.01 (0.10)	-0.05				
Season (summer)			0.04 (0.15)	0.24				
Season (autumn)			-0.12 (0.16)	-0.76				
Season (winter)			-0.07 (0.15)	-0.45				
TD sport			-0.12 (0.19)	-0.64				
Diff awake/test			**-0.00 (0.00)**	**-3.41**				
Group			**-0.64 (0.14)**	**-4.47**			**-0.79 (0.24)**	**-3.24**
ALE			-0.07 (0.17)	-0.43			-0.09 (0.31)	-0.30
Group x ALE			0.28 (0.22)	1.26			-0.67 (0.41)	1.63
ReactivitySlope	0.01(0.01)	1.47	**0.06 (0.01)**	**4.03**	**0.07 (0.01)**	**5.66**	**0.07 (0.02)**	**3.74**

Age			**-0.01 (0.00)**	**-3.58**				
Sex			**-0.03 (0.01)**	**-3.09**				
Season (summer)			**-0.05 (0.02)**	**-3.25**				
Season (autumm)			**-0.07 (0.02)**	**-4.27**				
Season (winter)			**-0.05 (0.02)**	**-2.95**				
TD sport			**0.04 (0.02)**	**2.14**				
Group			0.03 (0.01)	1.90			0.01 (0.03)	0.33
ALE			-0.01 (0.02)	-0.68			-0.04 (0.04)	-1.13
Group x ALE			-0.01 (0.02)	-0.59			0.03 (0.05)	-0.48
RecoverySlope	**-0.05 (0.00)**	**-9.37**	**-0.10 (0.01)**	**-7.81**	**-0.18 (0.01)**	**-16.75**	**-0.21 (0.02)**	**-11.88**
Sex			**0.04 (0.01)**	**3.79**				
Season (summer)			**0.04 (0.01)**	**3.09**				
Season (autumn)			**0.06 (0.01)**	**4.02**				
Season (winter)			**0.03 (0.01)**	**2.21**				
Group			0.01 (0.01)	0.79			0.04 (0.03)	1.43
ALE			-0.01 (0.02)	-0.84			0.04 (0.03)	1.24
Group x ALE			0.01 (0.02)	0.38			-0.03 (0.05)	-0.70

Note. **Bold** estimates are considered significant (t>2)

Due to indications from previous research that CPSUDs often report experiencing more ALEs (e.g. Hussong, et al., 2008; Johnson & Leff, 1999), and that early adverse experiences are related to blunted cortisol responses (e.g. Gunnar & Vazquez, 2001), we examined whether number of ALEs was related to cortisol levels in CPSUDs and in controls. As expected, CPSUDs reported having experienced significantly more ALEs, however number of ALEs was not related to cortisol levels, nor did it influence the relation between group and cortisol levels. There is tentative evidence that the HPA axis, ALEs and vulnerability to SUDs maybe interrelated (i.e. Schepis, Rao, Yadav, & Adinoff, 2011), however, it does not appear that the relation between CPSUD status and blunted HPA axis activity is accounted for by having experienced more environmental stress. Our findings corroborated those of Hardie et al. (2002) in which family disruption in CPSUDs and controls was not related to cortisol levels. Furthermore, an earlier study found that paternal alcoholism was a more important risk factor for alcohol use than environmental stressors (Chassin, et al., 1991). Thus, so far, it does not appear that early environmental adversity is the mechanism behind HPA axis dysregulation in CPSUDs.

What then, are potential mechanisms underlying this HPA axis dysregulation in CPSUDs? In our sample, about 47% of the patients were mothers diagnosed with SUDs, and these mothers may have been more likely to have used substances during pregnancy, which is known to affect the developing HPA axis (Huizink & Mulder, 2006). However, only six mothers reported having used substances during pregnancy, so this does not seem to be a likely explanation, at least, in our sample. Another possibility could be that mothers experienced a higher level of stress during pregnancy, which is also known to affect the developing HPA axis (Huizink, et al., 2004). Due to the known effect of stress on the development of SUDs (Sinha, 2008), it is plausible that the mothers of our participants experienced higher levels of stress at earlier points in their lives, which may have also occurred during pregnancy. We do bear in mind, though, that about half of our participants were the offspring of *fathers* with SUDs, and therefore do not expect that these pregnancy factors are the only relevant factors.

A more likely possibility is that parental factors influence HPA axis development in children, specifically parental abuse and neglect, which are substantially more common in CPSUDs (Ammerman, Kolko, Kirisci, Blackson, & Dawes, 1999), and have been shown to be related to HPA axis dysregulation in children (e.g. Gunnar & Donzella, 2002). In an exploratory

analysis, we examined the influence of adolescent-perceived parental rejection on diurnal and stress-evoked cortisol levels, but found this not to be a significantly influencing factor, though CPSUDs reported experiencing more rejection than controls (data available upon request). As we did not assess specifically parental abuse and neglect, perhaps being more severe than rejection, this remains a viable underlying mechanism, for which subsequent investigations will be necessary. Another likely mechanism is the genetic transmission of HPA axis dysregulation. At this phase in our study, we were unable to examine this possibility, but hope to do so in the future.

A final potential mechanism is that CPSUDs, themselves more vulnerable to risky substance use (Chassin, et al., 1991), used more substances than controls which led to HPA axis dysregulation. In patients and heavy users, research has shown that smoking and other substance use is related to altered stress-evoked cortisol levels (e.g. Lovallo, et al., 2000; Richards, et al., 2011). In the present study, substance use was strongly related to group in our sample, as could be expected (CPSUDs were more likely to have used substances). Therefore, in the present study, we were unable to disentangle group effects from potential effects of current substance use. It should be noted though, that adolescents, as in our sample, have not used substances as chronically or heavily as adults have in studies of patients and heavy users. We therefore do not expect substance use to have played a major role in HPA axis dysregulation, though such effects have been largely unstudied in CPSUDs.

This study should be evaluated in light of the following. First, pubertal stage may influence cortisol levels, beyond the effects of age. Although information on pubertal stage was available for most of our participants, it was not included at the beginning of the data collection phase, and therefore was structurally missing in the younger control participants. Because of this, we were unable to include it as a covariate in our models. Second, we were unable to standardize the time of day at which the experimental sessions took place. Planning all sessions at the same time of day, preferably in the afternoon, is ideal in cortisol studies, but was unfortunately not feasible in the current study, due to difficulty in recruiting CPSUD participants. Third, the tasks were not counterbalanced in our experimental session. Fourth, we included adolescents from a large age range. Due to the small sample size, we were unable to control for developmental effects, aside from including age as a covariate, which most likely play a role at these ages. Fifth,

as mentioned in the paragraph above, we did not examine potential influences of substance use on cortisol levels, due to a significant difference in substance use between groups.

An important final limitation is that we were unable to exclude CPSUDs whose parents had been diagnosed with psychopathology other than a SUD. For some parents such information was known (see Table 6.1), however, for many it was not. Due to this, and in light of a fairly large percentage of parents having other axis 1 disorder diagnoses, which is expected in SUD patients, we were unable to control for this factor in our analyses. Because the offspring of patients with other axis 1 disorders have also been reported to exhibit HPA axis dysregulation (e.g. Duffy, Lewitzka, Doucette, Andreazza, & Grof, 2012; Ellenbogen, Hodgins, Walker, Couture, & Adam, 2006), we do not know from the present study whether the difference in stress-evoked cortisol levels in CPSUDs as compared to controls is specific to CPSUDs, or a more general effect that is found in the offspring of parents with any psychopathology.

This study examined HPA axis activity as a potential vulnerability marker for SUDs. Implementing multilevel modeling, we examined diurnal and stress-evoked cortisol levels in CPSUDs and matched controls. Cortisol levels at onset of a psychosocial stress procedure, reflecting anticipatory stress, were lower in CPSUDs as compared to controls. Groups did not differ regarding diurnal cortisol levels, and reactivity and recovery slopes. Importantly, CPSUDs had experienced more adverse life events, but this did not influence cortisol levels. Our findings indicate that dysregulated HPA axis activity, but only in anticipation of stress, may reflect an inborn vulnerability to the development of SUDs.

ACKNOWLEDGEMENTS

The JOiN study is conducted by the Erasmus Medical Center, department of Child and Adolescent Psychiatry, and the Erasmus University, department of Clinical Psychology, in close collaboration with Bouman GGZ. We gratefully acknowledge the contribution of Ben van de Wetering, director of Bouman GGZ and treatment coordinators Jeannette Verveen, Piet Visser, Jannie Wolfert, Bob Pruissers, Daphne Kanner, Peter Tielemans, Peter Lever, and Michiel Boog

for their efforts to recruit CPSUDs. We are furthermore grateful to all participants of the JOiN Study and their parents. This project is supported by ZonMw grant 3116.0002.