Roots of Anxiety

The role of cardiovascular regulation and cortisol in the development of anxiety in early adolescence

Kirstin Greaves-Lord

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Roots of Anxiety

The role of cardiovascular regulation and cortisol in the development of anxiety in early adolecence

de Oorsprong van Angst

De rol van cardiovasculaire regulatie en cortisol in de ontwikkeling van angst gedurende de vroege adolescentie

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You live you learn
You love you learn
You cry you learn
You lose you learn
You bleed you learn
You scream you learn

You grieve you learn
You choke you learn
You laugh you learn
You choose you learn
You pray you learn
You ask you learn
You live you learn

Alanis Morissette

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1 General Introduction

Kirstin Greaves-Lord

Background

Anxiety problems in childhood and adolescence are common (Treffers and Öst, 2001; Verhulst et al., 1997), tend to persist across time (Ferdinand et al., 1999; Ferdinand and Verhulst, 1995; Pollack et al., 1996), and constitute a risk factor for future depression (Wittchen et al., 2000; Woodward and Fergusson, 2001). Since anxiety problems are -together with depression- the most frequent mental health problems in adulthood that put forth an enormous burden both in human and economic terms, it is important to develop and improve prevention and intervention programs. However, to develop and improve such programs, we first have to deepen our understanding of underlying aetiological mechanisms.

Increasing evidence underscores the importance of the role of physiological stress regulation in the aetiology of anxiety (Boyce et al., 2001; Dietrich et al., 2006; Feder et al., 2004; Gerra et al., 2000; Goenjian et al., 2003; Kagan et al., 1988; Mezzacappa et al., 1997; Smider et al., 2002). The functioning of two major physiological stress response systems; the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA)-axis has been associated with anxiety (Figure 1.1). To gain more insight in the exact role of these two physiological stress response systems in the aetiology of anxiety, individuals with clinical anxiety disorders and individuals at high risk for anxiety problems are examined at our department (Kallen, Dieleman, Diercks, Van der Vegt, personal communication). Yet, it is also important to examine the relation between ANS and HPA-axis functioning and anxiety levels of individuals from the general population, to gain more insight in the underlying vulnerability for future anxiety problems in healthy individuals. Therefore, the present study was conducted.

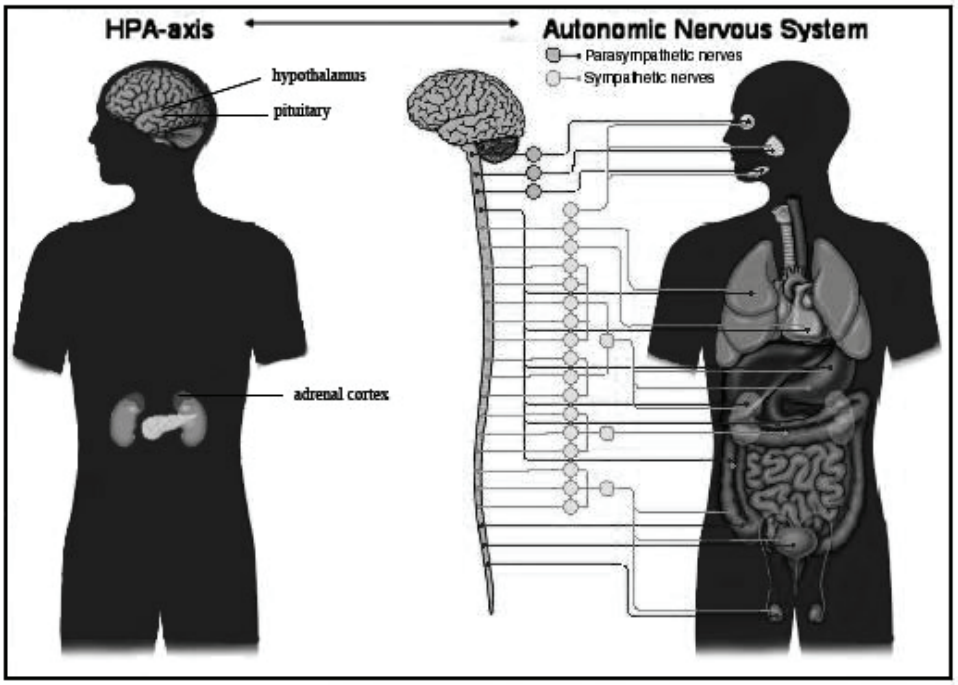
In this introduction, firstly ANS functioning, HPA-axis functioning, and the interaction between both systems will be described. Secondly, theories on the relation between these physiological stress systems and anxiety will be discussed. Thirdly, the aim of this thesis and the research questions will be formulated. Lastly, the sample and methods will be described, and the outline of this thesis will be given.

The Autonomic Nervous System (ANS)

Function

The ANS (Figure 1.1) is a physiological system that is involved in the quick response to stressors. It is part of the human nervous system and controls homeostasis, which means maintaining a stable, constant condition of the internal organs and other physiological regulation mechanisms. The ANS maintains homeostasis by controlling for instance cardiovascular, digestive, and respiratory functions, and salivation, perspiration, urination, and erection. This system is called 'autonomic' because for the most part it is not under conscious, voluntary control.

Figure 1.1: The Autonomic Nervous System and the HPA-axis



The ANS exists of two opposite, but complementary subsystems: the sympathetic and the parasympathetic system. In a metaphorical way, one may think of the sympathetic system as the accelerator, and of the parasympathetic system as the brake. The sympathetic system is sometimes called the 'fight and flight' system which refers to the quick response to stressors of this system. The sympathetic system is continuously active, but in response to stressful stimuli the activity comes to a maximum. The release of catecholamines (e.g. norepinephrine, NE) increases, heart rate (HR) and blood pressure (BP) increase, respiration accelerates, and in this way the body is prepared for action. The parasympathetic is sometimes called the 'rest and digest' system and mainly concerns the conservation of energy and maintenance of organ function during calm states. The parasympathetic system is constantly active, but activity also changes under stressful circumstances. This system can also be involved in the stress reaction; it 'pulls back', the 'brake' is removed, and the system retracts itself quickly to facilitate adaptation to environmental demands (Porges, 1995). Because parasympathetic activity reduces, HR and BP can increase. In this way, the body can prepare for action without the metabolical costs of sympathetic activation. The vagus nerve is an important part of the parasympathetic system, therefore this system is also referred to as the vagal system.

Measures

To gain insight in the activity of the ANS, one can assess measures of cardiovascular activity, such as HR and BP. Cardiovascular variability, particularly HR variability (HRV), is frequently analysed by means of power spectral analyses

to provide non-invasive estimates of sympathetic and parasympathetic regulation of the cardiovascular system (Akselrod et al., 1981). HRV in the high frequency band, usually around 0.15-0.40 Hz, is related to respiratory variations (respiratory sinus arrhythmia), and results from centrally mediated cardiac parasympathetic activity. HRV in the frequency domain around 0.10 Hz (low frequency band) reflects changes in sympathetic control, although an influence of parasympathetic activity has also been documented (Parati et al., 1995). Baroreflex sensitivity (BRS) is another well-known indicator of homeostatic cardiovascular regulation. The baroreflex is an important short-term BP control mechanism (Ketch, 2002). Pressure-sensitive baroreceptors, most of which are located in the aorta and carotid sinus, react on stretch or deformation of blood vessels associated with BP changes. As a result, HR changes to keep BP values between narrow limits. The index for BRS can be derived from measures of HRV and BP variability. This BRS index reflects both vagal and sympathetic influences (Dietrich et al., 2006).

The Hypothalamic-Pituitary-Adrenal (HPA)-axis

Function

The HPA-axis (Figure 1.1) is another important physiological system that is involved in the stress response, but this system is has a slower response to stressors. It is part of the human neuroendocrine system that regulates various bodily processes such as immunity and energy usage. The HPA-axis is constantly active, but if a stressful situation lasts longer than a few minutes, activity of the HPA-axis increases: the production of corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the hypothalamus (H=hypothalamus) increases, which in turn stimulates the release of adrenocorticotropin hormone (ACTH) in the anterior lobe of the pituitary (P=pituitary). As a consequence, cortisol secretion from the adrenal cortex (A=adrenal cortex) increases. Cortisol, a glucocorticoid, is the end-product of the HPA-axis, and is known to play a role in homeostasis. It stabilizes the body's internal environment during and after a stressful situation. Cortisol and ACTH in the bloodstream also activate the feedbackloop of the HPA-axis; the release of CRH is reduced when levels of cortisol and ACTH are high.

HPA-axis activity changes not only as a result of stress, it also fluctuates during the day, influenced by the sleep-wake cycle. In the morning, while waking up, cortisol levels rise quickly, reaching a peak after approximately half an hour (cortisol awaking response; Wust et al., 2000b, 2001). After this peak, cortisol levels begin to decrease, and continue to decrease during the day.

Measures

To assess an index for HPA-axis activity, one can measure cortisol levels. Cortisol can be assessed in either blood, urine or saliva. Collection of salivary cortisol is a relatively stress-free approach that avoids confounding by stress responses, e.g. as induced by venipuncture (Schmidt, 1997). According to several authors

correlations between saliva cortisol levels and serum cortisol concentrations are high (e.g. Kirschbaum and Hellhammer, 1989, 1994).

The interaction between the ANS and the HPA-axis

The HPA-axis and the sympathetic system are biologically intertwined (Stratakis and Chrousos, 1995). The NE and CRH producing components of the stress response systems connect at several brain sites. For instance, neurons that secrete CRH project from the lateral PVN in the hypothalamus to sympathetic hindbrain regions (Chrousos and Gold, 1992). Administration of CRH increases catecholaminergic activity and NE levels, and NE stimulates the release of CRH in the PVN (Brown et al., 1982; Dunn and Berridge, 1990). Further, cortisol has a complex and multilateral role in the immediate autonomic stress response. It probably first permits and stimulates the sympathetic stress response, and later supresses the ongoing stress response. Also, it might prepare the body for a putative subsequent stressor (Sapolsky et al., 2000). Since HPA-axis activity has such important effects on ANS activity, it possibly moderates the putative effects of ANS (re)activity on anxiety levels. Taken together, the ANS and the HPA-axis are physiologically interconnected, and may have joint effects on emotion and behaviour.

Theories on anxiety and ANS or HPA-axis activity

The (re)activity of the HPA-axis and the ANS possibly plays a role in the development of anxiety problems. A general belief is that anxious individuals are characterized by signs of hyperarousal (Clark and Watson, 1991, Table 1.1, line 1). Hyperarousal is a state of alertness and readiness to respond, involving activation of the ANS and HPA-axis which results in higher autonomic (re)activity and higher cortisol levels. According to Clark and Watson (1991) anxiety is associated with signs of hyperarousal, whereas depression is not associated with hyperarousal.

Table 1.1: Overview of theories on the association of ANS functioning and HPA-axis functioning with anxiety

Authors	Theories (propositions)
1 Clark and Watson (1991)	Hyperaurousal (i.e. higher ANS and HPA-axis (re)activity) ↔ anxiety disorders
2 Kagan et al. (1988)	Low threshold central nervous system activation → higher sympathetic and HPA-axis activation (hyperarousal) → withdrawal, avoidance, fearfulness → susceptibility to anxiety
3 Gunnar and Vazquez (2001)	Stressful events early in life → frequent elevations of cortisol → downregulation HPA-axis, low cortisol levels ↕ Stressful events early in life → increased risk anxiety (Goodyer and Altham,1991)
4 Porges et al. (2001)	Relatively low vagal (re)activity → increased risk physical and mental health problems, among which anxiety
5 Friedman and Thayer (1998)	Limited psychophysiological flexibility → relatively low heart rate variability ↔ anxiety disorders
6 Bauer et al. (2002)	Asymmetric activation patterns (HPA↑ + sympathetic↓/ HPA↓ + sympathetic↑) ↔ increased risk anxiety

More specifically, Kagan and colleagues (1988, Table 1.1, line 2) proposed that certain individuals might have an inborn tendency towards overarousal of the central nervous system (particularly the hypothalamus and the amygdala) due to a lower threshold for activation. As a consequence, reactivity of the HPA-axis and the sympathetic system is enhanced, resulting in elevated cortisol levels and elevated HR. These individuals compensate for this state of hyperarousal through withdrawal and avoidance of possible stressful situations, and they may become more fearful to end up in such situations. Withdrawn, avoidant, and fearful behaviours are characteristics of an inhibited temperament that has often been associated with anxiety (Rapee, 2002), and such characteristics may make an individual more susceptible to develop anxiety problems. Signs of relatively high HPA-axis and sympathetic (re)activity may therefore be associated with higher anxiety levels.

Gunnar and Vazquez (2001) hypothesized that stressful influences early in life may provoke frequent elevations in cortisol. These elevations would however eventually lead to down-regulation of components of the HPA-axis (Gunnar and Vazquez, 2001, Table 1.1, line 3). Based on their theory, and the assumption that stress in early life would also result in future anxiety (Goodyer and Altham, 1991; Horesh et al., 1997), one would expected that signs of relatively lower HPA-axis (re)activity may be associated with higher anxiety levels.

Porges and colleagues (Porges, 2001, Table 1.1, line 4) proposed the polyvagal theory. According to this theory, lowering vagal activity to a certain extent -so-called 'removal of the vagal brake'- provides the opportunity to easily adapt to environmental challenges without the severe biological cost of the metabolic excitation associated with sympathetic-adrenal activation. This adaptive mechanism is very functional for healthy individuals in stressful circumstances, but in some individuals the vagal brake may have been constantly less (re)active from birth, or may have become less (re)active during early development. These individuals might be more susceptible to many physical and mental health problems, among which anxiety problems. Hence, signs of relatively lower vagal (re)activity might be associated with higher anxiety levels.

Another belief concerning ANS reactivity is that anxiety is characterized by an ANS that lacks flexibility and adaptability (e.g. Friedman and Thayer, 1998a, Table 1.1, line 5). A rigid, non-dynamic ANS regulation may hinder the individual to adequately respond to a constantly changing environment. Thus, anxiety may be associated with signs of limited ANS flexibility.

Lastly, Bauer and colleagues (Bauer et al., 2002, Table 1.1, line 6) suggested that since the sympathetic system and the HPA-axis interact, they might be complementary in their influences on emotions and behaviour. Accordingly, optimal functioning is only possible when the activity of both systems is balanced, leading to an optimal medium level of arousal. The sympathetic system and the HPA-axis should show symmetric activation patterns to obtain an optimal level of arousal. If individuals show asymmetric activation patterns, for instance relatively

low sympathetic (re)activity together with relatively high HPA-axis (re)activity, they are at increased risk for either emotional or behavioural problems. Therefore, signs of asymmetric activation patterns of the ANS and HPA-axis might be associated with higher anxiety levels.

Aim & research questions

The aim of the present thesis is to extend the existing knowledge on the aetiology of anxiety. More specifically, the aim is to gain more insight in the role of the ANS and HPA-axis in the development of anxiety in early adolescence. The main research questions of this thesis are:

1. Is anxiety associated with signs of hyperarousal, whereas depression is not?
2. Are high cortisol levels associated with high anxiety levels? Is the persistence of anxiety problems associated with high cortisol levels?
3. Do measures of ANS (re)activity predict future anxiety levels? Are such associations different between boys and girls, and specific for anxiety, as apart from depression?
4. Do cortisol measures predict future anxiety levels? Are distinct developmental pathways of anxiety associated with different cortisol levels?
5. Do assymmetric activation patterns of ANS and HPA-axis activity predict future anxiety levels? Are such associations more evident in individuals with high familial vulnerability and different for boys and girls?

Sample & methods

The TRacking Adolescents' Individual Lives Survey (TRAILS) is a prospective cohort study of Dutch young adolescents initially aged 10-12 years, who are followed biennially, until the age of 24. The main objective of TRAILS is to chart and explain the development of physical and mental health problems at the level of underlying vulnerability and environmental risk factors. For the present thesis data from the first (2001-2002) and second (2003-2004) assessment waves were used. The target sample consisted of young adolescents from five municipalities in the North of the Netherlands, including both urban and rural areas. Of all individuals approached for participation in the study (n=3,145), 6.7% were excluded. The exclusion criteria were 1) an incapability to participate because of mental retardation or serious physical illness or handicap, and 2) no availability of a Dutch-speaking parent or parent surrogate, and no feasibility to administer a part of the measurements in the parent's own language. Of the remaining individuals (n=2,935), 76.0% participated in the study (n=2,230, mean age 11.09 years, SD .55, 50.8% girls). Participants did not differ from those who refused to participate with respect to the proportion of single parent families, the prevalence of teacher-rated problem behaviour, several socio-demographic variables, and mental health outcomes (de Winter et al., 2005). At the second assessment wave, information was obtained from 2,149 (96.4%) of those who participated at the

first assessment wave (mean age 13.56 years, SD .53, 51.0% girls). There was no selective attrition. The number of individuals that were included in analyses differs for the separate chapters of this thesis, depending on the measures that were used.

Outline

The role of the ANS and HPA-axis in the development of anxiety in early adolescence is investigated in several ways. In *Chapter 2* we investigate the cross-sectional association between measures of ANS activity and current anxiety versus depressive problems. In *Chapter 3* we examine the cross-sectional association between cortisol levels and current anxiety problems. The relation of cortisol levels and the persistency of anxiety -based on retrospective data- is also investigated in this chapter. In *Chapter 4* we test whether measures of ANS (re)activity predict anxiety levels two years later. Putative gender differences in these associations are investigated, and we test if associations are specific for anxiety, as apart from depression, or apply to the broader dimension of internalizing problems. The predictive value of cortisol measures for anxiety levels two years later is examined in *Chapter 5.* Possible gender differences, developmental pathways of anxiety, and specificity for anxiety are also looked into in this chapter. *Chapter 6* discusses the role of assymmetric activation patterns of ANS and HPA-axis reactivity in the development of anxiety. Interactions between measures of ANS reactivity and measures of HPA-axis reactivity are investigated as putative predictors for anxiety levels two years later. These associations are considered in individuals with low versus high familial vulnerability, and in boys versus girls, to gain more insight in the role of familial vulnerability and gender. Again the specificity for anxiety is examined by adjusting for co-occurring depressive problems. Finally, in *Chapter 7* the main findings and conclusions of chapters 2-6 are presented and discussed.

2 Testing the tripartite model in young adolescents: is hyperarousal specific for anxiety and not depression?

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Abstract

Background To clarify the distinction between anxiety and depression, the tripartite model was introduced. According to this model, physiological hyperarousal (PH, i.e. autonomic hyperactivity) is specific for anxiety and not depression. Research on the relation between anxiety, depression and physiological measures representing arousal is lacking.
Methods Parent- and self-reported anxiety and depressive problems were assessed using the CBCL and RCADS. Heart rate (HR), heart rate variability in the low frequency (HRV LF) and respiratory sinus arrythmia (RSA) were used as indices for autonomic arousal.
Results Parent-reported anxiety was associated with low RSA in supine posture. This association was also found for self-reported anxiety problems, but only in boys. These findings point towards high arousal in anxiety. Self-reported depressive problems were associated with low HRV LF in standing posture and high RSA in supine posture in boys, pointing towards low arousal in depression. However, self-reported depressive problems were also associated with high HR in standing posture and with low HRV LF in supine posture in girls, suggesting high arousal in depression.
Conclusions Some evidence was found for hyperarousal in anxiety, but also for hyperarousal in depression. Apparently, the idea of hyperarousal in anxiety and not in depression is too simple to reflect the more complex reality.

Introduction

Anxiety and depressive problems in childhood and adolescence occur frequently (Treffers, 2000; Verhulst et al., 1997), result in considerable suffering and impairment, and tend to persist (Ferdinand et al., 1999; Ferdinand and Verhulst, 1995; Pollack et al., 1996; Treffers and Öst, 2001). Therefore, it is important to investigate their aetiology, and to develop an adequate taxonomy, that can serve as a cornerstone for high quality assessment.

Since anxiety and depression often co-occur, it is doubted if the two represent distinct constructs (Angold et al., 1999; Axelson and Birmaher, 2001; Essau et al., 2000; Goodwin, 2002; Kessler et al., 1999; Stein et al., 2001). In an attempt to improve the taxonomy of anxiety and depression, Clark and Watson introduced the tripartite model (Clark and Watson, 1991). According to this model, anxiety and depression share negative affect (NA) as a common factor, whereas depression is specifically characterized by low levels of positive affect (PA), and anxiety by physiological hyperarousal (PH; i.e. autonomic hyperactivity, motor tension). Since the introduction of this model, it has been frequently used and tested, both in children as well as in adults, and it has become well known in the field of anxiety and depression research.

Empirical evidence for the usefulness and fit of the tripartite model was provided by studies that employed factor analyses (Chorpita, 2002; Laurent and Ettelson, 2001). While several studies investigated the association between anxiety or depression and NA or PA (Chorpita, 2002; Joiner et al., 1996; Laurent et al., 1999), fewer have investigated whether an association with PH was specific for anxiety and not for depression (Joiner et al., 1999; Laurent et al., 2004). In the studies that have investigated the association between PH and anxiety or depression, questionnaires were used to measure PH. However, the items that were used to measure PH tended to overlap with items tapping vegetative symptoms of anxiety, which might explain the associations that were found between PH and anxiety. In our opinion, physiological measures representing arousal would be more appropriate to measure PH (see also Laurent and Ettelson, 2001). To our knowledge, investigations aimed at testing the validity of the PH component of the tripartite model against physiological measures representing arousal, are lacking.

Physiological measures that give an impression of the activity of the autonomic nervous system (ANS) are highly related to arousal. In a state of high arousal, heart rate (HR) is high. HR is influenced by two competing autonomic branches. The sympathetic branch has the function of increasing HR. Hence high arousal is associated with high sympathetic activity. The parasympathetic/vagal branch takes care of decreasing HR. High arousal is therefore associated with low vagal activity. Thus, to assess levels of arousal, it is important to measure HR. In addition, it is important to obtain other estimates of ANS functioning to give an impression of the activity of the separate branches of the ANS.

Some studies have investigated the relation between anxiety and autonomic functioning in children or adolescents (Gerra et al., 2000; Kagan et al., 1988; Mezzacappa et al., 1997). All of these studies found associations between higher HR and higher levels of anxiety. However, these studies were confined to small, nonrepresentative samples. None of these studies focused on both anxiety and depression, so firm conclusions about the specific association between anxiety and autonomic measures representing arousal, in comparison to depression, could not be drawn.

In a previous study, we investigated the association between internalizing and externalizing problems and autonomic functioning in the TRacking Adolescents' Individual Lives Survey (TRAILS) general population sample (Dietrich et al., 2006). For instance, evidence was found for higher HR in individuals with internalizing problems (affective, anxiety and somatic problems taken together). In the present study, anxiety and depressive problems were investigated separately. The TRAILS study provided the opportunity to investigate associations with HR, but also with heart rate variability (HRV). HRV reflects changes in beat-to-beat variations in HR. HRV can be analyzed by means of spectral analysis, which portrays the variance in HR as a function of frequency. The frequency range can be divided into low frequency HRV (generally between 0.04 and 0.14 Hz), and high frequency HRV (above 0.14 Hz). Low frequency HRV (HRV LF) measured in standing position, is primarily sympathetically mediated and vagal effects are inhibited, whereas in the supine posture vagally mediated effects predominate. HRV in the high frequency band is often called respiratory sinus arrythmia (RSA), and is primarily vagally mediated (Mezzacappa et al., 1997). Therefore, based on the tripartite model, we expected anxiety problems to be associated with high HRV LF in standing posture and high HR in both postures. Further, we expected anxiety problems to be related to low HRV LF in supine posture and low RSA in both postures. No signs of hyperarousal were expected to be found in depression. Since the tripartite model does not further describe arousal in depression, it was unclear if we would expect no associations between depression and arousal at all, or even low arousal levels in depression.

In addition to our previous work that only concerned parent-reports, self-reported anxiety and depressive problems were investigated. Examining self-reported problems may be an important extension, since anxiety and depression are highly subjective, and some of the more unobservable symptoms are often under-reported by parents (Comer and Kendall, 2004).

In summary, the aim of the present study was to investigate the putative associations of both parent- and self-reported anxiety problems and depressive problems with physiological measures representing arousal (HR, HRV LF, and RSA) in a large population sample of young adolescents. Based on the tripartite model (Clark and Watson, 1991), we expected specific associations between these physiological measures representing arousal and anxiety, indicating hyperarousal in anxiety, but not in depression.

Methods

Sample and procedure

Participants were 10- to 13-year-old young adolescents who participated in the TRacking Adolescents' Individual Lives Survey (TRAILS), a large Dutch general population study (n=2,230). In the TRAILS study, not only young adolescents, but also their parents and their teachers participated. The young adolescents filled out questionnaires at school, in the classroom, under the supervision of one or more TRAILS assistants. In addition to that, a number of physiological and neurocognitive parameters, such as heart rate, were assessed in a separate room at school on another school day. Further, well-trained interviewers visited one of the parents or guardians (preferably the mother, 95.6%) at their homes to administer an interview covering a wide range of topics, for instance developmental history and somatic health. Besides the interview, the parent was asked to fill out some questionnaires concerning their child's mental health and behaviour. Teachers gave the opportunity to let the young adolescents participate during school hours and filled out a brief questionnaire for all TRAILS-children in their class. For the present manuscript, we included 1,027 boys and girls (47% vs 53%, mean age 11.0 years, SD=0.5) for whom reliable physiological measures could be computed.

To examine possible selective attrition, a stepwise regression analysis was performed. The 1,027 participants did not differ from the other 1,203 participants in the TRIALS study regarding gender, pubertal stage, Body Mass Index, and anxiety or depression scores. Age predicted attrition; older participants were less likely to take part in the physiological measurements. Nevertheless, the age difference (11.02 versus 11.15 years) and effect size (1.2%) were small. Written consent was obtained from the participant's parents. The study was approved by the Central Dutch Medical Ethics Committee. Detailed information about the TRAILS sample selection and characteristics has been reported elsewhere (de Winter et al., 2005).

Measures

Anxiety & depressive problems

Parent-reports (CBCL): The Child Behavior Checklist (CBCL) is a parent-report questionnaire for assessing problems in 6- to 18-year-olds and contains 120 items on behavioural or emotional problems in the past six months, that are scored on a 3-point scale (0 = not true, 1 = somewhat or sometimes true, 2 = very or often true). The good reliability and validity of the American version of the CBCL were confirmed for the Dutch translation (De Groot et al., 1994). The original empirical syndrome scales for the CBCL that were used in our previous study, were based on multivariate statistical analysis on data from large samples. To fit more closely with the clinical-diagnostic approach represented by the DSM (APA, 2006), six new DSM-IV scales were recently constructed: Affective Problems, Anxiety Problems, Somatic Problems, Attention Deficit/Hyperactivity Problems

(ADH), Oppositional Defiant Problems (OD), and Conduct Problems (CD) (Achenbach et al., 2003; Achenbach and Dumenci, 2001).

In the present study, we used the Anxiety Problems and Affective Problems scales to assess anxiety and depressive problems. The Anxiety Problems scale consists of 6 items that reflect symptoms of DSM-IV generalized anxiety disorder, separation anxiety disorder, and specific phobia. The Affective Problems scale consists of 13 items, and reflects symptoms of DSM-IV dysthymia and major depressive disorder. The Cronbach's alphas based on the present data set were .67 for the Anxiety Problems scale, and .66 for the Affective Problems scale. The ADH, OD and CD scales were summed and used as a measure of Disruptive Behaviour, to adjust for comorbidity.

Self-reports (RCADS): We assessed self-reported anxiety and depressive problems using the Revised Child Anxiety and Depression Scale (RCADS; Chorpita et al., 2000, 2005). The RCADS assesses anxiety and depressive symptoms thoroughly; it contains 47 items that are scored on a 4-point scale (0 = never, 1 = sometimes, 2 = often, 3 = always). The questionnaire covers six of the DSM-IV dimensions of anxiety disorders and depressive disorder: separation anxiety disorder, generalized anxiety disorder, social phobia, panic disorder, obsessive compulsive disorder, and major depressive disorder (MDD). In this study, a Total Anxiety scale was computed by summing the scores on the separate anxiety items and dividing this by the number of items that were completed. Similarly, a MDD scale was computed, and used as a measure of depressive problems. The Cronbach's alphas based on the present data set were .91 for the Total Anxiety scale, and .73 for the MDD scale.

Physiological measures

Heart rate (HR) measurements were performed in a quiet room at school, one child at a time. First, participants were asked to lie down. While supine, the procedure was explained to them. A three-lead electrocardiogram was applied to register HR. Participants were encouraged to relax and asked not to move or speak during data acquisition. Participants were in supine position for approximately 5 minutes before measurement began. Recordings did not start until signals had reached a stabilized steady-state. Then HR signals were registered for 4 minutes in supine position during spontaneous breathing, followed by 2 minutes in standing position, again after signals had stabilized. Recordings were digitized (sample rate 100Hz, using a DAS-12 data acquisition card for notebooks, Keithley Instruments, Cleveland, Ohio, USA) and stored on hard disk for off-line analyses. HR was calculated as 60,000 divided by the mean inter beat interval (IBI), expressed in beats per minute (bpm). Calculation of HRV LF and RSA was performed by spectral analysis in the CARSPAN software program using estimation techniques based on Fourier transformations (Robbe et al., 1987). The power spectrum for the frequency range was divided into low frequency (HRV LF; between 0.07 and 0.14 Hz), and high frequency (RSA; above 0.14 Hz). The

analyzed time series were checked and corrected for artefacts. More detailed information about these analysis and internal reliability of the data has been reported previously (Dietrich et al., 2006).

Statistical analyses

Descriptives were calculated for all anxiety, depression, and physiological measures. To approximate a normal distribution, HRV LF and RSA values were transformed to their natural logarithm. Linear regression analyses were performed with each physiological measure (HR, HRV LF, and RSA, both in supine and standing posture) as the dependent variable, and both CBCL Anxiety Problems and Affective Problems scales as predictors. In addition, gender and scores on Disruptive Behaviour (i.e. summed scores of the CBCL ADH, OD, and CD DSM-IV scales) were entered as covariates, to adjust for possible confounding effects of gender and comorbid externalizing problems. Finally, Gender * Anxiety Problems and Gender * Affective Problems interactions were added to the model. These interactions were only included in the final model if they yielded significant effects. Moreover, in case of a significant effect of one of these interaction terms, additional post hoc analyses were performed for boys and girls separately.

A similar set of analyses was conducted for the Total Anxiety and MDD scales of the RCADS. The RCADS does not assess disruptive behaviour problems. Therefore, the Disruptive Behaviour scores of the Youth Self-Report (YSR) were used as a covariate in these analyses. The YSR is a self-report questionnaire that was modeled on the CBCL and contains similar items.

Preliminary analyses indicated that there were no significant effects of Body Mass Index or pubertal stage on the physiological measures or the anxiety and depressive problems scores. Hence, these factors were not considered in the analyses. A p-value smaller than .05 was considered statistically significant. Since we performed several statistical tests, the results may suffer from capitalization on chance: one would expect some 5% of the associations examined to be significant merely on the basis of chance. Hence, a statistically significant result in this context does not have the same weight as significant results in a classical experimental design.

Results

Descriptives

Mean scores and standard deviations of all the anxiety, depression, and physiological measures are shown in Table 2.1:

Table 2.1: Descriptives of anxiety, depression, and physiological measures

Measures	**Boys**			**Girls**		
	n	Mean (SD)	Range	n	Mean (SD)	Range
CBCL Anxiety Problems	448	.31 (.30)	(.00-1.83)	501	.31 (.30)	(.00-1.50)
CBCL Affective Problems	448	.19 (.19)	(.00-1.00)	501	.18 (.19)	(.00-1.23)
RCADS Total Anxiety	478	.54 (.32)	(.00-1.89)	542	.61 (.33)	(.05-1.97)
RCADS MDD	478	.62 (.34)	(.00-2.10)	542	.62 (.32)	(.00-2.00)
HR supine (bpm)	484	75.8 (10.4)	(51.7-111.7)	543	79.2 (11.1)	(49.1-115.9)
HRV LF supine ($ln(ms^2)$)	484	6.54 (1.03)	(3.79-9.66)	543	6.30 (1.07)	(3.02-9.06)
RSA supine ($ln(ms^2)$)	484	7.47 (1.32)	(3.11-10.47)	543	7.21 (1.29)	(3.01-10.55)
HR standing (bpm)	484	92.7 (13.4)	(59.4-131.2)	543	95.5 (13.2)	(57.8-143.0)
HRV LF standing ($ln(ms^2)$)	484	6.25 (.97)	(2.87-8.85)	543	6.13 (.91)	(3.07-8.51)
RSA standing ($ln(ms^2)$)	484	6.00 (1.33)	(1.70-9.71)	543	5.92 (1.22)	(1.33-9.39)

Note: MDD=Major Depressive Disorder, HR=Heart Rate, HRV LF=Heart Rate Variability in the Low Frequency band, RSA=Respiratory Sinus Arrythmia.

Table 2.2: Associations of anxiety and depression with the physiological measures (HR, HRV LF, and RSA) in supine and standing posture

Dependent variable	**Significant predictors**	**Beta's, p-values, and effect sizes (R^2)**	
HRV LF supine	***RCADS***		
	Gender * Total Anxiety	β= -.18, p=.046, R^2=2.2%	girls; β=.05, p=0.360, R^2=1.0%
			boys; β= -.11, p=.079, R^2=0.9%
	Gender * MDD	β=.28, p=.005, R^2=2.2%	girls; β= -.14, p=0.023, R^2=1.0%
			boys; β=.11, p=0.081, R^2=0.9%
RSA supine	***CBCL***		
	Anxiety Problems	β= -.09, p=.020, R^2=2.3%	
	RCADS		
	Gender * Total Anxiety	β=.24, p=.015, R^2=1.9%	girls; β=.05, p=.357, R^2=0.2%
			boys; β= -.15, p=.018, R^2=1.7%
	Gender * MDD	β= -.21, p=.018, R^2=1.9%	girls; β= -.05, p=.389, R^2=0.2%
			boys; β=.15, p=.022, R^2=1.7%
HR standing	***RCADS***		
	MDD	β=.09, p=.038, R^2=1.6%	
HRV LF standing	***RCADS***		
	MDD	β= -.11, p=.013, R^2=1.0%	

Note: HR=Heart Rate, HRV LF=Heart Rate Variability in the Low Frequency band , RSA=Respiratory Sinus Arrythmia, MDD=Major Depressive Disorder.

Parent-reports (CBCL)

Table 2.2 shows the results of the linear regression analyses performed with the Anxiety Problems and Affective Problems scales of the CBCL as predictors, and physiological measures (HR, HRV LF, and RSA, in supine and standing posture) as dependent variables. Betas, p-values and effect sizes are presented. Betas show the direction of the association, while effect sizes give an idea of the magnitude of an association. According to Cohen (Cohen, 1988), effect sizes ≥ 13.8% are large, between 5.9% and 13.8% medium, and between 1.0% and 5.9% small.

Anxiety: Anxiety Problems were significantly associated with low RSA in supine posture, indicating relatively low vagal activity in anxious individuals.

Depression: No significant associations were found between any of the physiological measures and Affective Problems. This means that the association we found between Anxiety Problems and RSA in supine posture was specific for anxiety, and not depression.

Self-reports (RCADS)

The results of the linear regression analyses conducted with the RCADS scales Total Anxiety and MDD as candidate predictors, and the physiological measures (HR, HRV LF and RSA, in supine and standing posture) as dependent variables are also presented in Table 2.2.

Anxiety: A significant association was revealed between HRV LF in supine posture and the Gender * Total Anxiety interaction. When we performed the analyses for boys and girls separately, we found that the association between HRV LF in supine posture and Total Anxiety was not significant in either boys or girls, but it was negative in boys, and positive in girls. Furthermore, a significant association was revealed between RSA in supine posture and the Gender * Total Anxiety interaction. Analyses regarding the association between Total Anxiety and RSA in supine posture for each sex separately revealed that in boys anxiety was significantly associated with low RSA in supine posture, indicating relatively low vagal activity in anxious boys.

Depression: A significant association was revealed between HRV LF in supine posture and the Gender * MDD interaction. Analyses stratified for gender revealed that in girls, MDD was significantly associated with low HRV LF in supine posture. This finding suggests higher arousal in depressed girls. Also, a significant association was found between RSA in supine posture and the Gender * MDD interaction. Post-hoc analyses revealed high RSA in supine posture in depressed boys, indicating low arousal in depressed boys. MDD was associated with high HR in standing posture, pointing towards relatively high arousal in depression. In contrast, MDD was associated with low HRV LF in standing posture, which indicates low arousal in depression.

Discussion

In the present study, the tripartite model (Clark and Watson, 1991) was tested by investigating putative associations of both parent- and self-reported anxiety problems and depressive problems with physiological measures of arousal (HR, HRV LF, and RSA) in a large population sample of young adolescents. Based on the tripartite model, we expected to find specific associations between these physiological measures and anxiety problems, indicating hyperarousal in anxiety, but not in depression.

Evidence supporting the tripartite model

- Anxiety -

Although effect sizes were relatively small, our findings yielded some evidence for hyperarousal in anxiety. The analyses regarding parent-reported anxiety problems revealed that anxiety problems were associated to low RSA in supine posture, indicating relatively low vagal activity in anxious individuals. This association was found for self-reported anxiety problems as well, but only in boys. These findings point towards diminished vagal activity in individuals with anxiety problems. Decreased vagal activity in anxiety may reflect less influence of the 'vagal brake'. According to Porges (2001), in healthy individuals removal of the vagal brake provides the opportunity to easily adapt to environmental challenges (stress), without the severe biological cost of the metabolic excitation associated with sympathetic-adrenal activation. Where this adaptive mechanism is functional in stressful circumstances for healthy individuals, in anxious individuals this vagal brake may have been constantly less active from birth, or may have become less active across development.

- Depression -

No associations were found between parent-reported depressive problems and any of the physiological measures, indicating that the association that was found between RSA and anxiety problems was specific for anxiety, and not depression. Therefore, these findings in parent-reported data supported the concept of hyperarousal specifically in anxiety, and not depression, which is in accordance with the tripartite model (Clark and Watson, 1991; Joiner et al., 1999; Laurent and Ettelson, 2001).

The tripartite model only implicates that there is no hyperarousal in depression, but it does not provide suggestions about the expected arousal patterns in depression. Interestingly, in our analyses of self-reported depression, we found some evidence pointing towards low arousal in depression. A negative association was found between HRV LF in standing posture and MDD. Further, a positive association was found between RSA in supine posture and MDD in boys. These findings suggest that depression is associated with low arousal and raise the interesting idea of low arousal in depression versus hyperarousal in anxiety.

Evidence against the tripartite model

Some of the findings were inconsistent with the tripartite model. HR is a clear measure of arousal; the higher the HR, the higher the arousal level. Therefore, we expected to find specific positive associations between HR and anxiety problems. However, no such associations were revealed. Moreover, in contrast with the findings mentioned above, self-reported depression was associated with high HR in standing posture, suggesting high arousal in depression. In addition, in girls self-reported depression was associated with low HRV LF in supine posture, which also points towards higher arousal in depression. According to the tripartite model, hyperarousal is specific for anxiety and not depression. Therefore these findings do not support the tripartite model.

Sex differences

Our findings differed between boys and girls. For instance, findings with RSA in supine posture, i.e. vagal activity, were significant in boys, and not in girls. Also in an other study, associations of vagal function with, in this case coping style, were specific for young males, and not for females (Ramaekers et al., 1998). Interestingly, our findings in boys supported the idea of low arousal in depression versus hyperarousal in anxiety. It could be that, in boys, anxiety and depression are to a large extent associated with biological factors such as vagal activity, while in girls, the associations with vagal activity are not as strong, because their problems are to a larger extent associated with other, more social, environmental factors. For example, Rice and colleagues (Rice et al., 2002) found evidence for a stronger genetic component in self-reported depressive problems in boys than in girls. The study of Boomsma and colleagues (Boomsma et al., 2005) revealed a larger influence of environmental factors on the outcome of anxiety and depression in girls than in boys, but did not find significant sex differences for genetic influences on anxious or depressed outcome.

Differences across informants and measures

Findings were different between informants. In the parent-reports (CBCL) we found some evidence in favor of the tripartite model. However, findings were less robust in self-reports (RCADS). These varying findings stress the importance of using multiple informants, also while investigating biological substrates of anxiety and depression.

Further, different physiological measures led to different findings. As stated earlier, we expected to find specific associations between HR and anxiety, since HR is the most evident measure of arousal. However, no such associations were revealed. In our previous study (Dietrich et al., 2006), internalizing problems were associated with higher HR in supine posture. Nevertheless, when we performed the analyses for anxiety and depression separately for the present study, no significant associations were found. Apparently, HR in supine posture was only associated with broad-band internalizing problems, but not specifically with

anxiety. Most of the support we found for the tripartite model regarded low RSA in supine posture in relation to anxiety, i.e. decreased vagal activity in anxiety. Although RSA is not as closely related to arousal as HR, these negative associations could be expected according to the tripartite model, and according to the polyvagal theory (Porges, 2001). Decreased vagal activity points towards a less active vagal brake, which can lead to increased arousal in anxiety.

Dimensions of psychopathology and comorbidity

As mentioned above, findings can differ depending to which dimensions of psychopathology are used. Associations with HR were significant when we used to broad dimension of internalizing problems (Dietrich et al., 2006), while they were not when we split this dimension up into anxiety and depression. In literature there has been some debate on how the broad dimension of internalizing problems should be subdivided (e.g. Lahey et al., 2004). Lahey and colleagues have suggested that some anxiety problems, such as separation anxiety, fears, obsessions and compulsions, are reasonably distinct from depression, but that other types of anxiety, like generalized anxiety and social phobia, are highly correlated with depression. Therefore, one could hypothesize that the anxiety problems that are more distinct from depression would differ more from depression regarding physiological arousal than the depression-related anxiety problems. Yet, additional analyses based on this alternative method of defining subdimensions did not reveal different results (findings can be obtained from the author).

Of course, there is a lot of comorbidity of anxiety and depressive problems; 'pure' cases of individuals with only problems in one of these dimensions are rare, especially in a general population sample. Comorbidity can influence the results of a study. Also comorbidity with externalizing problems can play a role, since arousal is not only associated with anxiety, but also with these problems (Thayer and Lane, 2000). In the present study, we took into account the influence of comorbidity, by correcting for coexisting problems in our analyses. However, this only eliminates the variance associated with comorbid problems, but does not eliminate the comorbidity itself. To tackle the problem of comorbidity, future clinical studies could try to select participants with 'pure' psychopathology -scoring high on one dimension and low on the others-, although this will probably not be easy, since comorbidity is usually rather the rule than the exception.

Sample characteristics

The present study made use of data from the general population. The model of Clark and Watson (1991) was based on findings in clinical samples. Because of differences in symptom levels, different associations between internalizing problems and physiological measures might be found in clinical versus general

population samples. Thus, to gain more insight in the veracity of the model, future replication studies in clinical samples are needed.

Limitations

The validity of the CBCL DSM-IV scale Anxiety Problems can be questioned (Ferdinand, submitted). However, in spite of this, the association with RSA in supine posture was specific for the Anxiety Problems scale and not the Affective Problems scale. Furthermore, although HRV LF in standing posture is primarily controlled by sympathetic influences, and HRV LF in supine posture is primarily vagally mediated, the association of HRV LF in standing and supine posture with respectively sympathetic and vagal activity is not exclusive. In other words, it should be taken into account that HRV LF measures are not more or less than approaches of sympathetic and vagal activity. The same is true for RSA. Although RSA is better known and investigated as a measure of vagal activity than HRV LF in supine position, one could argue that it is important to use respiratory control procedures (Ritz and Dahme, 2006), to get an even better indication of vagal activity. In addition, pre-ejection-period (PEP) should be considered as a suitable measure for sympathetic activity (Berntson et al., 1994). Nevertheless, since associations were found between the HRV LF measures and anxiety problems in both the present study and the study of Mezzacappa and colleagues (1997), we can assume that these measures are also interesting correlates of anxiety problems.

Conclusions and implications

In the present study, we found some evidence for hyperarousal in anxiety, which supported the tripartite model of Clark and Watson (1991). Yet, some other findings pointed towards higher arousal in depression, which is in contrast with this model. Although effect sizes were small, our results illustrate that hyperarousal cannot differentiate between anxiety and depression. The idea that hyperarousal is specific for anxiety and not depression is too simple and does not reflect the more complex reality. Therefore, it might not be useful to make a distinction between anxiety and depression when considering signs of hyperarousal in the general population. This study investigated individuals from the general population. Findings might be different in clinical samples with participants with more severe problems. If, however, findings would be similar in clinical samples, this might imply that, although effective, ingredients of treatment protocols for specifically anxiety disorders that pertain to reducing hyperarousal to diminish anxiety levels might lack a scientific rationale. This, as said, does not mean that such ingredients are ineffective. It could even be the case that they might be helpful to tackle other types of problems as well. In any case, more research is needed to further unravel the specific associations between arousal and anxiety versus depression in samples with higher problem levels.

3 Higher cortisol awakening response in young adolescents with persistent anxiety problems

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Abstract

Objective The aims of the present study were to test the association between current anxiety problems and basal cortisol levels in a large population sample of young adolescents, and to test if HPA-axis activity differs between individuals with no, only current, or persistent anxiety problems.
Methods Cortisol levels of 1,768 10- to 12-year olds from the general population were measured on three time points during the day. A self-report questionnaire (RCADS) was used to assess current anxiety, and a parent-report questionnaire (TPBQ) was used to assess anxiety problems at age 4.
Results Associations between cortisol levels and current anxiety problems were not found. However, individuals with persistent anxiety problems had higher morning cortisol levels and a higher cortisol awakening response.
Conclusions Apparently, only persistent, and not current, anxiety problems are associated with higher HPA-axis activity. Alterations in HPA-axis activity might underlie persistent anxiety problems, or result from the stress accompanied by persistent anxiety problems.

Introduction

Anxiety problems occur frequently (Treffers, 2000; Verhulst et al., 1997), result in considerable suffering and impairment, and tend to persist across time (Ferdinand et al., 1999; Ferdinand and Verhulst, 1995; Pollack et al., 1996; Treffers and Öst, 2001). Therefore it is important to investigate putative aetiological mechanisms.

One of the biological systems that possibly plays a role in anxiety is the hypothalamic-pituitary-adrenal (HPA)-axis. Under stress, the HPA-axis is activated; the hypothalamic production of corticotropin-releasing hormone (CRH) increases, which stimulates the pituitary release of adrenocorticotropin hormone (ACTH) and, as a consequence, cortisol secretion by the adrenal cortex. The HPA-axis is also active in normal circumstances, showing a diurnal pattern of cortisol levels. In the morning, cortisol levels rise quickly during waking up (cortisol awaking response, (Wust et al., 2001). Then, approximately half an hour after awakening, cortisol levels begin to drop, and generally continue to decrease during the day.

Kagan and colleagues (1988) have suggested that some children are more vulnerable for anxiety problems, because they have a lower threshold for activation of the HPA-axis through environmental stimuli, which increases cortisol concentrations. According to this theory, one would expect anxiety problems to be associated with higher basal cortisol levels. Gunnar and Vazquez (2001) hypothesized that stressful influences in early life may provoke frequent elevations in cortisol. These elevations would however eventually lead to down-regulation of components of the HPA-axis. Based on their theory, and the assumption that stress in early life would also result in future anxiety, anxiety problems would expected to be associated with lower basal cortisol levels.

One of the most robust findings in biological psychiatry is that of higher basal cortisol levels in adult patients with major depression (Gunnar and Vazquez, 2001; Heim and Nemerhoff, 1999). In contrast, evidence has been found for lower cortisol levels in both adult and adolescent patients with post traumatic stress disorder (PTSD; Bonne et al., 2003; Bremner et al., 2003; Goenjian et al., 2003; Hageman et al., 2001). For other anxiety disorders, that are not caused by extreme environmental stressors, findings were inconclusive. For instance, some findings in adults suggested increased HPA-axis activation in panic disorder (e.g. Schreiber et al., 1996) while other findings did not support this hypothesis (e.g. Curtis et al., 1997).

In the few studies that investigated the association between anxiety problems and cortisol levels in children and adolescents, findings were as inconclusive as in adults. Kagan and colleagues (1987) found that basal cortisol levels were higher in inhibited than in uninhibited young children. An inhibited temperament overlaps with anxiety; both represent fearful and withdrawn behaviours (e.g. Rapee, 2002). On the other hand, Feder and colleagues found lower nighttime cortisol levels and a slower morning rise in anxious 6- to 12-year-

old children, compared to depressed children or controls (Feder et al., 2004). In another study, Martel and colleagues did not find differences in basal cortisol levels of social phobic adolescent girls versus matched controls (Martel et al., 1999). Previous studies were all confined to small, clinical samples, thereby limiting the generalizability of their findings. Furthermore, earlier work mostly concerned the association between HPA-axis activity and current anxiety problems. It can be argued however that, in those with an inborn lower threshold for activation of the HPA-axis (Kagan et al., 1988), chronically elevated anxiety levels can be found. Therefore, alterations in HPA-axis activity might be most evident in individuals with a long lasting history of anxiety problems. Finally, it is worth while mentioning that it is important to adjust for comorbid symptoms of major depression. Since symptoms of major depression have been associated with alterations in HPA-axis activity, such associations might explain or interfere with the associations with anxiety problems that are investigated.

In conclusion, data on the association of HPA-axis activity and anxiety problems in children or adolescents are hardly available and inconclusive. Further, studies that investigated the association between persistence of anxiety problems and HPA-axis activity in a large population sample, adjusting for comorbid major depression, are lacking. This study had two aims:1) to test the association between current anxiety problems and basal cortisol levels in a large population sample of pre-adolescents, 2) to test if HPA-axis activity differs between pre-adolescents with no, only current, or persistent anxiety problems.

Methods

Sample

Participants all took part in the TRacking Adolescents' Individual Lives Survey (TRAILS), a general population study of Dutch early adolescents aged 10-12 years, who were assessed from March 2001 to July 2002. The TRAILS target sample consisted of young adolescents from five municipalities in the North of the Netherlands, including both urban and rural areas. Of all individuals who were approached (n=3,145), 6.7% were excluded. Of the remaining 2,935 young adolescents, 76.0% participated in the study (n=2,230, mean age 11.09 years, SD .55, with 50.8% girls). Responders and non-responders did not differ with respect to the proportion of single parent families, or the prevalence of teacher-rated problem behaviour. Furthermore, no differences between responders and non-responders were found regarding associations between socio-demographic variables and mental health outcomes (de Winter et al., 2005).

Cortisol was obtained in 1,768 participants (79.3% of all TRAILS participants). There was no difference in Total Anxiety scores (RCADS, see below) between the participants who provided saliva samples versus those who did not (t=0.287; p=.774). Also, there were no differences in terms of gender (49.4% male versus 48.5% male, χ^2(df=1)=0.132; p=.7). The participants that did not provide saliva samples were slightly older (11.16 years versus 11.08 years,

t=-3.084; p=.002). Written consent was obtained from the young adolescent's parents after the procedure had been fully explained.

Measures

RCADS

The Revised Child Anxiety and Depression Scale (RCADS; Chorpita et al., 2000, 2005) is a revision of the Spence Children's Anxiety Scale (SCAS; Spence, 1997). The RCADS is a self-report questionnaire with 47 items, that are scored on a 4-point scale (0 = never, 1 = sometimes, 2 = often, 3 = always). The questionnaire covers six of the DSM-IV dimensions of anxiety disorders and depressive disorder: separation anxiety disorder, generalized anxiety disorder, social phobia, panic disorder, obsessive compulsive disorder and major depressive disorder (MDD). In this study, a Total Anxiety scale was computed by summing the scores on the separate anxiety items and dividing this by the number of items that were completed. Similarly, a MDD scale was computed, and was used to adjust for comorbid depressive problems. The psychometric properties of the RCADS in the TRAILS sample were reported elsewhere (Ferdinand et al., 2005).

TPBQ

The TRAILS Preschool-Behaviour Questionnaire (TPBQ) is a parent-report questionnaire developed by TRAILS to measure behaviours at preschool age (± 4 years old) retrospectively. Parents were asked to compare their child's behaviour with the behaviour of other children. The questionnaire contains 17 items about the occurrence of (problem) behaviours that are scored on a 5-point scale (1 = a lot less, 2 = less, 3 = as much, 4 = more, 5 = a lot more) and covers scales of different behaviours (e.g. aggressive, social). The subscale that covers anxiety problems was used for analyses. The items of this scale were: 1) anxious, 2) shy, 3) gloomy, and 4) afraid to go to school. The internal consistency of this subscale in the TRAILS sample was .76.

Cortisol

TRAILS participants collected three samples of saliva, by using the Salivette sampling device. Collection of salivary cortisol is a relatively stress-free approach that avoids confounding by stress responses, e.g. as induced by venipuncture (Schmidt, 1997). According to several authors correlations between saliva cortisol levels and serum cortisol concentrations are high (e.g. Kirschbaum and Hellhammer, 1989, 1994).

Participants and their parents were instructed to collect saliva at three time points during the day; directly after waking up (while still lying in bed, Cort 1), half an hour later (Cort 2), and at 8.00 P.M. (Cort 3). Parents of all the participants received both written and oral instructions. First, they received a letter, containing information about the purpose of saliva collection and some

background information about diurnal basal cortisol levels. Then, a member of our team visited them at home and gave further instructions. It was stressed that it was important to collect saliva on a normal day, during a normal week, without special events or stressful circumstances. Also, parents were told that their child should not be ill, have a cold, be menstruating, or take any medication at the day of saliva collection. With regard to the actual saliva sampling, it was stressed that participants should always rinse their mouth with tap water before sampling saliva, and not consume sour products or brush their teeth before sampling. Parents were encouraged to place the first salivette next to their child's bed, so that he or she could collect the first sample directly after waking up. Participants were told to not fall asleep and stay awake between providing the first sample and the second sample, and to provide the second sample exactly half an hour later. Finally, it was told that the salivettes should be placed in a freezer directly after saliva collection. All these instructions were also handed over on an instruction form. If any of the requirements were not met, parents could note this down on an accompanying form. Twenty-two pre-adolescents were excluded because of use of antibiotics or corticosteriods. Furthermore, a number of participants were excluded because of extreme cortisol values (>3SD), which eventually yielded n=1,666, n=1,683, and n=1,689 for Cort 1, Cort 2, and Cort 3 respectively. Since the schools that participated in TRAILS started at approximately the same time, the sampling-time variation of the morning samples among the children was limited and the estimated corresponding times are 0700 h for the first sample (Cort 1) and 0730 h for the second sample (Cort 2).

The saliva samples were stored at -20°C until analysis. Competitive solid phase time-resolved fluorescence immunoassays with fluorometric end point detection (DELFIA=dissociation-enhanced lanthanide fluorescent immunoassays) were used to determine cortisol concentrations in the saliva samples. The intra-assay coefficient of variation was between 4.0% and 6.7%, and the corresponding inter-assay coefficients of variation between 7.1% and 9.0% (details about the procedure in Rosmalen et al., 2005).

Other individual characteristics

Given possible confounding effects, information on gender, age, pubertal stage, medication, smoking habits, disruptive behaviours, height and weight (Body Mass Index, BMI) and perinatal variables, like pregnancy duration and birth weight, were also assessed. Pubertal stage was assessed using schematic drawings of secondary sex characteristics associated with the five standard Tanner stages of pubertal development (Marshall and Tanner, 1690, 1970). In a parent interview, the parent -usually the mother- was provided with gender-appropriate sketches, and asked which of the sketches 'looked most like their child'. These ratings have been widely used and have demonstrated good reliability and validity (Dorn et al., 1990). Information on medication and the perinatal variables was also

assessed during this parent-interview. Smoking habits and disruptive behaviours were assessed in a self-report questionnaire of which the anonymity was stressed.

Statistical analyses

Scores on the RCADS Total Anxiety scale, RCADS MDD scale and the TPBQ Anxiety scale were calculated by computing the sum of the separate items of the scale and dividing this sum by the number of items of that scale that were completed. If more than 33% of the items were missing, the scale score was coded as missing. To approximate a normal distribution, all anxiety and depression scales and all cortisol measures were root-transformed.

To obtain an index for the cortisol awakening response (CAR, Wust et al., 2001), we calculated the CAR measure using the following formula:
CAR = (Cort 1 + Cort 2)/2). The cortisol awakening response is a useful index of HPA-axis activity, which is rather consistent, shows good intraindividual stability across time and appears to be useful for assessing subtle changes in HPA-axis activity (Wust et al., 2001). Previous research provided evidence for a significant genetic influence on the cortisol awakening response and this response was found to be independent of the time of awakening, 'manner of awakening' (spontaneously or by an alarm clock), sleep duration, sleep quality, physical activity, or morning routines (Wust et al., 2001). Furthermore, the cortisol awakening response has proven to be a good index to uncover associations between HPA-axis activity and stress-related problems, such as worrying, social stress, persisting pain, and burnout (e.g. Wust et al., 2000a, 2000b, 2001).

To investigate the association between current anxiety problems and cortisol levels, several linear regression analyses were performed with scores on the RCADS Total Anxiety scale as the predictor and respectively, Cort 1, Cort 2, Cort 3, and CAR as dependent variables.

The putative impact of confounders was also investigated, by adding these variables to the regression model. Given gender influences on both anxiety levels and cortisol levels, first gender was taken into account as a confounding factor. In addition, because we found some correlations between pubertal stage and anxiety levels and between age and both anxiety levels and evening cortisol levels, we decided to take pubertal stage and age into account as possible confounders. As stated above, possible confounding effects of depressive problems (MDD) were also taken into account. The effects of perinatal variables, like pregnancy duration and birth weight, and of BMI were not taken into account, since Rosmalen and colleagues found no significant confounding effects of these variables on the cortisol measures in the TRAILS sample (Rosmalen et al., 2005). Moreover, disruptive behaviours were not taken into account, because no or very weak associations were found between these problems and cortisol levels in the TRAILS sample (Sondeijker et al., 2006). Given the age-range of our sample (10 to 12 years old), there was no use of oral contraceptives (.0 %) and a very low

number of smokers (.9% of our sample had smoked on occasion). Therefore these variables were not included in the analyses.

For each index of HPA-axis activity (Cort 1, Cort 2, Cort 3, and CAR) separately multiple linear regression analyses were conducted in which first, RCADS Total Anxiety was entered into the model as a predictor. Subsequently the following possibly confounding predictors were added: 1) gender, 2) pubertal stage, 3) age, 4) MDD.

To investigate if HPA-axis functioning differed between individuals with no, only current, or persistent anxiety problems, three groups were composed. The first group, with no anxiety problems, consisted of those participants who scored below the 50th percentile of the cumulative frequency distribution of the TPBQ Anxiety scale (<P50) and who scored <P50 on the RCADS Total Anxiety scale. The second group consisted of participants with only current anxiety problems. These participants scored <P50 on the TPBQ Anxiety scale, but above the 90th percentile of the cumulative frequency distribution (>P90) of the RCADS Total Anxiety scale. The third group, of participants with more persistent anxiety problems scored >P90 on both the TPBQ Anxiety scale and the RCADS Total Anxiety scale.

Mean daytime cortisol levels (Cort 1, Cort 2, Cort 3) were computed for each group to compose a figure of daytime cortisol levels. Then, differences between the groups on all cortisol measures (Cort 1, Cort 2, Cort 3 and CAR) were calculated using four ANCOVAs, with gender, pubertal stage, age and MDD as covariates. Post-hoc Bonferroni tests were computed to detect between-group differences.

Results

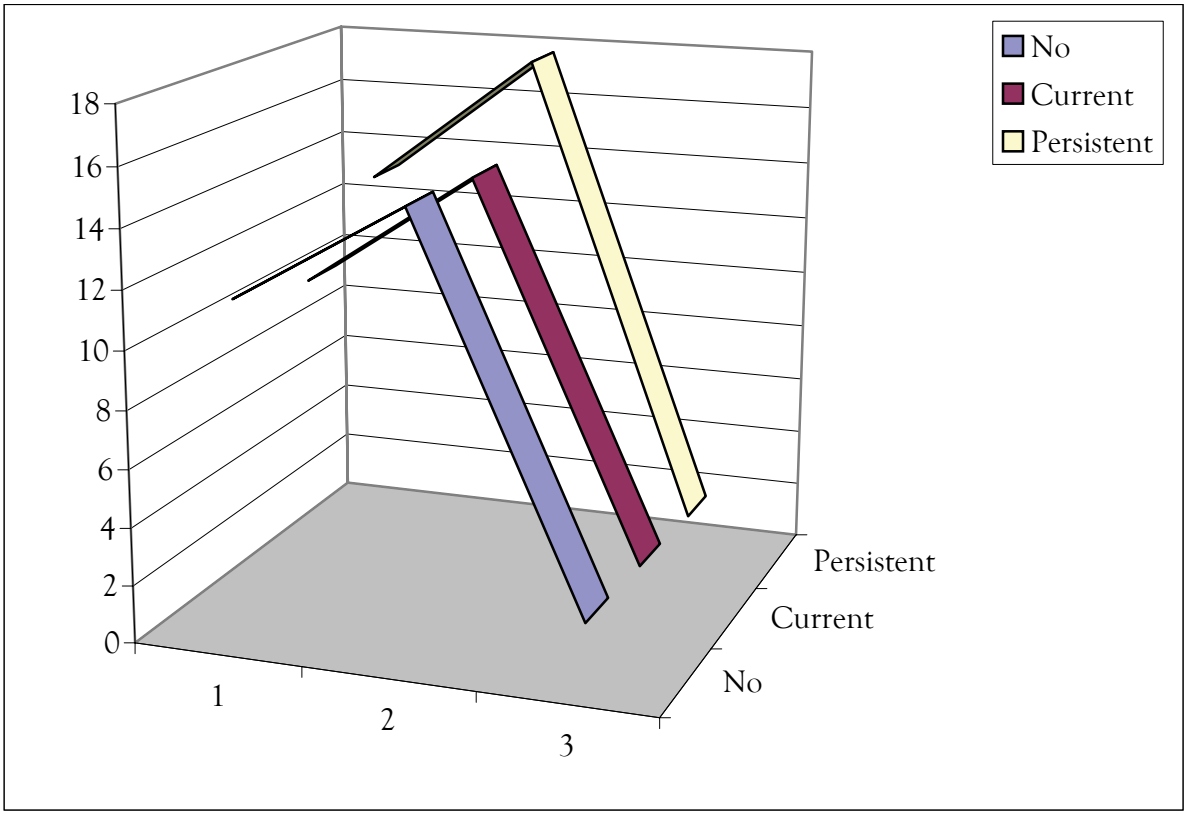
Linear regressions

There were no significant associations between RCADS Total Anxiety scores and any of the cortisol measures (Cort 1; β=.020, p=n.s. Cort 2; β=-,022 p=n.s. Cort 3; β=-.026, p=n.s. CAR; β=-.008, p=n.s.). The addition of gender, pubertal stage, age and MDD to the regression models improved the fit of the models, but the effect of the Total Anxiety scores on the cortisol measures remained insignificant.

One-way ANCOVA's

In Figure 3.1 mean daytime cortisol levels for each group are presented.

Figure 3.1: Day time cortisol levels (in nmol/l) for participants with no anxiety problems (n=324), only current anxiety problems (n=52), or persistent anxiety problems (n=30)



Note: 1=cortisol directly after awakening; 2=cortisol half an hour after awakening; 3=cortisol at 8.00 p.m.; The cortisol awakening response is the increase in cortisol levels between 1 and 2.

The three groups differed significantly with respect to Cort 1 levels (F=4.316, p=.014, effect size (R^2)=5.3%). According to Cohen (1988) effect sizes ≥ 13.8% are large, between 5.9% and 13.8% medium, and between 1.0% and 5.9% small. Post-hoc analyses indicated that this effect was due to higher Cort 1 levels in individuals with persistent anxiety problems versus only current anxiety problems (p=.049, R^2=1.4%). The groups did not differ with respect to Cort 2 levels (F=2.622, p=.074), although participants with persistent anxiety problems had non-significantly higher Cort 2 levels than participants with no anxiety problems. No significant group differences were found either for evening cortisol levels (Cort 3; F=0.496, p=.609).

The cortisol awakening response; CAR, was significantly higher in pre-adolescents with persistent anxiety problems than in pre-adolescents with no, or only current anxiety problems (F=6.678, p=.001, R^2=5.8%). Participants with persistent anxiety problems differed significantly from those with no anxiety problems (p=.007, R^2=2.8%) and from those with only current anxiety problems

(p=.003, R^2=2.8%). Participants with no, or only current anxiety problems did not differ from each other (p=.773).

Discussion

The present study investigated associations between level of anxiety problems and basal cortisol levels in a population sample of 1,768 pre-adolescents. The sample was large and not influenced by referral biases, which provided the opportunity to detect very small effects in a representative sample. Merely studying associations between cortisol levels and current anxiety problems did not yield significant effects. However, even though the effect sizes were small, individuals with high current anxiety levels and a history of anxiety problems early in life showed higher morning cortisol levels and a higher cortisol awakening response. Apparently, only the more persistent anxiety problems were associated with higher HPA-axis activity.

A possible explanation for the association between persistent anxiety problems and cortisol levels may be that inborn alterations in HPA-axis activity result in anxiety problems. Evidence is available indicating that HPA-axis functioning is at least partially determined by genetic (Bartels et al., 2003a, 2003b; Rosmond et al., 2001b) and prenatal factors (Heim and Nemerhoff, 1999). These factors may influence CRH secretion patterns (Arborelius et al., 1999), feedback effects of cortisol on central glucocorticoid receptors (Rosmond et al., 2001a), or both (Rosmond et al., 2001b). In this way, the sensitivity of an individual to stressful stimuli may be determined, which may influence risk for anxiety problems (Kagan et al., 1988).

Another explanation for our findings could be that anxiety problems influence HPA-axis functioning. It is likely that persistent anxiety problems are accompanied by high levels of stress, resulting in elevated cortisol concentrations. Prolonged stress-induced elevations in cortisol levels may influence HPA-axis functioning. These elevations could induce down-regulation of components of the HPA-axis, as suggested by Gunnar and Vazquez (2001). However, this would lead to lower cortisol levels which is not in accordance with the present study's findings. On the other hand, elevations in cortisol levels that persist across time could also tune HPA-axis activity to a higher level. For instance, elevated cortisol levels could result in damage of the hippocampal glucocorticoid receptors or even a loss of hippocampal neurons (Sapolsky et al., 1986), which would affect the glucocorticoid feedback inhibition of CRH secretion (Young et al., 1990) and would result in higher CRH and cortisol concentrations.

Interestingly, individuals with persistent anxiety problems had both higher basal morning cortisol levels (Cort 1) and higher awakening response levels (CAR). This points to a generally higher activated stress hormone release as well as a higher dynamic responsivity. In other words, not only basal HPA-axis functioning, but also the responsiveness of the HPA-axis is different in

individuals with persistent anxiety problems. A higher cortisol awakening response in individuals with persistent anxiety problems indicates a stronger physiological response to the process of awakening, but might also indicate a more general stronger physiological responsivity, that could result in a different stress reaction in these individuals. A possible explanation might lie in exposure to adversities early in life which may have altered HPA-axis functioning. In the present study only the relation between cortisol levels and anxiety was investigated. In future research, it would be interesting to also investigate cortisol responses to daily stresses, psychological stress tasks, or to stressful life-events.

The results of this study did not reveal a linear association between anxiety scores and cortisol levels. Kagan et al. (1988) did show an association between anxiety-related temperamental characteristics and cortisol. However, this study had a small sample size and only investigated children with extreme scores, limiting the generalizability of the findings. Our finding of no association between current anxiety scores and cortisol levels in a large population sample shows that cortisol levels are not related to current anxiety levels, which is of importance for the development of theories on the aetiology of anxiety. Since many anxiety problems develop later in life, it is possible that cortisol levels predict future anxiety problems. Future prospective longitudinal studies will have to address this topic.

In the current study, only retrospective data have been used to investigate the persistence of anxiety problems. Often this is regarded as a shortcoming, since it could be argued that parents of children with high levels of current anxiety may be more likely to indicate that their children were anxious as well earlier in life, known as 'recall bias'. However, the results of our study indicated that, within the group of all pre-adolescents with high current anxiety levels, only those with a parent-reported history of anxiety showed higher cortisol levels. We argue that recall bias based on presence of current anxiety is equally likely to be present in both individuals with only current anxiety problems as well as in individuals who also have a parent-reported history of anxiety, especially since both groups did not differ in the severity of the current anxiety problems ($F=0.005$, $p=.945$). Therefore, it is unlikely that recall bias has influenced the results of this study.

The present study indicates that HPA-axis functioning could be one of the factors that determine anxiety. It could be informative to measure HPA-axis functioning together with other important biological and environmental factors at a younger age than we did now, to investigate their role in the aetiology of anxiety problems. Longitudinal research is needed to gain more insight in the direction of the causal chain between HPA-axis functioning and anxiety problems.

4 Autonomic nervous system functioning as a predictor of anxiety in early adolescence

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Abstract

Objective Better indicators are needed to identify individuals at risk for anxiety problems. This study investigated whether measures of autonomic nervous system (ANS) (re)activity predicted the development of anxiety in early adolescence.
Methods This study is part of the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective cohort study of Dutch young adolescents initially aged 10-12 years, who are followed biennially. The present study used data from the first and second assessment waves. Wave 1 ANS (re)activity measures were available for 1,023 participants. A self-report questionnaire was used to assess anxiety levels at both assessment waves. Possible gender differences, and the role of confounders and co-occurring depressive problems were also examined.
Results ANS reactivity measures predicted anxiety levels two years later, but only in girls. Low Heart Rate Variability (spectral analyses: low and high frequency band power), and low Baroreflex Sensitivity predicted anxiety levels two years later in girls. Baseline Heart Rate Variability in the low frequency band predicted future anxiety levels in girls, but only when adjusted for co-occurring depressive problems. Most other effects were not specific for anxiety, but applied to the broader dimension of internalizing problems.
Conclusions In adolescent girls from the general population, signs of limited ANS flexibility predicted future anxiety levels. Effect sizes were small, hence the ANS alone might not play a major role in the aetiology of anxiety, but can be regarded as one additional and interactional factor in a complex and large group of joint biopsychosocial risk factors for anxiety.

Introduction

The onset of adolescence is characterized by high levels of anxiety, especially in girls (Verhulst et al., 1997). Since anxiety negatively affects psychological and social functioning (Stednitz and Epkins, 2006), it is important to investigate which aetiological mechanisms are involved in the development of anxiety in early adolescence.

One of the physiological mechanisms that may play a role in the development of anxiety is the autonomic nervous system (ANS). The ANS consists of two branches, the sympathetic and the parasympathetic nervous system, and controls the short-term regulation of variability in heart rate (HR) and blood pressure (BP). Cardiovascular variability, particularly HR variability (HRV), is frequently analysed by means of power spectral analyses to provide non-invasive estimates of sympathetic and parasympathetic regulation of the cardiovascular system (Akselrod et al., 1981). HRV in the high frequency band, usually around 0.15-0.40 Hz, is related to respiratory variations (respiratory sinus arrhythmia) and results from centrally mediated cardiac vagal (parasympathetic) activity. HRV in the frequency domain around 0.10 Hz (low frequency band) reflects changes in baroreflex-mediated sympathetic control, although an influence of vagal activity has also been documented (Parati et al., 1995). Baroreflex sensitivity (BRS) is another well-known indicator of homeostatic cardiovascular regulation. This measure reflects both vagal and sympathetic influences (Dietrich et al., 2006).

The general idea about the role of the ANS in the aetiology of anxiety is that anxious individuals are characterized by an ANS that lacks flexibility (e.g. Friedman and Thayer, 1998a). A rigid ANS regulation may hinder the individual to adequately respond to a constantly changing environment. More specific theories have also been formulated. Porges and colleagues (2001) proposed that some individuals might be characterized by relatively low vagal (re)activity, which makes them more vulnerable to develop various mental and physical health problems, among which anxiety. On the other hand, Kagan and colleagues (1988) suggested that some individuals might have a lower threshold for sympathetic activation, which makes them more susceptible to future anxiety problems.

Cross-sectional studies in adults with anxiety problems found evidence for signs of low HRV and low BRS (Friedman et al., 1993; Klein et al., 1995; Piccirillo et al., 1997; Tulen et al., 1996; Virtanen et al., 2003; Watkins et al., 1999) e.g. reduced flexibility. Although ANS function is known to be different in younger subjects (Yeragani et al., 1994), prospective studies on the relationship between ANS (re)activity and anxiety in younger subjects do not exist. A few cross-sectional studies in children or adolescents with anxiety related problems found evidence for a higher baseline HR (Gerra et al., 2000; Kagan et al., 1988; Mezzacappa et al., 1997). Only some studies in younger individuals also examined the role of more specific ANS markers, like HRV (Mezzacappa et al., 1997). Previous studies were mostly confined to small, nonrepresentative samples

limiting the generalizability of the findings. In a previous study (Dietrich et al., 2007), we investigated several parameters of ANS activity in a large population sample and found evidence for an association between internalizing problems and ANS activity. This study had a cross-sectional design and did not investigate gender differences. A longitudinal design gives more insight in the predictive value of ANS measures, and it is known to be important to examine the role of gender in aetiological studies of psychopathology (Rutter et al., 2003). Further, our previous work concerned broadband internalizing problems, while it is important to investigate anxiety apart from depression to clarify the specificity of the associations with ANS functioning (Greaves-Lord et al., 2007b, Chapter 2).

The aim of the present study was to investigate whether measures of ANS (re)activity predict future anxiety levels, using data from a large, prospective cohort study of adolescent boys and girls. We investigated the role of several ANS markers which were assessed during supine rest, and in reaction to an orthostatic challenge test. Possible gender differences, putative confounders, and the role of co-occurring depressive problems were also examined.

Methods

Sample and procedure

The present study was part of the TRacking Adolescents' Individual Lives Survey (TRAILS). TRAILS is a prospective cohort study of Dutch young adolescents, who were 10 to 13 years old at the first assessment wave (wave 1; 2001-2002). They were re-assessed two years later in 2003-2004 (wave 2). The target sample consisted of adolescents from five municipalities in the North of the Netherlands, including both urban and rural areas. Of all eligible individuals (n=2,935), 76.0% participated in the study (n=2,230, mean age 11.09 years, SD .55, 50.8% girls). Participants did not differ from those who refused with respect to the proportion of single parent families, the prevalence of teacher-rated problem behaviour, several socio-demographic variables, and mental health outcomes (de Winter et al., 2005).

At wave 2, information was obtained from 2,149 (96.4%) of those who participated at wave 1 (mean age 13.56 years, SD .53, 51.0% girls). Anxiety was assessed at wave 1 and wave 2 using the Revised Child Anxiety and Depression Scale (see below). Complete data for both assessment waves on this questionnaire was available from 2,081 individuals. ANS measures were determined from 1,868 individuals; 841 were excluded because their measurements were regarded as unsuitable (adequate BP signal recording failed) and 4 cases were excluded because they showed an abnormal decrease in HR after standing up (below 1 SD, most likely reflecting measurement error). This resulted in 1,023 boys and girls (47% vs 53%, mean age 11.0 years, SD=.51) from whom reliable ANS measures could be computed. In total, complete data for both wave 1 and wave 2 RCADS Total Anxiety scores, and reliable wave 1 ANS measures were available from 965 individuals.

To examine possible selective attrition a logistic regression analysis was performed with 'complete data yes/no' as the dependent variable and gender, pubertal stage (see below), socioeconomic status, HR reactivity, and wave 1 Total Anxiety scores as predictors. Low socioeconomic status predicted attrition, whereas the other predictors did not. The effect size of the entire model was small (Cox and Snell R^2=0.7%). Written consent was obtained from the children's parents. The study was approved by the Central Dutch Medical Ethics Committee.

Measures

Anxiety and co-occurring depressive problems

Anxiety levels were assessed using a self-report questionnaire; the Revised Child Anxiety and Depression Scale (RCADS; Chorpita et al., 2000). The RCADS assesses anxiety and depressive symptoms thoroughly; it contains 47 items that are scored on a 4-point scale (0 = never, 1 = sometimes, 2 = often, 3 = always). The questionnaire covers six of the DSM-IV dimensions of anxiety and depressive disorder: separation anxiety disorder, generalized anxiety disorder, social phobia, panic disorder, obsessive compulsive disorder, and major depressive disorder. In this study, Total Anxiety scores -the mean scores on all anxiety items- were computed by summing the scores on all anxiety items and dividing this by the number of items that were completed. Similarly, Depression scores were computed. The Cronbach's alphas based on the present data set were -respectively at wave 1/wave 2- .91/.93 for the Total Anxiety scale, and .72/.81 for the Depression scale.

ANS measures

Orthostatic challenge task: The orthostatic challenge test is a widely used standardised physical test that assesses ANS reactivity to the act of standing. In this test, psychological, motivational, and cognitive processes which play a role in mental stress tasks do not intervene. The orthostatic challenge tests were performed in a quiet room at school, one person at a time. Participants encouraged to relax and not to move or speak during data acquisition, then the procedure was explained to them. Subsequently, a cuff was fixed around the middle phalanx of the third finger of the right hand to non-invasively measure BP. An electrocardiogram (ECG) was recorded to register HR. The recordings did not start until the participants had been in supine position for approximately 5 minutes, and signals were stabilized. ECG and BP signals were registered for 4 minutes in supine rest during spontaneous breathing. Next, the participant was asked to stand up. Again, after signals were stabilized, signals were registered for another 2 minutes.

ANS analyses: HR was registered using a three-lead electrocardiogram, and spontaneous fluctuations in continuous beat-to-beat systolic finger BP were assessed using the Portapres device (FMS Finapres Medical Systems BV,

Amsterdam, the Netherlands). Recordings were digitized using a DAS-12 data acquisition card for notebooks (Keithley Instruments, Cleveland, Ohio, USA). The sample rate was 100 Hz. Recordings were stored on hard disk for off-line analysis. A special interpolation algoritm was used that increased the time resolution for R-peak detection by a factor of 2.5. This resulted in interbeat-intervals (=the intervals of time between two consecutive heart beats i.e. R-peaks, IBI's) with sufficient resolution for HRV determination. The analyzed time series were checked for stationarity and corrected for artifacts. More detailed information about the internal reliability of the data has been reported elsewhere (Dietrich et al., 2006).

HR was calculated as 60,000/mean IBI and expressed in beats per minute (bpm). HRV power in the low frequency band (HRV LF: 0.07-0.14 Hz) and HRV power in the high frequency band (HRV HF: 0.15-0.40 Hz) were computed by means of power spectral analysis (CARSPAN software program, (Mulder et al., 1988). CARSPAN allows for discrete Fourier transformation of non-equidistant systolic BP and IBI-series. Calculation of the BRS was also performed by means of spectral analysis, using the transfer function technique as previously described (Robbe et al., 1987). The BRS index was defined as the modulus between systolic BP and IBI in the .07-.14 Hz frequency band (ms/mmHg) with a coherence of more than .3. A coherence level of .3 has been found to be comparable to the frequently used level of .5 (Dietrich et al., 2006).

To approximate a normal distribution, ANS measures were transformed to their natural logarithm and centered. Reactivity measures were calculated by subtracting the values obtained during supine rest from the measures obtained during active standing.

Other individual characteristics

Some variables are associated with both anxiety and ANS (re)activity and might thus have confounding effects. In the present sample, gender and pubertal stage were associated with anxiety and ANS (re)activity, and were therefore taken into account as possible confounders. Information on gender and pubertal stage was obtained at wave 1. Pubertal stage was assessed using schematic drawings of secondary sex characteristics associated with the five standard Tanner stages of pubertal development (Marshall and Tanner, 1969, 1970). The parent -usually the mother- was provided with gender-appropriate sketches, and asked which of the sketches looked most like their child. These ratings have been widely used and have demonstrated good reliability and validity (Dorn et al., 1990).

Information regarding other possible confounders, like disruptive behaviour and Body Mass Index (BMI) was also assessed, but was not taken into account in the present study, because these factors were not associated with anxiety and ANS (re)activity, and did not markedly influence the relationship between the two.

Statistical analyses

Descriptives were computed for all variables. To investigate whether ANS measures predicted future anxiety levels, 8 regression analyses were performed with wave 2 Total Anxiety scores as the dependent variable. In each set of analyses, one of the ANS measures (HR, HRV LF, HRV HF, and BRS during supine rest, or in reaction to standing) was added as the predictor in the first block, together with gender, Tanner stage, and with wave 1 Total Anxiety scores which were added to examine the direction of the association (model 1). In the second block, the interaction between gender and the respective ANS measure was added to investigate possible gender differences (model 2). Lastly, to investigate whether associations were specific for anxiety, as apart from depression, wave 1 and wave 2 Depression scores were added in the last block (model 3).

In case of a significant effect of an interaction term between gender and a ANS measure, additional post hoc probing analyses (Holmbeck, 2002) were performed for boys and girls separately, from which illustrative figures were constructed.

Results

ANS measures

All ANS measures changed significantly in reaction to the orthostatic challenge test (change from supine rest to active standing; HR: $t=-58.7$, $p<0.01$, HRV LF: $t=7.1$, $p<0.01$, HRV HF: $t=36.9$, $p<0.01$, BRS: $t=27.9$, $p<0.01$). HR increased, whereas HRV LF, HRV HF and BRS decreased.

Descriptives

Mean scores and standard deviations of all independent and dependent variables are presented in Table 4.1.

Regression models

Table 4.2 shows results of the 8 linear regression analyses performed with wave 2 Total Anxiety scores as the dependent variable, and the separate ANS measures (HR, HRV LF, HRV HF, and BRS during rest, or in reaction to active standing) as the predictors (model 1). The results after addition of the interaction between gender and each ANS measure (model 2), and co-occurring depressive problems at wave 1 and 2 (model 3) are also shown.

Table 4.1: Descriptives of all ANS measures, Total Anxiety scores, Tanner stage, and Depression scores

Measures	**Boys**			**Girls**			**Total**		
	n	Mean (SD)	Range	n	Mean (SD)	Range	n	Mean (SD)	Range
HR supine (bpm)	482	75.7 (10.3)	52-116	541	79.1 (11)	49.1-115.9	1023	77.5 (11)	49-116
HRV LF supine (ln(ms^2))	482	6.53 (1.02)	3.79-9.66	541	6.31 (1.1)	3.02-9.06	1023	6.41 (1.1)	3.02-9.66
HRV HF supine (ln(ms^2))	482	7.46 (1.32)	3.11-10.5	541	7.21 (1.3)	3.01-10.55	1023	7.33 (1.3)	3.01-10.6
BRS supine ln(ms/mmHg)	482	2.64 (0.58)	0.95-4.20	541	2.49 (0.6)	0.83-4.29	1023	2.56 (0.6)	0.83-4.29
dHR (bpm)	482	17.06 (9.2)	-3.3-52.6	541	16.4 (9)	-9.0-49.2	1023	16.7 (9.1)	-9.0-52.6
dHRV LF (ln(ms^2))	482	-.29 (1.0)	-3.55-2.95	541	-.18 (1.1)	-3.73-3.69	1023	-.23 (1.0)	-3.73-3.69
dHRV HF (ln(ms^2))	482	-1.47 (1.2)	-6.35-1.79	541	-1.29 (1.2)	-5.31-2.11	1023	-1.38 (1.2)	-6.35-2.11
dBRS ln(ms/mmHg)	482	-.537 (0.6)	-3.03-1.49	541	-.49 (0.6)	-2.57-1.22	1023	-.513 (0.6)	-3.03-1.49
Total Anxiety wave 2	456	0.33 (0.24)	0.00-1.43	509	0.48 (0.3)	0.00-1.78	965	0.41 (0.3)	0.00-1.78
Total Anxiety wave 1	482	0.53 (0.32)	0.00-1.89	541	0.61 (0.3)	0.00-1.97	1023	0.57 (0.3)	0.00-1.97
Tanner stage wave 1	450	1.73 (0.56)	1-5	526	1.97 (0.8)	1-5	976	1.86 (0.7)	1-5
Depression wave 1	476	0.62 (0.34)	0.00-2.10	540	0.62 (0.3)	0.00-2.00	1016	0.62 (0.3)	0.00-2.10
Depression wave 2	456	0.33 (0.30)	0.00-1.60	509	0.47 (0.4)	0.00-1.90	965	0.41 (0.3)	0.00-1.90

Note: HR=Heart Rate, HRV LF=Heart Rate Variability in the Low Frequency band , HRV HF=Heart Rate Variability in the High Frequency band, BRS=Baro Receptor Sensitivity, d=difference between measures in supine and standing position, i.e. reactivity.

Table 4.2: Regression models with the ANS measures and their interactions with gender as the predictors and Total Anxiety scores two years later as the dependent variable

ANS Measure		Statistical values
HR supine	main effect (model 1)	B<.01, p=.77, R^2=27.6%
	interaction gender (model 2)	B<.01, p=.90, ΔR^2<.01%
	interaction gender, adjusted (model 3)	B<.01, p=.97, ΔR^2=28.8%
dHR	main effect (model 1)	B=.001, p=.45, R^2=27.6%
	interaction gender (model 2)	B= -.003, p=.07, ΔR^2=0.3%
	interaction gender, adjusted (model 3)	B= -.001, p=.46, ΔR^2=28.5%
HRV LF supine	main effect (model 1)	B=.007, p=.35, R^2=27.6%
	interaction gender (model 2)	B= -.022, p=.15, ΔR^2=0.2%
	interaction gender, adjusted (model 3)	B= -.024, p=.04, ΔR^2=28.9%*
dHRV LF	main effect (model 1)	B= -.009, p=.24, R^2=27.7%
	interaction gender (model 2)	B=.039, p =.01, ΔR^2=0.5%*
	interaction gender, adjusted (model 3)	B=.023, p=.05, ΔR^2=28.4%*
HRV HF supine	main effect (model 1)	B=.007, p=.23, R^2=27.7%
	interaction gender (model 2)	B= -.011, p=.39, ΔR^2=.01%
	interaction gender, adjusted (model 3)	B= -.009, p=.36, ΔR^2=28.8%
dHRV HF	main effect (model 1)	B= -.006, p=.36, R^2=27.6%
	interaction gender (model 2)	B=.032, p=.02, ΔR^2=0.4%*
	interaction gender, adjusted (model 3)	B=.011, p=.31, ΔR^2=28.4%
BRS supine	main effect (model 1)	B=.025, p=.07, R^2=27.8%
	interaction gender (model 2)	B= -.016, p=.55, ΔR^2<.01%
	interaction gender, adjusted (model 3)	B= -.016, p=.45, ΔR^2=28.7%
dBRS	main effect (model 1)	B= -.011, p=.43, R^2=27.6%
	interaction gender (model 2)	B= .062, p=.02, ΔR^2=0.4%*
	interaction gender, adjusted (model 3)	B=028, p=.18, ΔR^2=28.4%

Note: HR=Heart Rate, HRV LF=Heart Rate Variability in the low frequency band, HRV HF= Heart Rate Variability in the high frequency band, BRS=Baro Receptor Sensitivity, d=difference between measures in supine and standing posture i.e. reactivity, adjusted=adjusted for co-occuring depressive problems, *=p<.05.

Baseline

None of the ANS measures at rest significantly predicted wave 2 Total Anxiety scores. Only the effect of the interaction between gender and baseline HRV LF became significant after adjusting for co-occurring depressive problems. This means that there are gender differences in the way that baseline HRV LF specifically predicts anxiety levels two years later. These differences are discussed below.

Reactivity

The interaction terms gender * HRV LF reactivity, gender * HRV HF reactivity, and gender * BRS reactivity each predicted wave 2 Total Anxiety scores significantly in model 2. Thus, there are also gender differences in the association between these ANS reactivity measures and anxiety levels two years later. These are discussed and illustrated below.

Baseline versus reactivity

Reactivity can be diminished because of high baseline values (ceiling effect). Since one of the baseline measures was associated with wave 2 Total anxiety scores, we performed post hoc analyses in which we controlled for baseline measures in model 2. In these post-hoc analyses the effects of gender * ANS reactivity measures remained significant (gender * dHRV LF: B=.039, p=.01, gender * dHRV HF: B=.031, p=.02, gender * dBRS: B=.061, p=.02), meaning that the effects of reactivity were significant independently from baseline activity.

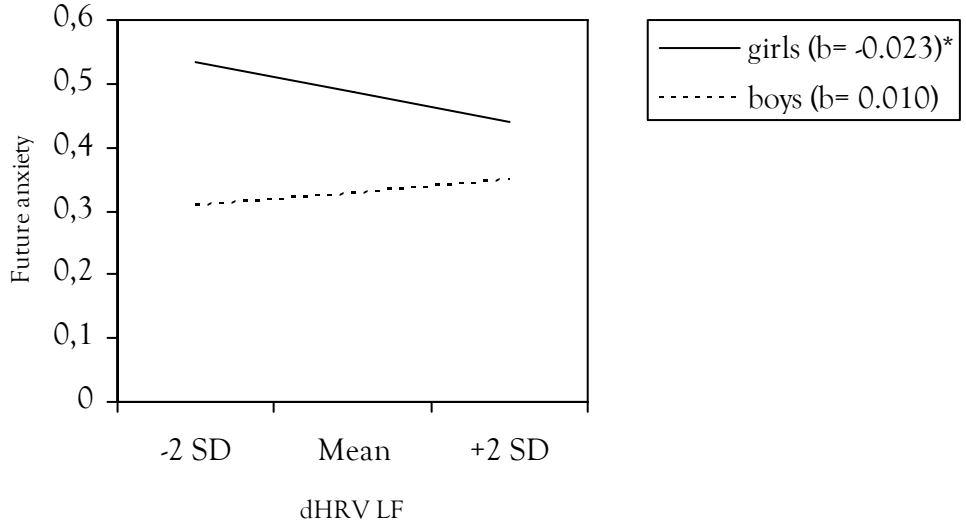
Gender differences

Results differed between boys and girls (Figure 4.1). In boys, none of the ANS reactivity measures significantly predicted anxiety levels two years later (baseline HRV LF: b= -.008, p>.05, for other values see Figure 4.1). However, in girls high baseline HRV LF (b=.02, p<.05), low dHRV LF (Figure 4.1a), low dHRV HF (Figure 4.1b) as well as low dBRS (Figure 4.1c) predicted anxiety levels two years later.

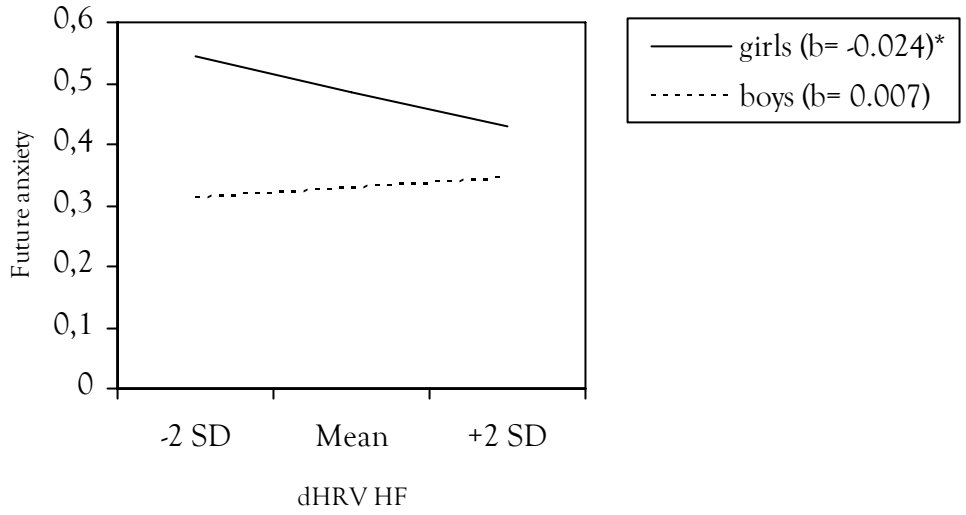
Specificity

The effect of the interaction between gender and baseline HRV LF became significant after adjusting for co-occurring depressive problems (model 3). The effect of the gender * HRV LF reactivity interaction term remained significant after adjusting for co-occurring depressive problems. Thus, effects for HRV LF were specific for anxiety as apart from depression. The effects of the interaction terms gender * HRV HF reactivity, and gender * BRS reactivity did not remain significant after adjusting for co-occurring depressive problems (model 3), and thus applied to broadband internalizing problems.

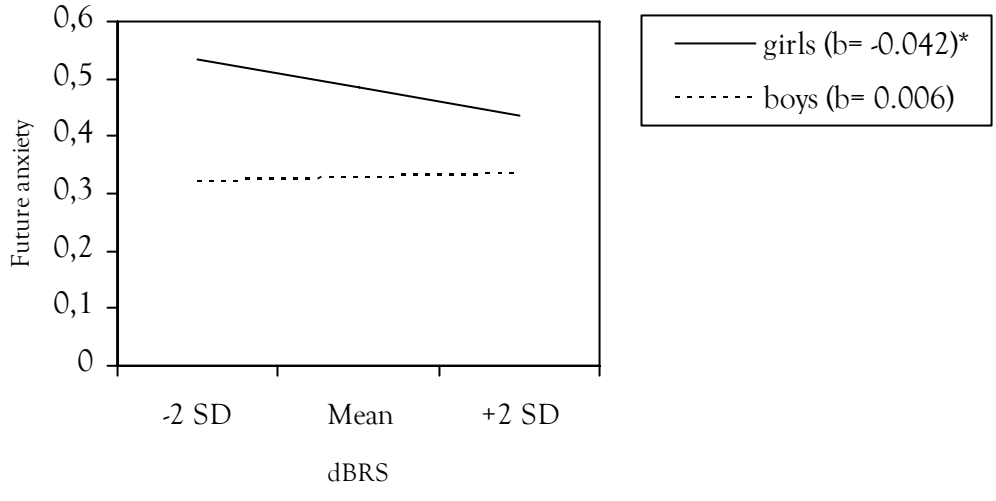
Figure 4.1: Gender differences in the relationship between ANS reactivity measures and Total Anxiety scores two years later



a): dHRV LF



b): dHRV HF



c): dBRS

Note: HRV LF=Heart Rate Variability in the Low Frequency band, HF=Heart Rate Variability in the High Frequency band , BRS=Baro Receptor Sensitivity, d=difference between measures in supine and standing position, i.e. reactivity, SD=standard deviation, b=unstandardized regression coefficient , i.e. simple slope, *=significant slope ($p<.05$).

Discussion

In the present study we investigated whether non-invasive cardiovascular measures of ANS regulation predicted the development of anxiety in early adolescence. Most ANS measures assessed during supine rest did not predict anxiety levels two years later. Only high baseline HRV LF predicted future anxiety levels in girls, after adjusting for co-occurring depressive problems. ANS reactivity measures to an orthostatic challenge test (low HRV LF, low HRV HF and low BRS) predicted future anxiety levels in girls. Most effects were not specific for anxiety. Only effects of HRV LF were specific for anxiety, as apart from depression.

ANS functioning: rest versus reactivity

Measures of ANS activity at rest mostly did not predict later anxiety problems, while most measures of ANS reactivity to an orthostatic challenge test did. Effects of reactivity measures remained significant after controlling for baseline activity. These results point in the direction that in the general population, measures of ANS reactivity probably are better indicators of future anxiety problems than measures of baseline ANS activity. We assessed ANS reactivity to a physical stressor. Although the orthostatic challenge test is a widely used standardised test to assess ANS reactivity to a physical stressor, measures of ANS reactivity to other stressors, like mental challenge, might yield different effects.

Interestingly, most reactivity measures yielded significant effects. HRV HF has been generally accepted as an index for vagal (re)activity. Thus, we may conclude that low vagal reactivity predicts future anxiety levels in girls. This corroborates the theory of Porges that relatively low vagal (re)activity may result in anxiety problems (Porges, 1995). Since opinions differ on what exactly HRV LF reactivity reflects, our finding that low HRV LF reactivity predicts future anxiety levels in girls is more difficult to interpret. Some authors suggested that HRV LF in reaction to standing is mainly an index of sympathetic activation (Yeragani, 1995). Yet, other authors state that HRV LF reflects vagal as well as sympathetic (re)activity (Parati et al., 1995). In our sample HRV LF decreased in reaction to standing, while sympathetic activity is known to increase in reaction to standing. This suggests that in our sample, HRV LF reactivity also reflects vagal reactivity to a considerable amount. Therefore, this finding only indicates that limited ANS reactivity predicts future anxiety levels in girls. Our finding concerning BRS also indicates that limited ANS flexibility predicts future anxiety levels in girls. Regulation of cardiovascular homeostasis, by fine tuning of the interrelationships of changes in HR and BP, may be less functional in anxiety prone girls.

Gender differences

The present study revealed gender differences in the relationship between ANS (re)activity and future anxiety levels. In girls, ANS measures predicted future anxiety levels. No associations were found in boys. In our previous study (Greaves-Lord et al., 2007b, Chapter 2), we found that baseline HRV HF was negatively associated with anxiety in boys but not in girls. Thus, whereas low vagal activity at rest was cross-sectionally associated with anxiety levels at age 10-13 in boys, low vagal reactivity was prospectively associated with anxiety levels at age 13-15 in girls. Possibly, the association between ANS functioning and anxiety changes across pubertal development. Both the incidence of anxiety problems, and ANS functioning are known to change across development and differ between sexes. Since the ANS and the gonodal system are known to interact (Stratakis and Chrousos, 1995), a certain level of sexspecific hormones may trigger limited ANS (re)activity to additionally increase the risk for future anxiety problems. Clearly, more research is needed to examine such a phenomenon.

Specificity

Most effects disappeared when we adjusted for co-occurring depressive problems at wave 2. This means that in general, associations probably apply for broadband internalizing problems, and are not specific for anxiety. Only associations between HRV LF and future anxiety levels in girls remained significant after adjustment for co-occurring depressive problems, and might therefore be specific for anxiety.

In this study, anxiety was investigated as one general concept. Previous studies among adults found differences in ANS activity of patients with panic attacks versus patients with blood phobia (Friedman et al., 1993) possibly reflecting differences in aetiology. However, since the present study was aimed at finding indicators for anxiety in general, and we did not find evidence for distinct types of anxiety symptoms in our sample (Ferdinand et al., 2006), we decided to take a more functional perspective.

Strenghts, weaknesses and important issues

To our knowledge, the present study is the first to prospectively investigate the relationship between ANS (re)activity and anxiety in early adolescence. This study examined a large, representative sample of both boys and girls, which enabled us to investigate gender differences. Several measures of ANS (re)activity were assessed and the role of confounders and co-occurring depressive problems was taken into account.

Of course, this study also has weaknesses. First, there was a lack of respiratory control in the assessment of the ANS measures, whereas it has been argued that it might be important to use such procedures (Ritz and Dahme, 2006). Furthermore, since ANS functioning was assessed at schools instead of in a standardized laboratory setting, the reliability of the measures of ANS (re)activity

can be influenced by several factors, such as noise or activities preceding the measurement. Thus, the reliability of the ANS measures might be limited to some extent. This might explain the small effect sizes we found. Effect sizes might be larger when ANS reactivity is measured under standardised conditions.

The present study concerned individuals from the general population. Hence, anxiety levels were relatively low. This might, at least for some part, explain the small effect sizes. Since participants were averagely 13.6 years old at wave 2, and many anxiety symptoms such as social phobia and panic are known to increase after the age of 15 (Thyer et al., 1985; Wittchen et al., 1998), possibly associations between ANS functioning and anxiety become clearer later in life. Further, it is known that anxiety emerges from a richly interconnected matrix of biopsychosocial variables (Friedman and Thayer, 1998b). Hence, ANS reactivity alone might not play a major role, but can be regarded as one additional, interactional factor in a large group of joint risk factors for anxiety.

Clinical implications

In this general population sample, the association between measures of ANS reactivity and future anxiety levels in girls were significant, but the effect sizes were small. Therefore, we do not believe that in the general population ANS measures can be used to identify individuals at risk for anxiety problems. If clinical studies will point out that ANS (re)activity plays a more important role in anxiety disorders -in the future- interventive programs aimed to normalise ANS functioning, such as relaxation training and slowed respiration, may be helpful (Sakakibara et al., 1996). Such programs might not only apply for specifically anxiety, but are probably also helpful in tackling depression.

5 Baseline cortisol measures and developmental pathways of anxiety in early adolescence

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Abstract

Objective Better indicators are needed to identify individuals at risk for anxiety problems. The present study investigated the significance of the hypothalamic-pituitary-adrenal (HPA) axis for the development of anxiety in early adolescence. We examined whether baseline cortisol measures predicted anxiety levels two years later, and we compared cortisol values of groups with different developmental pathways of anxiety.
Methods This study is part of the TRAILS study, a prospective cohort study of Dutch young adolescents initially aged 10-12 years old. Cortisol was assessed at the first assessment wave in 1,768 participants. A self-report questionnaire (RCADS) was used to assess anxiety levels at the first and second assessment waves.
Results In this general population sample, baseline cortisol measures did not predict anxiety levels two years later. Although we expected that individuals with persistent anxiety problems would show higher morning cortisol levels than those with persistently low, decreasing, or increasing anxiety levels, our analyses did not reveal such an effect. Instead, individuals with persistently high anxiety levels showed significantly lower evening cortisol levels than all other individuals. Further, participants with increasing anxiety levels showed higher morning cortisol levels than individuals with persistently low anxiety levels. Of all significant effects, the effect sizes were small.
Conclusions In the general population, daytime cortisol measures alone cannot be used to identify individuals at risk for anxiety problems. To what extent the HPA-axis by itself plays a role in the aetiology of anxiety is questionable. Interactions of the HPA-axis with other biological or environmental factors may be more important.

Introduction

The onset of adolescence is characterized by high levels of anxiety (Treffers, 2000; Treffers and Öst, 2001; Verhulst et al., 1997). Since these problems result in considerable suffering and social impairment (Stednitz and Epkins, 2006), it is important to investigate aetiological mechanisms and putative biological markers to identify individuals at risk. One of the biological systems that may play a role in the development of anxiety problems is the hypothalamic-pituitary-adrenal (HPA) axis. The HPA-axis is activated by stressful stimuli. Under stress, hypothalamic production of corticotropin-releasing hormone (CRH) increases, which in turn stimulates the pituitary release of adrenocorticotropin hormone (ACTH). As a consequence, cortisol secretion from the adrenal cortex increases. HPA-axis activity not only changes as a result of stress, it also fluctuates during non-stressful normal daily activities. In the morning, as a result of waking up, cortisol levels rise quickly, reaching a peak after approximately half an hour (cortisol awaking response; Wust et al., 2000b, 2001). After this peak, cortisol levels begin to decrease, and continue to decrease during the day.

Kagan and colleagues (1988) have suggested that some children are susceptible to develop anxiety problems, because of a low threshold for HPA-axis activation. In these children, stimuli would more easily increase HPA-axis activity, resulting in relatively high cortisol concentrations. In time, these children would show withdrawn, anxious behaviour, because they try to avoid the unpleasant stress reaction to stimuli. One could therefore expect relatively high cortisol levels in anxious individuals. Findings of Kagan and colleagues (1987) corroborated their hypothesis; they found that basal cortisol levels were higher in inhibited young children than in uninhibited ones. An inhibited temperament overlaps with anxiety; both represent fearful and withdrawn behaviours (e.g. Rapee, 2002).

However, Feder and colleagues (2004) found evidence for lower night time cortisol levels in anxious children. These findings can be interpreted in the light of a theory of Gunnar and Vazquez (2001), who hypothesized that stressful influences early in life may provoke frequent elevations in cortisol levels, which would eventually lead to down-regulation of components of the HPA-axis. On the basis of this theory, and of the assumption that stress in early life is also associated with a risk for anxiety problems (Goodyer and Altham, 1991; Horesh et. al., 1997), one could expect anxiety problems to be associated with relatively low cortisol levels. Taken together, there is still little consensus about whether relatively high or low cortisol levels underlie anxiety problems.

In a previous retrospective study (Greaves-Lord et al., 2007a, Chapter 3), we found that morning cortisol levels were higher in young adolescents with persistent anxiety problems (within ages 4 to 12) than in those with no or only current anxiety problems. But although these findings corroborated the theory of Kagan and colleagues, and did not fit in with the theory of Gunnar and Vazquez, it is still unclear whether the higher cortisol levels we found in individuals with persistent anxiety problems resulted from long lasting anxiety problems, or

reflected an underlying biological vulnerability that may have caused the anxiety problems. The use of retrospective data also means that our previous results should be interpreted with some caution.

Even though better insight in the direction of the association between cortisol measures and anxiety problems would be provided by prospective studies, only few studies investigated whether cortisol measures predicted future anxiety problems in children or adolescents. Smider and colleagues (2002) found that higher mean daytime cortisol levels at age 4.5 predicted internalizing problems (constituted by depression, generalized anxiety, and separation anxiety) at age 6. This indicates that higher basal daytime cortisol levels at a young age may be a risk factor for internalizing problems across a short interval. Many studies in adult patients with major depression indicated that symptoms of depression may cause an association between the broad dimension 'internalizing problems' and HPA-axis activity (Gunnar and Vazquez, 2001; Heim and Nemerhoff, 1999). Hence, it is important to distinguish anxiety from depression when investigating associations between anxiety and HPA-axis activity. To our knowledge, studies that investigated whether cortisol measures specifically predicted anxiety -apart from depression- in young adolescents are lacking.

The aim of the present study was to investigate the role of the HPA-axis in the development of anxiety in early adolescence. More specifically, we investigated whether baseline cortisol measures predicted anxiety levels two years later, and we compared cortisol values of groups with different developmental pathways of anxiety. Since there might be gender differences in the incidence of anxiety problems, and in HPA-axis functioning, we controlled for gender in our analyses and investigated possible gender differences in the association between the cortisol measures and future anxiety levels. We also took into account the role of co-occurring depressive problems, to examine whether the investigated associations were specific for anxiety or applied to the broader dimension of internalizing problems. Based on our previous findings, our hypotheses were that high cortisol levels predict high anxiety levels two years later, and that morning cortisol levels are higher in individuals with persistently high anxiety levels than in those with persistently low, decreasing, or increasing anxiety levels. Finally, we expected that cortisol measures would be specifically associated with anxiety -apart from depression-, since effects remained significant when we adjusted for co-occurring depressive problems in our previous study.

Methods

Sample and procedure

This study is part of the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective cohort study of Dutch (pre-)adolescents initially aged 10-12 years old, with the aim to chart and explain the development of mental health from pre-adolescence into adulthood, both at the level of psychopathology, and at the level of underlying vulnerability and environmental risk. The present study

involves data from the first and second assessment wave of TRAILS, which ran from, respectively, March 2001 to July 2002, and September 2003 to December 2004.

The TRAILS target sample consisted of young adolescents from five municipalities in the North of the Netherlands, including both urban and rural areas. Of all individuals who were approached (n=3,145), 6.7% were excluded. Of the remaining 2,935 young adolescents, 76.0% participated in the study (n=2,230, mean age 11.1 years, SD=0.6, 50.8% girls). Participants did not differ from those who refused to participate with respect to the proportion of single parent families, the prevalence of teacher-rated problem behaviour, several socio-demographic variables, and mental health outcomes (de Winter et al., 2005).

Of the 2,230 baseline participants, 96.4% (n=2,149, 51.0% girls) participated in the second assessment wave, two to three years after wave 1 (mean number of months 29.4, SD=5.4, range 16.7-48.1). Mean age at wave 2 was 13.6 (SD=0.5). The Revised Child Anxiety and Depression Scale (RCADS, see below) was completed at the first and second assessment wave by 2,081 individuals. At the first wave, we obtained cortisol samples three times on one day (sample 1: directly after waking up, sample 2: half an hour later, and sample 3: at 8.00 P.M.) in 1,768 participants. Twenty-two participants were excluded because of use of antibiotics or corticosteriods. To reduce the impact of outliers, cortisol values that were above 3 SD of the mean were also excluded. This yielded n=1,666 for sample 1 (21 excluded), n=1,683 for sample 2 (11 excluded), and n=1,689 for sample 3 (18 excluded). All in all, RCADS questionnaire data from both assessment waves and at least one cortisol sample were available for 1,623 participants.

To examine possible selective attrition, a stepwise logistic regression analysis was performed in which the 1,623 participants with available data for this study were compared with the other individuals who participated at wave 1. Gender, pubertal stage, socioeconomic status, the different cortisol measures (sample, 1, 2, and 3), and scores on the RCADS Total Anxiety and Depression (see below) scales were used as predictors. Low socioeconomic status significantly predicted attrition (β=.68, p<.00), whereas the other predictors did not. However, the effect size of the entire model was small (Cox and Snell R^2=1.5%). After the procedure had been fully explained, written consent was obtained from the young adolescents' parents at both assessments waves. The study was approved by the Central Dutch Medical Ethics Committee.

Measures

Anxiety and co-occurring depressive problems

The Revised Child Anxiety and Depression Scale (RCADS; Chorpita et al., 2000, 2005), a revision of the Spence Children's Anxiety Scale (SCAS; Spence, 1997), was used to measure anxiety levels and co-occurring depressive problems. The RCADS is a self-report questionnaire with 47 items that are scored on a 4-

point scale (0 = never, 1 = sometimes, 2 = often, 3 = always), and covers symptoms of the following DSM-IV disorders: separation anxiety disorder, generalized anxiety disorder, social phobia, panic disorder, obsessive compulsive disorder, and major depressive disorder (MDD). In this study, Total Anxiety scores were computed by summing the scores on all the anxiety items and dividing this sumscore by the number of items for which answers had been filled in, resulting in a mean score on all anxiety items. If more than 33% of the items were missing, the scale score was coded as missing. Similarly, Depression (MDD) scores were computed.

The internal consistencies of the scales that were used were -respectively at wave 1/wave 2- .91/.93 for the Total Anxiety scale, and .72/.81 for the Depression scale. The original factor structure, which was originally based on data from 1,641 children and adolescents from a community sample from Hawaii (Chorpita et al., 2000), was confirmed by confirmatory factor analysis in the TRAILS sample at wave 1 (fit indices of NNFI=.96, RMSEA=.05, and SRMR=.05, indicating an adequate fit to the sample data; Ferdinand et al., 2006).

Cortisol

TRAILS participants collected three samples of saliva with a Salivette sampling device. Collection of cortisol in saliva is a relatively stress-free approach that avoids confounding by stress responses, such as those induced by venipuncture (Schmidt, 1997). According to several authors, correlations between saliva cortisol levels and serum cortisol concentrations are high (Kirschbaum and Hellhammer, 1989, 1994).

Participants and their parents were instructed to collect saliva at two time points during the morning; directly after waking up (while still lying in bed, sample 1), and half an hour later (sample 2). In addition, saliva was collected at 8.00 P.M. (sample 3). Parents of all participants received written and oral instructions. First, they received a letter containing information about the purpose of saliva collection and some background information about diurnal basal cortisol levels. Then, a member of the team visited them at home and gave further instructions. It was stressed that it was important to collect saliva on a normal day, during a normal week, without special events or stressful circumstances. Parents were also told that their child should not be ill, have a cold, be menstruating, or take any medication on the day of saliva collection. Furthermore, it was explained that participants should rinse their mouth with tap water before sampling saliva, and not consume sour products or brush their teeth before sampling. Parents were encouraged to place the first salivette next to their child's bed, so that he or she could collect the first sample directly after waking up. In addition, it was stressed that the salivettes should be placed in a freezer directly after saliva collection, and mailed to the institute as soon as possible (but not on Fridays or Saturdays in order to prevent unnecessary delay

due to the weekend). Finally, all instructions were also handed out on an instruction form. If any of the requirements were not met, parents could note this down on an accompanying form. Participants who did not return the salivettes within a couple of months were sent a reminder letter. In all, saliva samples of 1,768 children (79.3% of all TRAILS participants) were received. For more characteristics of this study population see Rosmalen et. al. (2005).The saliva samples were stored at -20°C until analysis. Previous studies suggested that salivary cortisol levels are stable for prolonged periods of time at -20°C (Aardal and Holm, 1995). After completion of the data collection, all samples were sent in one batch (frozen, by courier) to the laboratory (Department of Clinical and Theoretical Psychobiology, University of Trier, Germany) for analyses.

Cortisol levels were determined with a competitive solid phase time-resolved fluorescene immunoassay with fluorometric end-point detection (DELFIA=dissociation-enhanced lanthanide fluorescent immunoassays). Ninety-six-well Maxisorb microtiterplates (Nunc) were used, coated with rabbit-anti-ovine immunoglobulin. After an incubation period of 48 hours at 4°C, the plates were washed with washbuffer (pH=7.4) coated with an ovine anti-cortisol antibody, and then incubated again. Synthetic saliva mixed with cortisol in a range from 0-100 nmol/l served as standards. Standards, controls (saliva pools) and samples were tested in duplicate wells. Fifty µl of biotin-conjugated cortisol was added, and after 30 minutes of incubation the non-binding cortisol/biotin-conjugated cortisol was removed by washing. Two-hundred µl europium-streptavidin (Wallac, Turku, Finnland) was added to each well and after 30 minutes and 6 times of washing, 200 µl enhancement solution was added (Pharmacia, Freiburg, Germany). Within 15 minutes on a shaker, the enhancement solution induced fluorescene that could be detected with a DELFIA-Fluorometer (Wallac, Turku, Finnland). A standard curve was generated and the cortisol concentrations of the samples were calculated with a computer program. The intra-assay coefficient of variation was between 4.0% and 6.7%, and the corresponding inter-assay coefficients of variation between 7.1% and 9.0% (Rosmalen et al., 2005).

To obtain an index for the cortisol awakening response, we calculated the area under the curve (AUC) of the two morning cortisol samples (Wust et al., 2000a, 2001). The cortisol awakening response is a useful index of HPA-axis activity, which is rather consistent, shows good intra individual stability across time, and appears to be useful for assessing subtle changes in HPA-axis activity (Pruessner et al., 1997; Wust et al., 2000a). Previous research has provided evidence for a significant genetic influence on the cortisol awakening response and this response was found to be independent of the time of awakening, 'manner of awakening' (spontaneously or by an alarm clock), sleep duration, sleep quality, physical activity, or morning routines (Pruessner et al., 1997; Wust et al., 2000a, 2000b). Furthermore, the cortisol awakening response has proved to be a good index for uncovering associations between HPA-axis activity and stress-related

problems, such as worrying, social stress, persisting pain, and burnout (Wust et al., 2001). Sample 3 was used to investigate possible associations with evening cortisol levels.

Other individual characteristics

Some variables might be associated with both cortisol and anxiety, and might therefore play a confounding role in relationship between cortisol levels and anxiety levels. In the present sample, gender and wave 2 Depression were significantly associated with the index for the cortisol awakening response (AUC) and with wave 2 Total Anxiety scores. Therefore, gender and wave 2 Depression were taken into account as possible confounders.

Information regarding other possible confounders, such as age, pubertal stage, perinatal variables (pregnancy duration, birth weight), Body Mass Index (BMI), and wave 1 and 2 disruptive behaviours was also assessed in the TRAILS study, but was not taken into account for the present manuscript, since these factors were not significantly associated with the cortisol measures and with wave 2 Total Anxiety scores, and since addition of these factors to the model did not change the investigated associations markedly. For the same reasons we did not take into account wave 1 Total Anxiety and Depression scores. Given the age-range of our sample at wave 1 (10 to 12 years old), there was no use of oral contraceptives (0 %), and there was only a very low number of smokers (87% of the selected sample had never smoked, only 1% had smoked 7 times or more) at the time of cortisol sampling. Therefore these variables were also not included in this study.

Statistical analyses

The cortisol measures were root-transformed and centered to approximate a normal distribution and to improve the interpretation of betas.

To test whether cortisol measures predicted future anxiety levels, two sets of multiple linear regression analyses were performed with wave 2 Total Anxiety scores as the dependent variable. In the first block of the first set of regression analyses AUC and gender were added as predictors. To investigate possible gender differences in the association between AUC and wave 2 Total Anxiety scores, the interaction between gender and AUC was added as a predictor in the second block. To investigate whether the association was specific for anxiety, wave 2 Depression scores were added as a predictor in the last block. The second set of analyses was performed similarly, but now evening cortisol levels -instead of AUC- and the interaction between evening cortisol levels and gender were added to the model.

Because our interest particularly concerned the association between cortisol measures and the persistence of anxiety, we tested whether cortisol values differed between individuals with persistently low, decreasing, increasing, or persistently high anxiety levels. Four groups were composed, using cut-off scores

based on the 50th percentile (P50, Total Anxiety =0.51) and the 80th percentile (P80, Total Anxiety =0.81) of the cumulative frequency distribution of the wave 1 Total Anxiety scores. The first group, which consisted of individuals with persistently low anxiety levels, scored below P50 on the Total Anxiety scale at wave 1 and at wave 2. The second group, with decreasing anxiety levels scored above P80 on the Total Anxiety scale at wave 1 and below P50 on the Total Anxiety scale at wave 2. The third group consisted of individuals with increasing anxiety levels, who scored below P50 on the Total Anxiety scale at wave 1, but above P80 on the Total Anxiety scale at wave 2. The fourth group consisted of individuals with persistently high anxiety levels who scored above P80 on the Total Anxiety scale at wave 1 and at wave 2. For each group, descriptives were computed. Differences between the groups concerning AUC and evening cortisol levels were calculated using one-way analyses of covariance, with gender, and wave 2 Depression scores as covariates. To detect differences between the group with persistently high anxiety levels and the other groups, contrasts were calculated with the group with persistently high anxiety levels as the reference group.

Results

Regression models

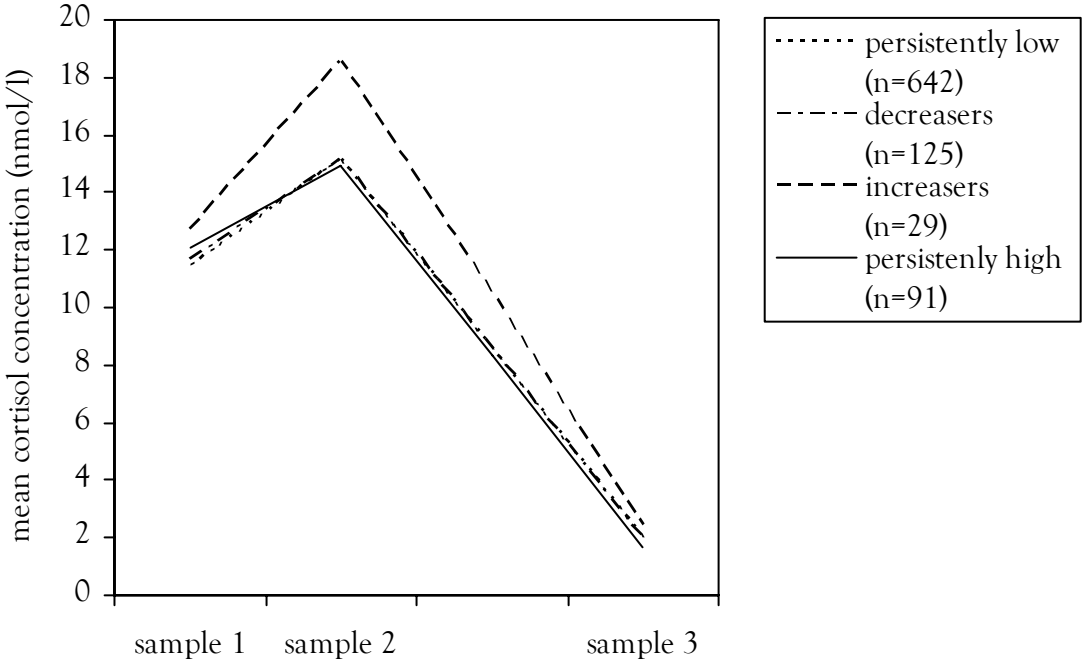
The index for the cortisol awakening response (AUC) did not significantly predict wave 2 Total Anxiety scores in the first block (Bauc=.017, p=0.32). Adding the interaction between AUC and gender to the model did not reveal significant gender differences (block 2: Baucsex=.019, p=.59). Addition of wave 2 Depression scores, did not reveal such gender differences either (block 3: Baucsex=-.017, p=.47).

Evening cortisol levels also did not significantly predict wave 2 Total Anxiety scores in the first block (Bsample3=-.007, p=.65). Adding the interaction between evening cortisol levels and gender to the model also did not reveal significant gender differences (block 2: Bsample3sex=-.024, p=.45). Addition of wave 2 Depression scores, did not reveal such gender differences either (block 3: Bsample3sex=-.012, p=.59).

Table 5.1: Descriptives for the groups with different developmental pathways of anxiety (persistently low, decreasing, increasing and persistently high anxiety levels)

Measures	**Persistently low Anxiety** (n=642)		**Decreasing Anxiety** (n=125)		**Increasing Anxiety** (n=29)		**Persistently high Anxiety** (n=91)	
	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Total Anxiety wave 1	0.30 (0.13)	0.00 - 0.49	1.05 (0.22)	0.81 - 1.97	0.38 (0.07)	0.22 - 0.49	1.13 (0.28)	0.81 - 2.08
Total Anxiety wave 2	0.21 (0.14)	0.00 - 0.49	0.31 (0.13)	0.00 - 0.49	0.98 (0.21)	0.81 - 1.59	1.10 (0.27)	0.81 - 2.14
Depression wave 2	0.23 (0.22)	0.00 - 1.10	0.38 (0.27)	0.00 - 1.20	0.92 (0.45)	0.30 - 2.20	1.02 (0.44)	0.10 - 2.50
Gender (% girls)	42%	-	45%	-	76%	-	71%	-

Figure 5.1. Mean cortisol concentrations throughout the day for individuals with persistently low, decreasing, increasing, or persistently high anxiety levels



Note: sample 1=directly after waking up, sample 2=half an hour later, sample 3=8 o'clock in the evening.

Group differences

Table 5.1 shows descriptives for each group (persistently low, decreasing, increasing and persistently high anxiety levels). Mean cortisol concentrations throughout the day are shown for each group in Figure 5.1.

The one-way analyses of covariance did not reveal an overall difference in AUC between the four groups ($F(3)=1.88$, $p=.13$, $\eta^2=0.7\%$), but post-hoc contrasts showed that morning cortisol levels (AUC) were significantly lower in individuals with persistently high anxiety levels than in those with increasing anxiety levels ($p=.02$). Individuals with persistently high anxiety levels did not differ with respect to AUC compared to the other groups (persistently low, decreasing). As Figure 5.1 shows, morning cortisol levels do not only seem to be higher in individuals with increasing anxiety levels compared to those with persistently high anxiety levels, but also compared with those with persistently low or decreasing anxiety levels. We tested this difference for significance by repeating the analyses, but this time the group with increasing anxiety levels was the reference group. These post-hoc analyses revealed that morning cortisol levels were also higher in individuals with increasing anxiety levels when we compared

this group to those with decreasing anxiety levels (p=.05) or those with persistently low anxiety levels (p=.05). Further, Table 5.1 shows that mean anxiety levels at wave 1 are higher in individuals with increasing anxiety levels than in the group with persistently low anxiety levels. This is the case, while both groups were composed using the same wave 1 cut-off point (below P50 of the Total Anxiety scale). Because this difference could be important for the interpretation of our findings, we tested this difference for significance by means of post-hoc one-way analysis of covariance. We found that participants with increasing anxiety levels had significantly higher baseline anxiety levels than those with persistently low anxiety levels ($F(1)=10.47$, $p<.01$, $\eta^2=1.5\%$).

The second set of one-way analyses of covariance revealed an overall difference in evening cortisol levels between the four groups ($F(3)=2.97$, $p=.03$, $\eta^2= 1\%$). Post-hoc contrasts showed that evening cortisol levels were lower in individuals with persistently high anxiety levels than in those with persistently low (p=.02), decreasing (p=.04) or increasing (p=.01) anxiety levels. These group-differences are also illustrated in Figure 5.1.

Discussion

This study investigated the role of the HPA-axis in the development of anxiety in early adolescence. We hypothesized that high cortisol levels predict high anxiety levels two years later, and that morning cortisol levels are higher in individuals with persistently high anxiety levels, than in those with persistently low, decreasing, or increasing anxiety levels. We expected that cortisol measures would be specifically associated with anxiety, as apart from depression.

Our analyses revealed that baseline cortisol measures did not predict anxiety levels two years later in this general population sample. Moreover, individuals with persistent anxiety problems did not have higher morning cortisol levels than those with persistently low, decreasing, or increasing levels of anxiety. Hence, although the TRAILS study provided the opportunity to investigate associations between cortisol measures and future anxiety levels in a large, representative population sample -and thus had the power to detect small effects- the effects we expected were not found. Two significant group-differences were found: The group with increasing anxiety levels had significantly higher morning cortisol levels than the other groups (persistently low, decreasing, persistently high). Further, the group with persistently high anxiety levels had significantly lower evening cortisol levels than the other groups (persistently low, decreasing, increasing). Yet, the sizes of these effects were small. Overall, we therefore conclude that in the general population, cortisol measures alone cannot be used to predict future anxiety problems.

Our findings do not provide clear evidence for either the theory of Kagan and colleagues (1988) who suggest that high cortisol levels may underlie future anxiety problems, or the theory of Gunnar and Vazquez (2001) that implies that stress during early development may lead to lower cortisol levels. Having said

this, our findings of higher morning cortisol levels in participants with increasing anxiety levels, and of lower evening cortisol levels in individuals with persistently high anxiety levels, are noteworthy. In the group with increasing anxiety levels, apparently an increase in anxiety levels involved higher morning cortisol levels. This could mean two things. First, it could be that, in this particular group, higher HPA-axis activity did eventually lead to an increase of anxiety levels, which would be in line with the theory of Kagan and colleagues (1988). This, however, is not very likely, since our regression analyses did not reveal that cortisol measures predicted future anxiety levels. Secondly, it could be that, in this particular group, an increase of anxiety levels went hand in hand with an increase in morning cortisol levels. In other words, as anxiety levels increased, so did the morning cortisol levels. Since this group already had somewhat higher baseline anxiety levels than the group with persistently low anxiety levels, this explanation seems more likely. Thus, this finding might indicate that a rise in anxiety levels across time may be reflected in a rise in morning cortisol levels. The finding of lower evening cortisol levels in participants with persistently high anxiety levels could be considered as supportive for the theory of Gunnar and Vazquez (2001). Percistence of anxiety may first have resulted in higher cortisol levels, and, as a consequence, in down-regulation of components of the HPA-axis in the long run. This may explain the lower evening cortisol levels in those individuals with persistent anxiety. However, only evening cortisol levels were lower, whereas morning levels were not. Hence, the results do not support the Gunnar and Vazquez theory unequivocally.

Since Feder and colleagues (2004) also found lower evening cortisol levels in anxious children, it might be fruitful to further investigate this phenomenon. Both higher morning cortisol levels and lower evening cortisol levels have been associated with anxiety. Previous research has shown that morning cortisol levels are mainly determined by genetic influences, while evening cortisol levels are mainly influenced by environmental factors (Bartels et. al., 2003, Wust et. al., 2000a). Therefore, it is possible that the theories of Kagan and colleagues and of Gunnar and Vazquez both apply, be it for different aspects of HPA-axis functioning.

Co-occurring depressive problems were taken into account in all analyses. The associations between the cortisol measures and certain developmental trajectories of anxiety (increasing anxiety, persistent anxiety) seemed to be specific for anxiety, because those associations did not disappear when adjusted for co-occurring depressive problems. Apparently, associations between cortisol and anxiety are not only explained by the broad dimension of internalizing problems, but are specific for anxiety, apart from depression.

One may question why individuals with persistently high anxiety levels did not have relatively high cortisol levels, like we expected. It could be that, due to down-regulation of components of the HPA-axis, the cortisol levels of individuals with persistent anxiety problems drop again (Gunnar and Vazquez, 2001). Yet,

we did find that morning cortisol levels were higher in individuals with persistent anxiety problems in our previous study (Greaves-Lord et al., 2007b, Chapter 2). This previous study concerned persistency of anxiety problems over a 6 to 8 year period from early childhood (4 years old) to early adolescence (10 to 12 years old), after which cortisol levels were obtained. Anxiety levels at age 4 were assessed retrospectively. In the present study, the persistency of anxiety was measured prospectively and covered a 2-year period of early adolescence. Cortisol levels were assessed at the start of this period. It is possible that the association between HPA-axis activity and anxiety changes throughout development. Therefore, it is important to investigate this association during different developmental periods. Further, since the period over which persistency of anxiety was measured was longer in the previous study, it might be that the association between cortisol measures and the persistence of anxiety becomes more evident when the persistence of anxiety is measured over a longer period of time. Possibly, cortisol levels increase after a long period of anxiety problems, but high cortisol levels do not predict the persistence of anxiety in the future. Lastly, one should bear in mind that our previous study relied on retrospective data, while the current study was a prospective one, which implies that the reliability of the anxietydata used in the current study was almost certainly higher. Thus, findings might differ between the studies due to some important methodological differences.

The present study was carried out in a general population sample. The theory of Kagan et. al. (1988) was based on findings in small, select groups and other studies often focussed on clinical samples (Gerra et al., 2000). Thus, because anxiety levels are relatively low in our sample, associations between cortisol levels and anxiety might be different in a sample with symptoms in the (sub-)clinical range. Further, basal daytime cortisol measures were assessed. Although our index of the cortisol awakening response reflects the response to a so-called physical stressor -waking up- measures of reactivity to relatively more stressful stimuli, such as mental stress, might reveal different results. Finally, the present study had some limitations. For instance, cortisol samples were collected at home, which may have limited the reliability of the time points at which cortisol samples were assessed. It is possible that the small effect sizes we found have been influenced by a relatively lower reliability of the cortisoldata. However, since the effect sizes were particularly small in this study, we may also conclude that anxiety levels in young adolescents are probably associated with many other factors as well. For instance, the HPA-axis works in alliance with many other physiological systems, such as the immune system and the gonodal axis (Stratakis and Chrousos, 1995), HPA-axis activity is triggered by environmental influences, and HPA-axis functioning is at least partially determined by genetic factors (Bartels et al., 2003a; Rosmond et al., 2001b; Wust et al., 2000a). Therefore, focusing too much on HPA-axis functioning alone in the search for aetiological mechanisms may not prove to be very fruitful.

The present study did not reveal strong evidence for a predictive association between baseline daytime cortisol measures and the development of anxiety in young adolescents from the general population. Therefore, we conclude that in the general population, daytime cortisol measures alone cannot be used to identify individuals at risk for anxiety problems. We question the extent to which the HPA-axis in itself plays an important role in the aetiology of anxiety, and we expect that the interaction of the HPA-axis with other biological or environmental factors will play a more important role.

6 Physiological reactivity, familial vulnerability and the development of anxiety in early adolescence: an interactive multisystem approach

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Abstract

Objective To gain more insight in the aetiology of anxiety, the present study investigated whether the interaction between measures of hypothalamic-pituitary-adrenal (HPA)-axis and autonomic nervous system (ANS) reactivity serves as a predictor for anxiety levels two years later. The role of familial vulnerability and gender was also investigated.
Methods This study is part of the TRAILS study, a prospective cohort study of Dutch young adolescents initially aged 10-12 years. HPA-axis and ANS reactivity measures were assessed at the first assessment wave, and a self-report questionnaire (RCADS) was used to assess anxiety levels at the first and second assessment waves. Complete data were available for 753 participants. Linear regression analyses were performed for individuals that scored low versus high on parental internalizing problems, and for boys and girls separately.
Results In the group with low parental internalizing problems, anxiety was associated with reduced ANS flexibility in girls, and anxiety was associated with higher morning cortisol levels in combination with a higher heart rate in boys. Yet, the effect sizes were small. In the group with high parental internalizing problems, effects were much stronger; anxiety-prone girls showed higher morning cortisol levels, and anxiety prone boys showed higher morning cortisol levels in combination with higher vagal reactivity.
Conclusions Apparently, physiological risk factors for future anxiety are more evident in individuals with a high familial vulnerability. Further, patterns of physiological risk for future anxiety are different in boys and girls. Therefore, it is important to take into account familial vulnerability and gender when investigating putative physiological risk factors for future anxiety.

Introduction

Anxiety levels are high during early adolescence, especially in girls (Treffers and Öst, 2001; Verhulst et al., 1997). Since anxiety problems put forth an enormous burden both in human and economic terms, it is important to investigate which aetiological mechanisms are involved in the development of anxiety problems during early adolescence.

The hypothalamic-pituitary-adrenal (HPA)-axis and the autonomic nervous system (ANS) are two physiological stress response systems that have been associated with anxiety problems (Boyce et al., 2001; Feder et al., 2004; Friedman and Thayer, 1998a; Greaves-Lord et al., 2007a, 2007b, Chapter 2 & 3; Kagan et al., 1987; Mezzacappa et al., 1997; Smider et al., 2002; Tulen et al., 1996; Virtanen et al., 2003; Watkins et al., 1999). The HPA-axis is a physiological system that is activated in response to stress. Under stress, the hypothalamic production of corticotropin-releasing hormone (CRH) increases, which in turn stimulates the pituitary release of adrenocorticotropin hormone (ACTH). As a consequence, cortisol secretion from the adrenal cortex increases. Changes in HPA-axis activity do not only occur as a result of stress, also during non-stressful normal daily activities the activity of the HPA-axis fluctuates. In the morning, as a response to waking up, cortisol levels rise quickly, and reach a peak after approximately half an hour (cortisol awaking response; Wust et al., 2000b, 2001)). After this peak, cortisol levels begin to decrease, and remain decreasing during the day. The autonomic nervous system (ANS) is another physiological system that is activated in response to stress. The ANS controls the heart and consists of two branches; the sympathetic and the parasympathetic nervous system. The sympathetic system prepares the body for action and increases heart rate (HR), whereas the parasympathetic or vagal system maintains homeostasis and slows HR. Under stress, sympathetic activity increases and vagal activity decreases. Under non-stressful circumstances, both systems are mutually active.

The (re)activity of the HPA-axis and the ANS may play a role in the development of anxiety problems. A general belief is that anxious individuals are characterized by signs of hyperarousal (Clark and Watson, 1991), such as high muscular tension and high autonomic reactivity. More specifically, Kagan and colleagues (1988) proposed that certain individuals might have an inborn tendency towards overarousal of the central nervous system (particularly the hypothalamus and the amygdala) due to a lower threshold for activation. As a consequence, reactivity of the HPA-axis and the sympathetic system enhances, resulting in elevated cortisol levels and elevated HR. These children compensate for this hyperarousal through withdrawal and avoidance, which makes them more susceptible to anxiety problems. Signs of relatively higher HPA-axis and sympathetic (re)activity may therefore predict future anxiety levels. Porges and colleagues (2001) suggested that individuals that show relatively low vagal (re)activity might be more susceptible to many physical and mental health

problems, among which anxiety problems. Signs of relatively lower vagal (re)activity might therefore also predict future anxiety levels.

In our previous work, we investigated the association between anxiety and HPA-axis functioning on the one hand and between anxiety and ANS functioning on the other hand (Greaves-Lord et al., 2007a, 2007b, Chapter 2 & 3). We found some signs of hyperarousal in anxiety, i.e. higher morning cortisol levels and lower vagal activity, but findings were inconsistent and effects were small. According to Bauer and colleagues (2002), the role of physiological systems in emotional and behavioural problems is best investigated by using an interactive multisystem approach. Effects of the HPA-axis and the sympathetic system might not be additive, but interactional, since both systems are biologically intertwined (Stratakis and Chrousos, 1995). Components of both systems connect at several brain sites (Brown et al., 1982; Chrousos and Gold, 1992; Dunn and Berridge, 1990), and have joint effects in response to stressors. Cortisol has a complex and multilateral role in the immediate autonomic stress response. It probably first permits, and stimulates the sympathetic stress response, and later supresses the ongoing stress response, and it might prepare the body for a putative subsequent stressor (Sapolsky et al., 2000). Since cortisol has such important effects on ANS activity, it possibly moderates the effects of ANS (re)activity on anxiety levels.

Bauer and colleagues (2002) suggested that the sympathetic system and the HPA-axis might be complementary in their actions and their influences on emotions and behaviour. Accordingly, optimal functioning is only possible when the activity of both systems is balanced, leading to an optimal, medium level of arousal. The sympathetic system and the HPA-axis should show symmetric activation patterns to obtain an optimal level of arousal. If individuals show asymmetric activation patterns, thus relatively low HPA-axis (re)activity together with relatively high sympathetic reactivity, or relatively high HPA-axis (re)activity together with relatively low sympathetic reactivity, they are at increased risk for either emotional or behavioural problems. To investigate this hypothesis, we can use the interaction between reactivity measures of both systems as a predictor to investigate whether the interaction between these two systems is a better and more consistent predictor for anxiety problems than the two systems separately.

Not only the sympathetic system and the HPA-axis interact, the vagal system also influences the sympathetic system and the HPA-axis (Stratakis and Chrousos, 1995). Therefore it is important to investigate the role of the vagal system as well. Further, other putative risk factors, such as familial vulnerability may also play a role. HPA-axis and/or ANS (dys)functioning may only be a valid indicator of anxiety once a certain threshold has been exceeded. A certain familial vulnerability may underlie ANS and/or HPA-axis dysfunctioning or putative activation asymmetries between both systems that in turn increases the risk for future anxiety problems. Therefore, the association of measures of ANS and HPA-axis functioning and their interaction with anxiety problems might

only manifest itself in individuals with a high familial vulnerability for anxiety, e.g. high parental internalizing problems. In addition, one can assume that individuals with a high familial vulnerability might not only have an increased risk for future anxiety problems, but may also lack resources, for instance social support, to compensate for possible physiological risk factors. In individuals with a low familial vulnerability, such resources may buffer the effects of physiological factors. Thus, since relations between putative physiological risk factors and anxiety may only appear in individuals with a high familial vulnerability, it is important to investigate the role of familial vulnerability. Further, when investigating putative risk factors for mental health problems, it is important to take into account possible gender differences (Rutter et al., 2003). Finally, since pubertal stage and co-occurring depressive problems are known to influence the relation of HPA-axis and ANS (re)activity with anxiety (Greaves-Lord et al., 2007a, 2007b, Chapter 2 & 3), the influence of these factors should be investigated.

Recently, Gordis and colleagues (2006) found that the interaction between the HPA-axis and the sympathetic system was associated with parent-reported aggression in a cross-sectional study in adolescents. To our knowledge, the relation of the interaction between HPA-axis and ANS reactivity with anxiety levels in young adolescents has not yet been investigated.

The aim of the present study was to investigate whether the interaction between HPA-axis and ANS reactivity predicts the development of anxiety in early adolescence. Prospective data from a large population sample of both boys and girls were used. In the analyses, indices of HPA-axis and ANS reactivity and their interactions were used as predictors for anxiety levels two years later. Associations between physiological measures and depression are sometimes in an opposite direction than associations between physiological measures and anxiety (Greaves-Lord et al., 2007b, Chapter 2; Gunnar and Vazquez, 2001). Therefore, the role of co-occurring depressive problems was investigated by examining whether associations were specific for anxiety or applied to the broader dimension of internalizing problems. To investigate the role of familial vulnerability and gender, the analyses were conducted for individuals with low versus high scores on parental internalizing problems, and for boys and girls separately.

Methods

Sample and procedure

The present study was part of the TRacking Adolescents' Individual Lives Survey (TRAILS). TRAILS is a prospective cohort study of Dutch young adolescents, who were 10 to 13 years old at the first assessment wave (wave 1), which took place in 2001-2002. They were re-assessed two years later in 2003-2004 (wave 2). The target sample consisted of young adolescents from five municipalities in the North of the Netherlands, including both urban and rural areas. Of all eligible

individuals (n=2,935), 76.0% participated in the study (n=2,230, mean age 11.09 years, SD .55, 50.8% girls).

Participants did not differ from those who refused to participate with respect to the proportion of single parent families, the prevalence of teacher-rated problem behaviour, several socio-demographic variables, and mental health outcomes (de Winter et al., 2005). At wave 2, information was obtained from 2,149 (96.4%) of those who participated at wave 1 (mean age 13.56 years, SD .53, 51.0% girls). Anxiety was assessed at wave 1 and wave 2 using the Revised Child Anxiety and Depression Scale (RCADS). Complete data for both assessment waves on this questionnaire was available for 2,081 individuals.

Cortisol was assessed at wave 1 in 1,768 participants. Twenty-two pre-adolescents were excluded because of use of antibiotics or corticosteriods. Furthermore, cortisol values that were above 3 SD of the mean were excluded, to reduce the impact of outliers. This yielded n=1,666 for sample 1 (21 excluded), and n=1,683 for sample 2 (11 excluded). ANS measures were determined for 1,868 individuals, of whom 841 were excluded because their measurements were regarded as unsuitable (adequate signal recording failed) and 4 cases were excluded because they showed an abnormal decrease in heart rate after standing up (below 1 SD), probably reflecting measurement error. This resulted in 1,023 boys and girls (47% vs 53%, mean age 11.0 years, SD=.51) for whom reliable ANS reactivity measures could be computed. In total, complete data for wave 1 and wave 2 RCADS Total Anxiety scores, and cortisol and ANS measures were available for 753 individuals.

To examine possible selective attrition, a stepwise logistic regression analysis was performed with 'complete data yes/no?' as a dependent variable and gender, socioeconomic status, pubertal stage (see below), AUC (index cortisol awakening response, see below), heart rate reactivity, and wave 1 and wave 2 RCADS Total Anxiety scores (see below) as predictors. None of these factors predicted attrition. Written consent was obtained from the children's parents. The study was approved by the Central Dutch Medical Ethics Committee.

Measures

Anxiety and co-occurring depressive problems

Anxiety levels were assessed using a self-report questionnaire; the Revised Child Anxiety and Depression Scale (RCADS; Chorpita et al., 2000). The RCADS assesses anxiety symptoms thoroughly; it contains 47 items, that are scored on a 4-point scale (0 = never, 1 = sometimes, 2 = often, 3 = always). The questionnaire covers six of the DSM-IV dimensions of anxiety disorders and depressive disorder: separation anxiety disorder, generalized anxiety disorder, social phobia, panic disorder, obsessive compulsive disorder and major depressive disorder. In this study, Total Anxiety scores -the mean scores on all anxiety items- were computed by summing the scores on the separate anxiety items and dividing this by the number of items that were completed. Similarly, Depression scores were

computed, and used as a measure for co-occurring depressive problems. The Cronbach's alphas based on the present data set were -respectively at wave 1/wave 2- .91/.93 for the Total Anxiety scores, and .72/.81 for the Depression scores.

Cortisol

TRAILS participants collected three samples of saliva, with a Salivette sampling device. Collection of salivary cortisol is a relatively stress-free approach that avoids confounding by stress responses, as for instance induced by venipuncture (Schmidt, 1997). According to several authors correlations between saliva cortisol levels and serum cortisol concentrations are high (Kirschbaum and Hellhammer, 1989, 1994).

Participants and their parents were instructed to collect saliva at two time points during the morning; directly after waking up (while still lying in bed, sample 1), and half an hour later (sample 2). Parents of all participants received written and oral instructions. First, they received a letter, containing information about the purpose of saliva collection and some background information about diurnal basal cortisol levels. Then, a member of the team visited them at home and gave further instructions. It was stressed that it was important to collect saliva on a normal day, during a normal week, without special events or stressful circumstances. Also, parents were told that their child should not be ill, have a cold, be menstruating, or take any medication on the day of saliva collection. Furthermore, it was explained that participants should rinse their mouth with tap water before sampling saliva, and not consume sour products or brush their teeth before sampling. Parents were encouraged to place the first salivette next to their child's bed, so that he or she could collect the first sample directly after waking up. In addition, it was stressed that the salivettes should be placed in a freezer directly after saliva collection, and mailed to the institute as soon as possible (but not on Fridays or Saturdays in order to prevent unnecessary delay due to the weekend). Finally, all instructions were also handed over on an instruction form. If any of the requirements were not met, parents could note this down on an accompanying form. Participants who did not return the salivettes within a couple of months were sent a reminder letter. We received saliva samples of 1,768 children (79.3% of all TRAILS participants). For more characteristics of this study population see Rosmalen et al. (2005).

The saliva samples were stored at -20°C until analysis. Previous studies suggested that salivary cortisol levels are stable for prolonged periods of time at -20°C (Aardal and Holm, 1995). After completion of the data collection, all samples were sent in one batch (frozen, by courier) to the laboratory (Department of Clinical and Theoretical Psychobiology, University of Trier, Germany) for analyses.

Cortisol levels were determined with a competitive solid phase time-resolved fluorescene immunoassay with fluorometric end point detection

(DELFIA=dissociation-enhanced lanthanide fluorescent immunoassays). Ninety-six well Maxisorb microtiterplates (Nunc) were used, that were coated with rabbit-anti-ovine immunoglobulin. After an incubation period of 48 hours at 4°C, the plates were washed with washbuffer (pH=7.4), coated with an ovine anti-cortisol antibody and incubated again. Synthetic saliva mixed with cortisol in a range from 0-100 nmol/l served as standards. Standards, controls (saliva pools) and samples were tested in duplicate wells. Fifty µl of biotin-conjugated cortisol was added and after 30 minutes of incubation the non-binding cortisol/biotin-conjugated cortisol was removed by washing. Two-hundred µl europium-streptavidin (Wallac, Turku, Finnland) was added to each well and after 30 minutes and 6 times of washing 200 µl enhancement solution was added (Pharmacia, Freiburg, Germany). Within 15 minutes on a shaker the enhancement solution induced fluorescene that could be detected with a DELFIA-Fluorometer (Wallac, Turku, Finnland). A standard curve was generated and the cortisol concentrations of the samples were calculated with a computer program. The intra-assay coefficient of variation was between 4.0% and 6.7%, and the corresponding inter-assay coefficients of variation between 7.1% and 9.0% (Rosmalen et al., 2005).

To obtain an index for the cortisol awakening response (Wust et al., 2000a, 2001), we calculated the area under the curve (AUC) of the two morning cortisol samples. The cortisol awakening response is a useful index of HPA-axis activity, which is rather consistent, shows good intra individual stability across time and appears to be useful for assessing subtle changes in HPA-axis activity (Pruessner et al., 1997; Wust et al., 2000a). Previous research provided evidence for a significant genetic influence on the cortisol awakening response and this response was found to be independent of the time of awakening, 'manner of awakening' (spontaneously or by an alarm clock), sleep duration, sleep quality, physical activity, or morning routines (Pruessner et al., 1997; Wust et al., 2000a, 2000b). Furthermore, the cortisol awakening response has proven to be a good index to uncover associations between HPA-axis activity and stress-related problems, such as worrying, social stress, persisting pain, and burnout (Wust et al., 2001).

ANS measures

Orthostatic challenge task: The orthostatic challenge test was performed in a quiet room at school, one person at a time. Participants were first asked to lie down and relax. Whilst supine, the procedure was explained to them. They were encouraged to relax and asked not to move or speak during data acquisition. A three-lead electrocardiogram (ECG) was recorded to register HR. The recordings did not start until the participants had been in supine position for approximately 5 minutes, and signals were stabilized. Then, ECG signals were registered for 4 minutes in supine rest during spontaneous breathing. Next, the participant was asked to stand up, to assess the ANS reaction to the act of active standing. Again,

after the signals were stabilized, the signals were registered for another 2 minutes in standing position.

Analyses of ANS measures: The ECG recordings were digitized using a DAS-12 data acquisition card for notebooks (Keithley Instruments, Cleveland, Ohio, USA). The sample rate was 100 Hz. Recordings were stored on hard disk for off-line analysis. A special interpolation algorithm was used that increased the time resolution for R-peak detection by a factor of 2.5. This resulted in interbeat-intervals (the intervals of time between two consecutive heart beats i.e. R-peaks, =IBI's) with sufficient resolution for HRV determination (see below). The analyzed time series were then checked for stationarity and corrected for artifacts.

HR was calculated as 60,000/mean IBI and expressed in beats per minute (bpm). To assess two other more specific indices for ANS reactivity, Heart Rate Variability (fluctuations in HR, HRV) was calculated. HRV was analyzed by means of power spectral analyses (CARSPAN software program, (Mulder et al., 1988) to provide non-invasive estimates of sympathetic and parasympathetic regulation of the cardiovascular system (Akselrod et al., 1981; Malliani et al., 1991; Parati et al., 1995). CARSPAN allows for discrete Fourier transformation of non-equidistant systolic BP and IBI-series. HRV in the high frequency band (HRV HF), from 0.15 to 0.40 Hz, is related to respiratory variations (respiratory sinus arrhythmia) and results from centrally mediated cardiac vagal (parasympathetic) activity. HRV in the low frequency band (HRV LF), from 0.07 to 0.14 Hz, reflects changes in baroreflex-mediated sympathetic control, although an influence of vagal activity has also been documented (Parati et al., 1995). More detailed information on the data-analyses is reported elsewhere (Dietrich et al., 2006).

Familial vulnerability

Lifetime parental internalizing problems were assessed during a parent interview at the first assessment wave by means of the TRAILS Family History Interview (FHI; Ormel et al., 2005). Anxiety and depression were assessed, using a vignette for each of these two dimensions which described the main DSM-IV characteristics of each dimension, followed by a series of questions assessing lifetime occurrence, professional treatment, and medication. The interview assessed lifetime parental internalizing problems for each biological parent separately, using a single informant -usually the mother. For each dimension, we assigned each parent one of the following scores: 0 = (probably) never had an episode, 1 = (probably) had an episode, 2 = had one or more episodes plus treatment and/or medication. A familial vulnerability index was constructed based on these scores, and on the findings of Kendler and colleagues (2003). These investigators performed multivariate twin modeling to investigate the structure of genetic risk factors for common psychiatric disorders. The path coefficients they found were used as regression coefficients to construct the familial liability index for internalizing problems. The regression coefficient for

depression was .54. The regression coefficient for anxiety was constructed as the mean of the path coefficients for generalized anxiety disorder (.53) and phobia (.33). The following regression equation was used: genetic risk for internalizing disorder =.54 (depression mother + depression father) + .43 (anxiety mother + anxiety father). For further details please see Ormel et al. (2005) and Veenstra et al. (2005). For the present manuscript, we calulated a cut-off point that was one standard deviation above the mean score on the familial vulnerability index. Participants were classified as 'low parental internalizing problems' if they scored lower than this cut-off point, and they were classified as 'high parental internalizing problems' if they scored above this cut-off point.

Gender & pubertal stage

Information on gender and pubertal stage was obtained at wave 1. Pubertal stage was assessed using schematic drawings of secondary sex characteristics associated with the five standard Tanner stages of pubertal development (Marshall and Tanner, 1969, 1970). During the parent interview, the parent -usually the mother- was provided with gender-appropriate sketches, and asked which of the sketches looked most like their child. These ratings have been widely used and have demonstrated good reliability and validity (Dorn et al., 1990).

Information regarding other possible confounders, like disruptive behaviour and Body Mass Index (BMI) was also assessed, but was not taken into account in the present study, since these factors did not markedly change the association of the interaction of the AUC and the ANS reactivity measures with wave 2 Total Anxiety scores. For more information on the associations between all variables, please see Table 6.1 and 6.2.

Statistical analyses

To approximate a normal distribution, AUC was root-transformed, and the ANS measures (HR, HRV LF, and HRV HF) were transformed to their natural logarithm. The ANS reactivity measures were calculated by subtracting the measures obtained in supine rest from the measures obtained in standing position (dHR, dHRV LF, and dHRV HF). All physiological measures when than centered for the regression analyses. Descriptives were computed for each category (boys-low parental internalizing problems, girls-low parental internalizing problems, boys-high parental internalizing problems, and girls-high parental internalizing problems).

Table 6.1: Correlations between all predictors, the outcome variable, and all possible confounders in girls

Measures	Tanx1	Tanx2	Dep1	Dep2	AUC	dHR	dHRVLF	dHRVHF	Tanner	BMI	Ext1	Ext2
Tanx1	1	0.47*	0.69*	0.42*	0.01	0.02	0.02	-0.05	<-0.01	<0.01	0.40*	0.29*
Tanx2	0.47*	1	0.29*	0.66*	0.07	0.06	-0.10	-0.09	0.12*	0.12*	0.22*	0.45*
Dep1	0.69*	0.29*	1	0.46*	0.05	0.06	0.03	-0.10	-0.03	0.03	0.47*	0.34*
Dep2	0.42*	0.66*	0.46*	1	0.01	0.11	-0.06	-0.12*	0.14*	0.15*	0.29*	0.59*
AUC	0.01	0.07	0.05	0.01	1	-0.38	-0.38	0.01	<0.01	0.04	0.08	0.02
dHR	0.02	0.06	0.06	0.11	-0.38	1	-0.41*	-0.63*	0.18*	<-0.01	0.06	0.14*
dHRVLF	0.02	-0.10	0.03	-0.06	-0.38	-0.41*	1	0.70*	0.06	0.12	0.16	-0.13*
dHRVHF	-0.05	-0.09	-0.10	-0.12*	0.01	-0.63*	0.70*	1	<-0.01	0.14*	-0.05	-0.22*
Tanner	<-0.01	0.12*	-0.03	0.14*	<0.01	0.18	0.06	<-0.01	1	0.43*	0.06	0.11
BMI	<0.01	0.12*	0.03	0.15*	0.04	<-0.01	0.12	0.14*	0.43*	1	0.09	0.09
Ext1	0.40*	0.22*	0.47*	0.29*	0.08	0.06	0.16	-0.05	0.06	0.09	1	0.44*
Ext2	0.29*	0.45*	0.34*	0.59*	0.02	0.14*	-0.13*	-0.22*	0.11	0.09	0.44*	1

Note: Tanx1=wave 1 Total Anxiety scores, Tanx2=wave 2 Total Anxiety scores, Dep1=wave 1 Depression scores, Dep2=wave 2 Depression scores, AUC=Area under the curve, the index for the cortisol awakening response, d=difference between measures in supine and standing position, HR=Heart Rate, HRVLF=Heart Rate Variability in the Low Frequency band , HRVHF=Heart Rate Variability in the High Frequency band, Tanner=Tanner stage, i.e. pubertal stage, BMI=Body Mass Index, Ext1=wave 1 externalizing problems, Ext2=wave 2 externalizing problems.

Table 6.2: Correlations between all predictors, the outcome variable, and all possible confounders in boys

Measures	Tanx1	Tanx2	Dep1	Dep2	AUC	dHR	dHRVLF	dHRVHF	Tanner	BMI	Ext1	Ext2
Tanx1	1	0.47*	0.69*	0.37*	-0.01	0.03	0.03	0.04	0.08	-0.07	0.47*	0.31*
Tanx2	0.47*	1	0.39*	0.68*	-0.02	-0.04	0.07	0.08	<0.01	-0.13*	0.33*	0.52*
Dep1	0.69*	0.39*	1	0.48*	-0.06	0.06	-0.05	-0.06	0.10	-0.07	0.50*	0.36*
Dep2	0.37*	0.68*	0.48*	1	-0.01	0.03	-0.02	0.01	0.02	-0.02	0.38*	0.58*
AUC	-0.01	-0.02	-0.06	-0.01	1	0.01	0.02	0.03	0.06	-0.07	-0.14*	-0.08
dHR	0.03	0.07	-0.05	-0.02	0.02	1	-0.39*	-0.62*	0.08	0.07	0.01	0.04
dHRVLF	0.03	0.07	-0.05	-0.02	0.02	-0.39*	1	0.70*	-0.04	-0.05	-0.03	-0.07
dHRVHF	0.04	0.08	-0.06	0.01	0.03	-0.62*	0.70*	1	-0.04	-0.06	-0.04	-0.08
Tanner	0.08	<0.01	0.10	0.02	0.06	0.08	-0.04	-0.04	1	0.01	0.02	0.01
BMI	-0.07	-0.13*	-0.07	-0.02	-0.07	0.07	-0.05	-0.06	0.01	1	<-0.01	0.02
Ext1	0.47*	0.33*	0.50*	0.38*	-0.14*	0.01	-0.03	-0.04	0.02	<-0.01	1	0.56*
Ext2	0.31*	0.52*	0.36*	0.58*	-0.08	0.04	-0.07	-0.08	0.01	0.02	0.56*	1

Note: Tanx1=wave 1 Total Anxiety scores, Tanx2=wave 2 Total Anxiety scores, Dep1=wave 1 Depression scores, Dep2=wave 2 Depression scores, AUC=Area under the curve, the index for the cortisol awakening response, d=difference between measures in supine and standing position, HR=Heart Rate, HRVLF=Heart Rate Variability in the Low Frequency band , HRVHF=Heart Rate Variability in the High Frequency band, Tanner=Tanner stage, i.e. pubertal stage, BMI=Body Mass Index, Ext1=wave 1 externalizing Problems, Ext2=wave 2 externalizing problems.

To investigate whether the interaction between HPA-axis and ANS reactivity predicted future anxiety levels, 3x4 regression analyses were performed for the 3 separate ANS reactivity measures (dHR, dHRV LF, and dHRV HF), in all 4 categories (boys-low parental internalizing problems, girls-low parental internalizing problems, boys-high parental internalizing problems, and girls-high parental internalizing problems). In all analyses, wave 2 Total Anxiety scores were used as the dependent variable. Covariates were added as predictors in the first block: we corrected for baseline Total Anxiety and Depression scores, to investigate the direction of the association, and we adjusted for Tanner stage in this block. In the second block, AUC and one of the respective ANS reactivity measures (dHR, dHRV LF or dHRV HF) were added as predictors. In the third block, the interaction between AUC and the respective ANS reactivity measure was added as a predictor. If a significant effect was found, post-hoc probing tests (Holmbeck, 2002) were performed to create illustrative figures. Lastly, Depression scores at wave 2 were added to the model, to investigate whether associations were specific for anxiety, as apart from depression.

Results

Descriptives for each category for all variables are given in Table 6.3. Table 6.4 shows the results for the regression analyses for each category.

As shown in Table 6.4, for individuals with low parental internalizing problems, there is a significant effect of the interaction between AUC and dHR in boys, but only after adjusting for co-occurring depressive problems. Post-hoc analyses revealed that high morning cortisol levels together with high HR reactivity predicted anxiety levels two years later in boys with low parental internalizing problems (B dHR=-.004, $p<.05$). There is also a significant main effect of dHRV LF in girls with low parental internalizing problems. Low HRV LF reactivity predicted anxiety levels two years later in these girls. The sizes of the effects in the in individuals with low parental internalizing problems were small (Table 6.4).

For individuals with high parental internalizing problems, there is a main effect of the AUC for girls in all analyses. This effect is illustrated in Figure 6.1, showing that higher morning cortisol levels results in relatively higher anxiety levels two years later in girls with high parental internalizing problems. In boys with high parental internalizing problems there is a significant effect of the interaction between AUC and dHRV HF (Table 6.3). This effect is illustrated in Figure 6.2, showing that high morning cortisol levels in combination with high vagal reactivity results in relatively higher anxiety levels two years later in boys with high parental internalizing problems. The sizes of these effects are medium to large (Table 6.4).

Table 6.3: Descriptive for all covariates, predictors and outcome variables split up between individuals with low or high parental internalizing problems and split up between boys and girls

Measures		**Low parental internalizing problems**		**High parental internalizing problems**	
		Boys (n=303)	Girls (n=338)	Boys (n=53)	Girls (n=59)
		Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Covariates:	Tanner Stage	1.72 (0.53)	1.96 (0.84)	1.81 (0.53)	2.08 (0.86)
baseline Total Anxiety scores		0.54 (0.32)	0.61 (0.32)	0.53 (0.32)	0.63 (0.37)
baseline Depression scores		0.60 (0.34)	0.62 (0.30)	0.64 (0.29)	0.63 (0.37)
wave 2 Depression scores		0.34 (0.31)	0.46 (0.35)	0.34 (0.27)	0.51 (0.33)
Predictors:	AUC (nmol/l)	13.19 (4.60)	13.92 (4.25)	12.65 (4.20)	14.32 (4.48)
	dHR (bpm)	17.14 (8.87)	16.55 (9.20)	14.07 (9.68)	16.78 (9.75)
	dHRVLF	-.26 (1.05)	-0.19 (1.04)	-.033 (1.10)	-0.38 (1.11)
	dHRVHF	-1.44 (1.15)	-1.30 (1.15)	-1.39 (1.39)	-1.47 (1.34)
Outcome:	Total Anxiety scores wave 2	0.33 (0.24)	0.47 (0.28)	0.37 (0.27)	0.50 (0.28)

Note: AUC=Area under the curve, the index for the cortisol awakening response, d=difference between measures in supine and standing position, HR=Heart Rate, HRVLF=Heart Rate Variability in the Low Frequency band , HRVHF=Heart Rate Variability in the High Frequency band.

Table 6.4: Regression models for the main effects and interaction effects of the HPA-axis and ANS reactivity measures split up between individuals with low or high parental internalizing problems and split up between boys and girls

Measures	**Low parental internalizing problems**				**High parental internalizing problems**			
	Boys (n=303)		Girls (n=338)		Boys (n=53)		Girls (n=59)	
Block 1: Covariates		ΔR^2 =.230		ΔR^2 =.239		ΔR^2 =.268		ΔR^2 =.231
Block 2: AUC	B= -0.016	ΔR^2 =.004	B= 0.002	ΔR^2 =.002	B= 0.065	ΔR^2 =.016	B= 0.297**	ΔR^2 =.184
dHR	B= -0.001		B= 0.001		B= -0.003		B= 0.003	
Block 3: CAR x dHR	B= -0.004	ΔR^2 =.005	B= 0.007	ΔR^2 =.008	B= -0.007	ΔR^2 =.010	B= -0.003	ΔR^2 =.001
Block 4: AUC x dHR[ad]	B= -0.005	ΔR^2 =.292*	B= 0.004	ΔR^2 =.273	B= -0.007	ΔR^2 =.324	B= 0.005	ΔR^2 =.201
Block 1: Covariates		ΔR^2 =.230		ΔR^2 =.239		ΔR^2 =.268		ΔR^2 =.231
Block 2: AUC	B= -0.018	ΔR^2 =.004	B= -0.001	ΔR^2 =.015	B= 0.064	ΔR^2 =.030	B= 0.283**	ΔR^2 =.192
dHRV LF	B= 0.012		B= -0.033*		B= 0.037		B= -0.033	
Block 3: AUC x dHRVLF	B= -0.006	ΔR^2 <.001	B= 0.055	ΔR^2 =.006	B= 0.084	ΔR^2 =.024	B= -0.023	ΔR^2 =.001
Block 4: AUC x dHRVLF[ad]	B= -0.020	ΔR^2 =.292	B= 0.025	ΔR^2 =.264	B= 0.025	ΔR^2 =.283	B= -0.085	ΔR^2 =.217
Block 1: Covariates		ΔR^2 =.230		ΔR^2 =.239		ΔR^2 =.268		ΔR^2 =.231
Block 2: AUC	B= -0.019	ΔR^2 =.004	B= 0.001	ΔR^2 =.005	B= 0.061	ΔR^2 =.054	B= 0.290**	ΔR^2 =.192
dHRV HF	B= 0.012		B= -0.017		B= 0.043		B= -0.029	
Block 3: AUC x dHRVHF	B= 0.013	ΔR^2 =.001	B= -0.011	ΔR^2 <.001	B= 0.106*	ΔR^2 =.068	B= 0.023	ΔR^2 =.001
Block 4: AUC x dHRVHF[ad]	B= 0.004	ΔR^2 =.289	B= -0.004	ΔR^2 =.275	B= 0.043	ΔR^2 =.227	B= -0.023	ΔR^2 =.200

Note: AUC=Area under the curve, the index for the cortisol awakening response, d=difference between measures in supine and standing position, HR =Heart Rate, HRVLF=Heart Rate Variability in the Low Frequency band , HRVHF=Heart Rate Variability in the High Frequency band, ad=adjusted for co-occurring depressive problems, *p<.05, **p<.01.

Discussion

The present study investigated whether HPA-axis and ANS reactivity predicted the development of anxiety in early adolescence. We used an interactive multisystem approach to test whether an asymmetric activation pattern of the HPA-axis and ANS increases risk for anxiety problems, as suggested by Bauer and colleagues (2002). The role of familial vulnerability was examined, since we hypothesized that associations might only appear in individuals with high scores on parental internalizing problems either due to a threshold effect or due to reduced buffering effects. Gender differences were investigated as boys and girls might show differences in their vulnerability towards anxiety. Lastly, the specificity of the associations was investigated by adjusting for co-occurring depressive problems.

Evidence was found for higher morning cortisol levels in combination with higher HR reactivity as a precursor of higher anxiety levels two years later in boys with low parental internalizing problems. In addition, limited ANS flexibility was a precursor of higher anxiety levels two years later in girls with low parental internalizing problems. Yet, the effect sizes of these associations in individuals with a low familial vulnerability were small. In individuals with high parental internalizing problems, higher morning cortisol levels predicted higher anxiety levels two years later in girls. Further, higher morning cortisol levels in combination with higher baseline vagal reactivity predicted higher anxiety levels two years later in boys with high parental internalizing problems. The effect sizes of associations in individuals with a high familial vulnerability were medium to large.

Interactive multisystem approach

Our finding of higher morning cortisol levels in combination with higher HR reactivity as a precursor of higher anxiety levels two years later in boys with low parental internalizing problems is in contrast with the idea of activation asymmetries of the HPA-axis and sympathetic system as proposed by Bauer and colleagues (2002). It seems that both HPA-axis activity and sympathetic reactivity are higher in this group. The finding of higher morning cortisol levels in combination with higher vagal reactivity as a precursor of higher anxiety levels two years later in boys with high parental internalizing problems is an example of an interaction between HPA-axis activity and ANS reactivity that increases risk for future anxiety problems. Yet, Bauer and colleagues only described asymmetric patterns of HPA-axis and sympathetic activation as a putative risk factor for future mental health problems, and did not discuss the particular role of the vagal system. High HPA-axis activation together with low sympathetic activation was described as an activation pattern that increases risk. Of course, low sympathetic activation usually leads to the same arousal levels as high vagal activation; both decrease HR. Therefore, a pattern of higher HPA-axis activity in combination with higher vagal reactivity that increases risk for future anxiety

problems does seem to be in line with the theory of Bauer and colleagues. Yet, we only found this activation pattern in boys with high parental internalizing problems and effects were different in boys with low parental internalizing problems. Therefore, we may conclude that combined measures of HPA-axis and ANS activity might apply as an indicator for future anxiety in boys, but more research is needed to clarify whether effects are additive, interactional, or both.

Familial vulnerability

Findings of physiological risk for future anxiety were more evident in individuals with high parental internalizing problems than in individuals with low parental internalizing problems. Therefore, our hypothesis that physiological vulnerability for future anxiety might only manifest itself in individuals with high a familial vulnerability because of an underlying familial mechanism or because of less resources to buffer for physiological risk factors as compared to individuals with healthy parents seems to be validated to some extent. Since the present study did not investigate putative buffering we can only speculate on the role of such factors. Further, it is possible that individuals with a high familial vulnerability have an increased risk due to an underlying genetic make-up. Although genetic factors were also not investigated, Table 6.1 shows that morning cortisol levels were highest in girls with a high familial vulnerability. The difference with the other categories turned out to be significant, when we performed post hoc analyses (ANCOVA: $F(3)=3.13$, $p<.05$). It is possible that due to underlying genetic factors, girls with a high familial vulnerability show higher morning cortisol levels. These levels might exceed a certain threshold which makes them more susceptible for future anxiety problems. Evidence is available indicating that especially morning cortisol levels are at least partially determined by genetic factors (Bartels et al., 2003a, 2003b; Rosmond et al., 2001b; Wust et al., 2000a). These factors may influence CRH secretion patterns (Arborelius et al., 1999), feedback effects of cortisol on central glucocorticoid receptors (Rosmond et al., 2001a), or both (Rosmond et al., 2001b). In this way, the sensitivity of an individual to stressful stimuli may be determined, which may increase the risk for anxiety problems.

Of course, heredity only provides one particular explanation of our findings. Other non-genetic familial factors might also be involved. Possibly, parents with high levels of internalizing problems influence their children's vulnerability to anxiety problems through controlling, overprotective parenting behaviours that have been associated with increased risk for anxiety problems in children (Mcleod et al., 2007; Wood et al., 2003). Such parenting behaviours might increase feelings of no control and helplessness in the child which in turn have been associated with increased cortisol levels (Dickerson and Kemeny, 2004; Lewis et al., 2006). Together, feelings of no control and increased cortisol levels might increase the risk for future anxiety problems. Possibly, genetic and parenting factors interact. If a child shows more stressed or anxious behaviour

due to a genetic vulnerability, the parent might be inclined to be even more controlling and overprotective which in turn again increases cortisol and anxiety levels of the child. Investigating the role of genetic and non-genetic familial factors and their interactions was beyond the scope of the present study, but might reveal interesting results in future research.

Gender differences

Different patterns of physiological risk for future anxiety problems were found for boys and girls. In girls we only found main effects of either ANS reactivity or HPA-axis activation, while in boys we found interaction effects of HPA-axis activation and ANS reactivity. Apparently, in girls both systems have their own separate effects depending on the level of parental internalizing problems whereas in boys the interaction between the two systems plays a more important role. This might mean, that in boys a number of biological factors (e.g. genetics, HPA-axis functioning, vagal reactivity) are required to have an additive or interactional effect before the risk for future anxiety problems increases, while in girls the main effect of one separate system (i.e. the HPA-axis) might be stronger. Of course, further research is needed to deepen our knowledge on the particular role of several biological and environmental factors in the development of anxiety in boys and girls.

Specificity

When we adjusted for co-occurring depressive problems in our analyses, the interaction effect of AUC and dHR in boys with low parental internalizing problems became significant. This means that the effect is specific for anxiety, as apart from depression. On the other hand, the effect of interaction between AUC and dHRV HF in boys with high parental internalizing problems did not remain significant after adjusting for co-occurring depressive problems. This reflects that the effect is not specific for anxiety, but probably applies to the broader dimension of internalizing problems. The main effect of AUC in girls with high scores on parental internalizing problems also remained significant in model 4. This means that the effects of morning cortisol levels on the anxiety levels of these individuals are specific for anxiety, as apart from depression. Possibly, the role of the HPA-axis in anxiety is specific for anxiety (Greaves-Lord et al., 2007a, Chapter 3), but the role of the vagal system does not seems to be specific for anxiety (Greaves-Lord et al., 2007b, Chapter 2).

Strenghts & limitations

The present study investigated the relation of HPA-axis and ANS reactivity with anxiety levels two years later in a large sample of both boys and girls, which provided the opportunity to investigate gender differences. Further, because information on co-occurring depressive problems, pubertal stage and familial vulnerability was also assessed, the role of these factors could also be examined.

Unfortunately, in this study, HPA-axis and ANS reactivity were not assessed at the same time in response to the same stressor. Thus, future studies that will assess both measures simultaneously might reveal different results. The fact that we split our sample into categories of low versus high parental internalizing problems and boys versus girls, provided the opportunity to gain more insight in the role of familial vulnerability and gender, but of course also made the findings more vulnerable to chance and less suitable for generalisation. Our findings reveal associations that are specific for these categories of individuals and cannot be generalized to other populations. Yet, when investigating certain risk factors for a problem that is known to be influenced by several other factors as well, it might not be possible to generalise the findings. It might be important to use several factors to define (sub-)samples to gain more insight in the role of specific factors that might only have effects under certain circumstances. For instance, this study revealed that physiological risk for future anxiety is most evident in individuals with a high familial vulnerability and differs between boys and girls. Thus, when investigating putative physiological risk factors for future anxiety problems, the role of familial vulnerability and gender should be taken into account. Future studies should therefore investigate the role of -and interactions with- several biological and environmental factors to gain more insight in the aetiology of anxiety.

7 General Discussion

Kirstin Greaves-Lord

Introduction

The aim of the present thesis was to extend the existing knowledge on the aetiology of anxiety. More specifically, the purpose was to gain more insight in the role of the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA)-axis in the development of anxiety in early adolescence. Below, first the research questions of this thesis are repeated and the main results and conclusions are summarized. Then, findings are interpreted in the light of a broad theoretical framework and other important issues are discussed.

Main results and conclusions

The answers to the research questions and the main findings are summarized in Table 7.1. Below, the findings are discussed in more detail and main conclusions are drawn.

The first research question was: *1) Is anxiety associated with signs of hyperarousal, whereas depression is not?* Chapter 2 shows that anxiety is associated with signs of hyperarousal; low vagal activity is associated with anxiety, mostly in boys. However, this association is not specific for anxiety. We also found associations between depression and signs of hyperarousal; high heart rate (HR) was associated with depression in both sexes, and low vagal activity was associated with depression in girls. Interestingly, analyses also revealed that depression was associated with signs of low arousal (e.g. high vagal activity) in boys. Apparently, the idea that hyperarousal is specific for anxiety and not depression is too simple to reflect the more complex reality.

Secondly, the next research questions were examined: *2 a) Are high cortisol levels associated with high anxiety levels?,* and *b) Is the persistence of anxiety problems associated with high cortisol levels?* Daytime cortisol levels were not associated with current anxiety levels, as demonstrated in Chapter 3. Yet, morning cortisol levels were higher in individuals with persistent anxiety problems. Thus, although cortisol levels are not associated with current anxiety levels, they are associated with the persistence of anxiety. Possibly, underlying physiological mechanisms only become evident in individuals with persistent anxiety problems, because this group is characterised by an inborn vulnerability. However, another possibility is that the higher morning cortisol levels are the result of the stress that accompanied the anxiety problems. It is likely that persistent anxiety problems are accompanied by high levels of stress, resulting in elevated cortisol concentrations. Elevations in cortisol levels that persist across time possibly tune HPA-axis activity to a higher level. For instance, elevated cortisol levels could result in damage of the hippocampal glucocorticoid receptors or even a loss of hippocampal neurons (Sapolsky et al., 1986), which would affect the glucocorticoid feedback inhibition of CRH secretion (Young et al., 1990) and would result in higher CRH and cortisol concentrations.

Table 7.1: Overview theories on the association of ANS functioning and HPA-axis functioning with anxiety

Research Questions	Answers & Main findings
1) Is anxiety associated with signs of hyperarousal, whereas depression is not?	No, anxiety is cross-sectionally associated with signs of hyperarousal, but also depression is sometimes cross-sectionally associated with signs of hyperarousal ($0\% < R^2 < 2\%$)
2) a. Are high cortisol levels associated with high anxiety levels? b. Is the persistence of anxiety problems associated with high cortisol levels?	a. No, there is no cross-sectional association b. Yes, retrospective data show that individuals with persistent anxiety problems have higher morning cortisol levels than individuals with no, or only current anxiety problems ($R^2 = 3\%$)
3) a. Do measures of ANS (re)activity predict future anxiety levels? b. Do such associations differ between boys and girls? c. Are such associations are specific for anxiety, as apart from depression?	a. Yes, measures of ANS reactivity predict anxiety levels two years later, but only in girls ($0\% < R^2 < 1\%$) b. Yes, associations only exist in girls c. Mostly not, only associations with HRV LF seem to be specific for anxiety as apart from depression
4) a. Do cortisol measures predict future anxiety levels? b. Are distinct developmental pathways of anxiety associated with different cortisol levels?	a. No, there is no longitudinal association b. Yes, prospective data show that individuals with increasing anxiety levels have higher morning cortisol levels than those with persistently low, decreasing or persistently high anxiety levels (R^2=1%) and that individuals with persistently high anxiety levels have lower evening cortisol levels than all other individuals (R^2=1%)
5) a. Do asymmetric activation patterns of ANS and HPA-axis activity predict future anxiety levels? b. Are such associations more evident in individuals with a high familial vulnerability for internalizing problems, i.e. with high parental internalizing problems? c. Are such associations different for boys and girls?	a. Sometimes, only in boys b. Yes, in boys with a high familial vulnerability we find that high morning cortisol in combination with high vagal reactivity predict anxiety levels two years later (R^2=7%), and in girls with a high familial vulnerability there is a main effect of morning cortisol levels (R^2=19%) c. Yes, see above

Further we investigated whether *3 a) measures of ANS (re)activity predict future anxiety levels?,* and whether *b) such associations differ between boys and girls?,* and whether *c) such associations are specific for anxiety, as apart from depression?* Chapter 4 reveals that measures of ANS reactivity predict anxiety levels two years later, but only in girls, and effect sizes are small. The associations we uncovered were mostly not specific for anxiety, but applied to the broader dimension of internalizing problems. One measure of ANS activity (i.e. HRV LF in supine rest) was specific for anxiety: associations were only uncovered when we adjusted for co-occurring depressive problems. It appears that ANS reactivity is one additional or interactional factor in the development of anxiety problems in girls, which does not apply for anxiety only, but probably for the broader band of internalizing problems.

Additional research questions were: *4 a) Do cortisol measures predict future anxiety levels?,* and *b) Are distinct developmental pathways of anxiety associated with different cortisol levels?* Daytime cortisol levels do not predict future anxiety levels, as illustrated in Chapter 5. Yet, morning cortisol levels were higher in individuals with increasing anxiety levels, and evening cortisol levels were lower in individuals with persistently high anxiety levels. Clearly, anxiety is not associated with either higher or lower cortisol levels, but it rather seems that several aspects of HPA-axis activity are differently associated with anxiety. These different aspects and their mechanisms will be discussed below when findings are discussed in the light of the theories that were introduced in the general introduction.

Our final research questions were: *5 a) Do asymmetric activation patterns of ANS and HPA-axis activity predict future anxiety levels?,* and *b) Are such associations more evident in individuals with high familial vulnerability for internalizing problems, i.e. with high parental internalizing problems?,* and *c) Are such associations different for boys and girls?* As Chapter 6 demonstrates, the interaction between indices for vagal and HPA-axis reactivity predicted future anxiety levels, but only in boys with a high familial vulnerability. This association might only appear in boys with a high familial vulnerability due to an increased physiological risk and/or less buffering factors in this specific group of individuals with a high familial vulnerability. Results differed for boys and girls, which shows -again- that it is important to take into account gender differences when investigating putative indicators of increased risk for future anxiety problems. The importance of gender differences and putative underlying mechanisms will be further discussed below.

Table 7.2: Overview theories on the association of ANS functioning and HPA-axis functioning with anxiety, and there support or non-support by the findings of this thesis

Authors	Theories (propositions)	Support?
1) Clark and Watson (1991)	Hyperaurousal (i.e. higher ANS and HPA-axis (re)activity) ↔ anxiety disorders	Few (Chapter 2), but mostly not
2) Kagan et al. (1988)	Low threshold central nervous system activation → higher sympathetic and HPA-axis activation (hyperarousal) → withdrawal, avoidance, fearfulness → susceptibility to anxiety	Some (Chapter 3,6)
3) Gunnar and Vazquez (2001)	Stressful events early in life → frequent elevations in cortisol levels → downregulation HPA-axis, low cortisol levels ↕ Stressful events early in life → increased risk anxiety problems (Goodyer and Altham,1991)	Some (Chapter 5)
4) Porges et al. (2001)	Relatively low vagal (re)activity → increased risk physical and mental health problems, among which anxiety	Some (Chapter 4), but Chapter 6 condradicts
5) Friedman and Thayer (1998)	Limited psychophysiological flexibility → relatively low heart rate variability ↔ anxiety disorders	Some (Chapter 4), but Chapter 6 condradicts
6) Bauer et al. (2002)	Asymmetric activation patterns (HPA↑ and sympathatic↓, or HPA↓ and sympathetic ↑) ↔ increased risk anxiety	Few (Chapter 6)

Theoretical framework

In the general introduction six important theories on the association of ANS and HPA-axis functioning with anxiety were introduced. Below, the theories are repeated in Table 7.2 with an extra column indicating the support, or non-support, through the present findings. Then, the support, rejection, or integration of all theories is discussed in more detail.

Firstly, in Chapter 2 we found evidence for and against the theory of Clark and Watson (1991). Anxiety was associated with signs of hyperarousal, but also depression was associated with our measures of arousal. Interestingly, we found some evidence for an association between low arousal and depression in boys. Although the tripartite model of Clark and Watson only implicates that there is no hyperarousal in depression, and does not provide suggestions about the expected arousal patterns in depression, our findings put forward the interesting idea of high arousal in anxiety versus low arousal in depression. Yet, we also found associations between measures of high arousal and depression. Therefore, we conclude that the idea of hyperarousal in anxiety and not in depression is too simple to reflect the more complex reality.

Secondly, we found some evidence for the theory of Kagan et al. (1988). Morning cortisol levels were higher in individuals with persistent anxiety problems (Chapter 3), and in individuals with increasing anxiety levels (Chapter 5). Also, higher morning cortisol levels predicted anxiety levels two years later in girls with a high familial vulnerability (Chapter 6). Yet, because initial anxiety levels (wave 1) were already higher in all these groups of individuals, we cannot rule out the possibility that cortisol levels were higher in these individuals due to anxiety problems that already existed. Possibly, anxiety problems are accompanied by high levels of stress, resulting in elevated cortisol concentrations as discussed above. Therefore, we cannot solely conclude that high morning cortisol levels underlie anxiety. Probably, there is a two-way relationship in which high anxiety levels increase morning cortisol levels, and higher morning cortisol levels might in turn lead to (even) higher anxiety levels.

Thirdly, the theory of Gunnar and Vazquez (2001) should be discussed in some more detail. At first, the findings of higher morning cortisol levels discussed above seem to contradict this theory. However, we also found evidence for lower evening cortisol levels in individuals with persistently high anxiety levels (Chapter 5). Further, although the theory of Kagan and colleagues and of Gunnar and Vazquez seem to contradict, actually, they do not rule each other out. In fact, it is possible that initially an inborn tendency towards higher morning cortisol levels can lead to higher anxiety levels, while prolonged stress that accompanies high anxiety levels, might first lead to (further) elevations in cortisol levels and eventually lead to down-regulation of components of the HPA-axis. In a recent meta-analysis, Miller and colleagues (2007) showed that much of the variability in findings on HPA-axis activity (high versus low) and chronic stress is attributable to stressor features and person features. Timing especially is

an important element, as HPA-axis activity is elevated at stressor onset, but reduces as time passes. Further, HPA-axis activity seems to be shaped by a person's response to the situation; it increases with subjective distress, but is lower in persons with post traumatic stress. Our findings in Chapter 5 support this view. Morning cortisol levels are higher in individuals with increasing anxiety levels (problems just started), while evening cortisol levels are lower in individuals with persistently high anxiety levels (prolonged problems). Previous research has also shown that morning cortisol levels are mainly determined by genetic influences, while evening cortisol levels are mainly influenced by environmental factors (Bartels et. al., 2003, Wust et. al., 2000a). Thus, whereas recent anxiety problems might be associated with genetically influenced aspects of HPA-axis activity, persistent anxiety problems might be associated with mainly environmentally influenced aspects of HPA-axis activity. Our findings in Chapter 3 do not completely fit this idea. Yet, since the retrospective data might not be as reliable as prospective data, and possibly mostly reflect the current anxiety levels due to recall-bias, it is possible that the anxiety problems in this group might not have existed for such a very long period after all, and cortisol levels are still elevated in this group, but will eventually drop after down-regulation of components of the HPA-axis.

Fourthly, in Chapter 4 some evidence was found for the theory of Porges et al. (2001). Low vagal reactivity predicted anxiety levels two years later in girls. Yet, in Chapter 6, we found that in boys with a high familial vulnerability, high vagal reactivity in combination with higher morning cortisol levels predicted anxiety levels two years later. Chapter 2 shows that vagal activity might be differently associated with symptoms of anxiety versus symptoms of depression. Since anxiety and depression often co-occur, it is difficult to disentagle the different mechanisms, and thus more research is needed to further investigate the theory of Porges et al. (2001).

Fifthly, the findings in Chapter 4 support the theory of Friedman and Thayer (1998) that limited psychophysiological flexibility, as for instance reflected in low HRV reactivity, is associated with anxiety problems. Measures of low ANS reactivity predicted anxiety levels two years later in girls. Yet, as discussed above, in Chapter 6 higher vagal reactivity predicted anxiety levels two years later. Thus, although our findings in Chapter 4 support this theory, the interactions with other physiological systems of ANS reactivity should first be further investigated, before the complex interplay between all these factors can be fully understood.

Lastly, our findings in Chapter 6 partly support the theory of Bauer et al. (2002), since the interaction between high cortisol levels and low vagal reactivity predicted anxiety levels two years later in boys with a high familial vulnerability. However, the findings in boys with a low familial vulnerability contradicts with this theory, and no effects of interactions between ANS and HPA-axis reactivity were found in girls. Recently, Gordis and colleagues (2006) found that the interaction between the HPA-axis and the sympathetic system was associated

with parent-reported aggression in a cross-sectional study in adolescents. In a recent study of Lewis and colleagues (2006) of infants, anger was related to increased heart rate, but not related to cortisol, whereas sadness was related to increased cortisol, but not related to heart rate. Yet, they did not find evidence for interactions between the two systems in relation to anger or sadness. Since only few studies have addressed the theory of Bauer and colleagues, it is important to investigate their hypothesis to a lager extent.

Predictive role of physiological measures for future anxiety problems

Although cortisol levels were associated with different developmental pathways of anxiety, in general, daytime cortisol levels did not predict anxiety levels two years later in the present general population sample of young adolescents. Associations were stronger in girls with a high familial vulnerability for internalizing problems. Apparently, in combination with other factors (gender, familial vulnerability), morning cortisol levels are better indicators for anxiety levels two years later. Measures of ANS (re)activity predicted future anxiety levels in girls. Yet, effects were specific for girls, and effect sizes were small, indicating that these findings are of theoretical relevance, but do not have strong clinical implications within our general population sample.

The interaction between measures of ANS and HPA-axis activity was a predictor of future anxiety levels, but only in boys, and the effect was stronger in boys with a high familial vulnerability than in boys with a low familial vulnerability. High morning cortisol levels in combination with high vagal reactivity explained 6.8% of the variation in the wave 2 anxiety levels in boys with a high familial vulnerability. Clearly, high morning cortisol levels, and high vagal reactivity increase the risk for anxiety problems only in a certain subsample that was defined on the basis of gender and familial vulnerability. Thus, to gain a clearer insight in the predictive role of physiological factors for the development of anxiety problems, physiological factors might be best investigated in subsamples that are defined on the basis of other factors that are known to play a role in the aetiology of anxiety as well.

Gender differences

Findings concerning HPA-axis activity mostly did not differ between boys and girls. Associations between cortisol levels and developmental pathways of anxiety pertained to both boys and girls. Yet, morning cortisol levels are only a strong predictor of anxiety levels two years later in girls with a high familial vulnerability. Cortisol levels might be higher in girls due to the influence of female sex steroids. Estrogen directly stimulates the CRH gene, which may explain the slightly higher cortisol levels in girls and their susceptibility to anxiety (Magiakou et al., 1997).

Associations between measures of ANS activity and anxiety versus depression differed between boys and girls. Associations of low RSA in supine position with

anxiety were specific for boys, and not for girls. On the other hand, the association of low HRV LF in supine position with depression was specific for girls, and not for boys. Further, measures of ANS (re)activity only predicted future anxiety levels in girls, and not in boys. Whereas low vagal activity at supine rest was associated with anxiety levels at age 10-13 in boys, low vagal reactivity was associated with anxiety levels at age 13-15 in girls. Maybe, the association between ANS functioning and anxiety changes across adolescent development. Both the incidence of anxiety problems, and ANS functioning change across pubertal development. Since the ANS and sex hormonal systems are known to interact (Stratakis and Chrousos, 1995), a certain level of either male or female sex hormones may trigger limited ANS (re)activity to become an additional risk factor for the development of anxiety problems. For instance, in a study of 5 to 17 year old individuals from the general population, norepinephrine (NE) levels increased significantly with advancing puberty and increasing testosterone levels in boys (Weise et al., 2002). Concentrations of epinephrine, another catecholamine that plays an important role in the sympathetic system, decreased significantly with advancing puberty and were higher in boys than in girls. Further, epinephrine levels were correlated with estradiol and testosterone levels. In the present investigations, preliminary analyses indicated that Tanner stage was associated with some of the ANS measures and with anxiety levels at wave 2. Addition of Tanner Stage also improved the fit of the models. Tanner stage reflects physical development and might in some part reflect levels of sex steriods that play a role in the relationship between ANS (re)activity and anxiety.

Taken together, there is a complex interplay between gonodal and stress-related physiological systems that also changes across pubertal development. Clearly, more research is needed to gain a better understanding of the role of sex hormones and stress physiology in the development of anxiety. Thus, although the exact gender differences in physiological risk for future anxiety still need further examination, our results clearly demonstrate that it is very important to take into account gender differences when investigating aetiological mechanisms that may underlie anxiety.

Specificity

Associations of cortisol levels with particular developmental pathways of anxiety were specific for anxiety, as apart from depression. On the other hand, most associations between measures of ANS (re)activity were not as specific for anxiety, but mainly applied to the broader dimension of internalizing problems. Only the effects of HRV LF were specific for anxiety: associations with measures in rest were uncovered when we adjusted for co-occurring depressive problems, and associations with measures of reactivity remained significant after adjusting for co-occurring depressive problems.

The effect of the interaction between morning cortisol levels and vagal reactivity in boys with a high familial vulnerability did not remain significant after adjusting for co-occurring depressive problems. Yet, the significance of the association of anxiety with the interaction between morning cortisol levels and HR reactivity was only uncovered after adjusting for co-occurring depressive problems. Evidently, the association of interactions between measures of HPA-axis and ANS reactivity with anxiety versus depression are very complex, and need to be investigated further, before firm conclusions can be drawn. It seems that the relation with the activity of the HPA-axis, is mostly specific for anxiety, whereas associations with the (re)activity of the ANS mostly apply to a broader dimension of problems, e.g. internalizing problems, but clearly more research is needed to unravel the exact relationships.

Basal activity *versus* reactivity to a physical stressor *versus* reactivity to mental stress

Most of our measures of HPA-axis or ANS activity concerned basal activity. For instance, the separate cortisol samples (1,2, and 3) were all measures of basal HPA-axis activity. The measures of ANS activity in supine rest also concerned basal ANS activity. The AUC can be regarded as an index of the cortisol awakening response, which reflects the response to a physical stressor, i.e. waking up. Similarly, the measures of ANS reactivity to the act of standing up can be regarded as an index for ANS reactivity to a physical stressor. The reaction to other stressors, such as mental or emotional stressors, was not assessed in the present studies.

In our studies, we did not find clear evidence for an association between basal activity of either the ANS or the HPA-axis and anxiety. We did find some evidence for a relationship between responsiveness of the two systems and anxiety. Our index for the cortisol awakening response was associated with both persistent and increasing anxiety problems and with anxiety levels two years later in girls with a high familial vulnerability. Measures of ANS reactivity predicted anxiety levels two years later in girls. However, effect sizes were small. Since effects were more evident for measures of responsiveness to physical stressors than for measures of basal activity, we expect that measures of reactivity to other stressors, such as mental or psychological stressors, might reveal stronger associations with anxiety. For example, effect sizes were larger in a study of reactivity to several mental stress tasks in children with either internalizing, externalizing or no problems (Boyce et al., 2001). However, many other studies (e.g. Dorn et al., 2003) do not report effect sizes. To understand the clinical relevance of findings, it is important to report effect sizes. Small effects can still be interesting and of theoretical relevance, but cannot hold strong clinical implications.

Familial vulnerability

Associations between morning cortisol levels and anxiety levels two years later were more evident in the group of girls with a high familial vulnerability. Moreover, the effect of the interaction between vagal and HPA-axis reactivity on anxiety levels two years later only appeared in boys with a high familial vulnerability. Effects were not as strong in individuals with a low familial vulnerability. These findings indicate that analyses in subsamples with a high familial vulnerability can uncover associations between measures of physiological (re)activity and anxiety. Thus, it is important to take into account the role of familial vulnerability in studies on the aetiology of anxiety.

General population samples versus (sub-)clinical samples

Many analyses in our general population sample did not reveal significant effects. If significant effects were found, effect sizes were small. Only when we selected the individuals with a high familial vulnerability, effects were stronger. Most previous studies were confined to clinical samples (Boyce et al., 2001; Dorn et al., 2003; Feder et al., 2004; Garralda et al., 1991; Gerra et al., 2000; Goenjian et al., 2003; Yeragani et al., 2001) and although the findings cannot be generalized due to possible selection bias, associations are more evident in such samples of individuals with higher anxiety levels. This might mean two things. First, associations might only be uncovered in individuals with higher anxiety levels. Secondly, stress physiology might only be altered in individuals with more severe and sustaining problems, and these alterations are not in the first place an underlying cause of the anxiety problems, but might rather be an effect of accumulating problems and risk factors that in turn might increase anxiety levels.

Limitations

The specific limitations of each study have been discussed in the previous chapters. Therefore, some more general limitations are discussed in this section. First of all, when assessing measures of ANS or HPA-axis (re)activity, one should always bear in mind the reliability of such measures. Since these measures reflect stress, and assessment of physiological (re)activity might somewhat increase stress levels in the participants, one can never rule out the effects of stress totally. However, cortisol levels were assessed at home, and stress levels might be maximally reduced in this setting. ANS (re)activity was assessed at school, which might be less stressful than in a laboratory setting. Yet, the home and school environment do also have some disadvantages. The exact timing of the cortisol sampling cannot be inspected. Further, the influence of possible physical activities before or during data collection cannot be ruled out. Thus, although assessment of physiological measures at home or school reduces the influence of stress, it might influence the reliability of the data in other ways.

Secondly, only in our first study (Chapter 2) we used data of both the child and their parent. Although we acknowledge the importance of using multiple

informants to obtain a complete view on (pre)adolesent's problems (van der Ende and Verhulst, 2005; Verhulst, 1995), since no parent version of the RCADS is available, and the CBCL does not make a clear distinction between anxiety and depression, we decided to focus our investigations on self-reports.

Lastly, re-assessment of anxiety levels was after a period of only two years. Maybe, this period was too short to observe a clear change in anxiety levels. Many symptoms of anxiety are known to increase after the age of 15 (Thyer et al., 1985; Wittchen et al., 1998), so it is possible that associations will become more evident after a longer period of time. Fortunately, the TRAILS cohort will be re-assessed biennially until the age of 24, so future investigations of this sample can test this hypothesis.

Recent studies at the Department of Child & Adolescent Psychiatry Erasmus MC Sophia

Recently, other members of our department also investigated ANS and HPA-axis functioning in a sample with clinical anxiety disorders (Kallen, Dieleman, Dierckx, personal communication) and in a high risk population (Van der Vegt, personal communication). Kallen and colleagues found evidence for low vagal reactivity and high sympathetic reactivity to a stress task in children with an anxiety disorder. Further, they found that interactions between measures of HPA-axis and sympathetic activity were strongly associated with anxiety disorders (Kallen, personal communication). Preliminary findings suggest an important role of early adversities and pregnancy (Van der Vegt, personal communication). Further, Sondeijker and colleagues previously investigated the relation of ANS and HPA-axis functioning with disruptive behaviours in the TRAILS sample (Sondeijker et al., 2006, submitted a+b), and stressed the importance of the interaction between physiological, and other biological, or environmental factors in the development of psychiatric problems in adolescence.

State of the art

Taken together, the present and previous findings and their interpretation in a broad theoretical framework lead to a very complex and extensive picture. Figure 7.1 shows a schematic overview of factors related to the development of anxiety. As can be seen in this Figure, the present thesis only considered part of a complex interplay of many factors. This thesis mostly focussed on investigating the associations j, k and m. By investigating the role of co-occurring depressive problems we also partly investigated the associations h and i. Some other associations have been the focus of previous research, but clearly, much more research is needed to clarify all these (and possibly other) associations and to integrate the findings.

Implications and recommendations for future research

Although the findings in this thesis are of great theoretical relevance, there are little direct clinical implications, since most effect sizes are small. We do not believe that in the general population measures of basal ANS or HPA-axis activity, or reactivity measures to a physical stressor, can be used to identify individuals at risk for anxiety problems. However, we did not investigate reactivity to stress tasks. Investigations of reactivity to for instance a mental stress task might reveal stronger effects. Also, findings might be different when clinical samples including participants with more severe problems are investigated. If clinical studies will point out that ANS or HPA-axis (re)activity plays a more important role in anxiety disorders -in the future- interventive programs aimed at normalising ANS activity, such as relaxation training and slowed respiration, may be helpful (Sakakibara et al., 1996). If, however, findings would be similar in clinical samples, this would imply that, although effective, ingredients of treatment protocols that pertain to reducing hyperarousal to diminish anxiety levels might lack a scientific rationale. This, as said, does not mean that such ingredients are ineffective. Since we found that associations mostly were not specific for anxiety, but probably applied to depression as well, it could even be the case that such treatments might be helpful to also tackle depression, or even other types of problems as well. As the brain is capable of profound plasticity during adolescence, this developmental period might be suitable for interventions, and might withhold opportunities to extenuate the negative consequences of earlier adversities (Romeo and Mcewen, 2006). Yet, first ANS and HPA-axis (re)activity must be further investigated in clinical samples, to obtain a clearer view of possible clinical implications. Moreover, future investigations of the role of ANS and HPA-axis functioning in the development of anxiety should take into account the complex underlying interplay of genetic, physiological, psychological, and environmental factors that is shown in Figure 7.1. The findings in this thesis provide an interesting point of departure for such future investigations.

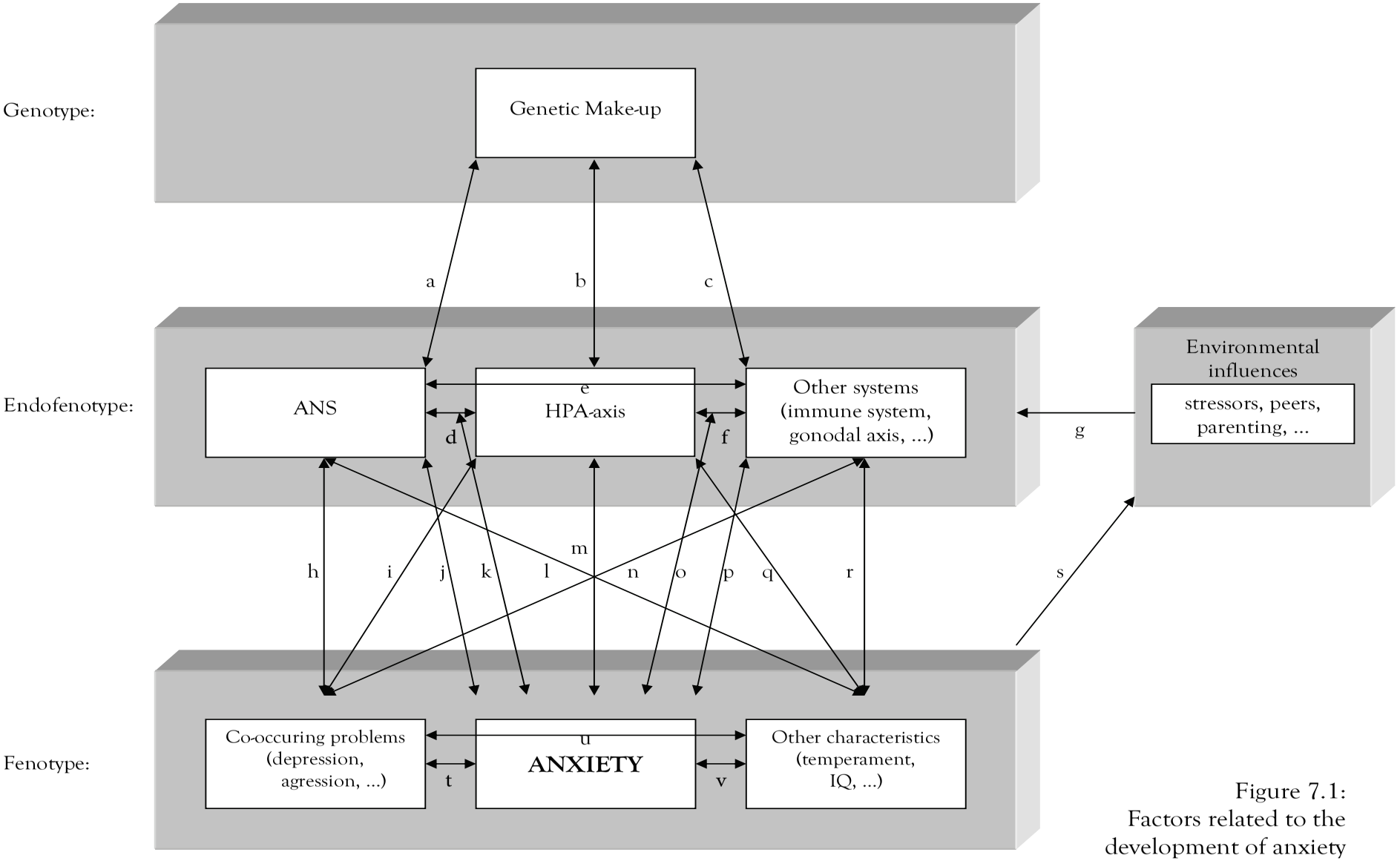

Figure 7.1:
Factors related to the development of anxiety

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Summary & Samenvatting

Summary

Since anxiety problems occur frequently, result in considerable suffering and impairment, and tend to persist over time, it is important to investigate putative underlying mechanisms. The aim of the present thesis was to extend the existing knowledge on the aetiology of anxiety by examining the role of two physiological stress response systems, the autonomic nervous system (ANS; consisting of the sympathetic and parasympathetic/vagal branche) and the hypothalamic-pituitary-adrenal (HPA)-axis, in the development of anxiety in early adolescence. The general idea is that some individuals are characterised by increased (re)activity of these two systems; they are more sensitive to stressors than others (so called 'hyperaroused'). This increased sensitivity might put them at risk for future anxiety problems.

In *Chapter 1*, the theoretical background and the main research questions were presented. The main research questions were: 1) Is anxiety associated with signs of hyperarousal, whereas depression is not? 2) Are high cortisol levels associated with high anxiety levels and is the persistence of anxiety problems associated with high cortisol levels? 3) Do measures of ANS (re)activity predict future anxiety levels, and are such associations different between boys and girls, and specific for anxiety, as apart from depression? 4) Do cortisol measures predict future anxiety levels, and are distinct developmental pathways of anxiety associated with different cortisol levels? 5) Do assymmetric activation patterns of ANS and HPA-axis activity predict future anxiety levels, are such associations more evident in individuals with high familial vulnerability, and different for boys and girls? To answer these questions the data of the first two assessment waves of TRAILS, a prospective cohort study of Dutch young adolescents, were used.

In *Chapter 2,* we examined whether signs of hyperarousal were specific for anxiety, and not depression. This chapter shows that anxiety is associated with signs of hyperarousal; low vagal activity was associated with anxiety, mostly in boys. However, this association was not specific for anxiety. We also found associations between depression and signs of hyperarousal; high heart rate (HR) was associated with depression in both sexes, and low vagal activity was associated with depression in girls. Interestingly, analyses also revealed that depression was associated with signs of low arousal (e.g. high vagal activity) in boys. Apparently, there are some interesting gender differences and the idea that hyperarousal is specific for anxiety and not depression is too simple to reflect the more complex reality.

In *Chapter 3,* we investigated whether high cortisol levels were associated with high anxiety levels and whether the persistence of anxiety problems was associated with high cortisol levels. Cortisol levels were not associated with current anxiety levels. Yet, morning cortisol levels were higher in individuals with persistent anxiety problems. Thus, although cortisol levels were not associated with current anxiety levels, they were associated with the persistence of

anxiety. Possibly, underlying physiological mechanisms only become evident in individuals with persistent anxiety problems, because this group is characterised by an inborn vulnerability. However, another possibility is that the higher morning cortisol levels are the result of the stress that the anxiety problems were accompanied by. Thus, it still remains difficult to disentangle cause and effect in the complex relationship between HPA-axis activity and anxiety.

In *Chapter 4*, it was investigated whether measures of ANS (re)activity predicted future anxiety levels, and whether such associations were different between boys and girls, and specific for anxiety, as apart from depression. The analyses revealed that measures of ANS reactivity predicted anxiety levels two years later, but only in girls. The associations we uncovered were mostly not specific for anxiety, but applied to the broader dimension of internalizing problems. Since the effect sizes were small, it appears that ANS reactivity is one additional or interactional factor in the development of anxiety problems in girls, which does not apply for anxiety only, but probably for the broader band of internalizing problems.

In *Chapter 5,* it was examined whether cortisol measures predicted future anxiety levels, and whether distinct developmental pathways of anxiety were associated with different cortisol levels. Cortisol levels did not predict future anxiety levels. However, morning cortisol levels were higher in individuals with increasing anxiety levels, and evening cortisol levels were lower in individuals with persistently high anxiety levels. Clearly, anxiety is not associated with only higher or only lower cortisol levels, but it rather seems that different aspects of HPA-axis activity (e.g. genetic/environmental) are differently associated with anxiety.

In *Chapter 6*, we investigated whether asymmetric activation patterns of ANS and HPA-axis activity predicted future anxiety levels, whether such associations were more evident in individuals with high familial vulnerability for internalizing problems, and whether such associations were different for boys and girls. This chapter demonstrates that the interaction between indices for vagal and HPA-axis reactivity predicted future anxiety levels, but only in boys with a high familial vulnerability. This association might have only appeared in boys with a high familial vulnerability due to an increased physiological risk and/or less buffering factors in this specific group of individuals with a high familial vulnerability. Results differed for boys and girls, which shows that it is important to take into account gender differences when investigating putative indicators of increased risk for future anxiety problems.

Finally, in *Chapter 7* the main findings and conclusions of this thesis were presented and discussed in the light of the theoretical framework and recent research. We discussed that we did not find clear evidence for the existing theories on physiological arousal and anxiety that are often too restricted or simplistic to reflect a more complex reality. We concluded that although some theoretically relevant associations were found, in clinical practice physiological

measures alone cannot be used as indicators for future anxiety problems in adolescents from the general population. We put forward that associations are not specific for anxiety, but probably apply to other affective or even behavioural problems as well. For future research we suggest that samples with higher anxiety levels should be investigated, possibly revealing different results. In addition, physiological measures should be investigated in combination with other biopsychosocial factors, such as familial vulnerability, to gain more insight in the complex underlying mechanisms of anxiety. We propose that measures of physiological reactivity (either to physical or to psychological stressors) might be better indicators for future anxiety problems than measures of basal activity. We -again- underscore the importance of taking into account and further investigating gender differences. The findings described in the present thesis provide an interesting point of departure for future investigations.

Samenvatting

Het doel van dit proefschrift is om de bestaande kennis over het ontstaan van angstklachten te vergroten. Angstklachten komen veel voor en ze beperken een persoon in zijn psychische, cognitieve en sociale functioneren. Het is daarom belangrijk om te onderzoeken hoe angstklachten ontstaan, zodat in de toekomst angstklachten voorkomen of beter behandeld kunnen worden.

Er zijn vele biologische, psychologische en sociale factoren die een rol spelen in het ontstaan van angstklachten. In dit proefschrift worden met name de biologische factoren onder de loep genomen. De werking van twee zogenaamde 'stress systemen' in het menselijk lichaam hangt mogelijk samen met het ontstaan van angstklachten. Daarom wordt in dit proefschrift de rol van deze twee stress systemen in het ontstaan van angstklachten verder onderzocht.

Ten eerste wordt de rol van het autonome zenuwstelsel onderzocht. Dit stress systeem is onderdeel van het centrale zenuwstelsel (zie figuur 1.1) en is betrokken bij de snelle reactie op stress. Het bestaat uit twee tegengestelde, maar elkaar aanvullende deelsystemen; het sympatische en het parasympatische zenuwstelsel. Het sympatische systeem is vergelijkbaar met een gaspedaal. Het activeert het lichaam in geval van stress. De hartslag stijgt bijvoorbeeld, zodat de persoon beter in staat is te vluchten of te vechten in reactie op de 'stressor' (zeg een inbreker). Het parasympatische systeem daarentegen kan vergeleken worden met de rem. Dit systeem vertraagt allerlei lichamelijke processen, waaronder de cardiovasculaire regulatie (hartslag en bloeddruk). Zo kan het lichaam weer ontspannen na de stress. Samen reageren deze twee deelsystemen op allerlei veranderingen in het lichaam of de omgeving ('stressoren') en passen ze de lichamelijke processen zo goed mogelijk hierop aan.

Ten tweede wordt de rol van de hypofyse-bijnier-as in het ontstaan van angstklachten onderzocht. De hypofyse-bijnier-as wordt in het Engels de 'hypothalamic-pituitary-adrenal-axis' genoemd, ofwel de HPA-axis en in het Nederlands ook wel de HPA-as genoemd (zie figuur 1.1). De HPA-as is het wat langzamer werkende stress systeem waar uiteindelijk het 'stress hormoon' cortisol wordt aangemaakt. Cortisol zorgt ervoor dat allerlei processen in het lichaam in balans komen en blijven. Bij stress, zoals veranderingen in het lichaam of de omgeving, wordt er meer cortisol aangemaakt om ervoor te zorgen dat het lichaam in balans blijft.

Het algemene idee is dat de werking van het autonome zenuwstelsel en de HPA-as een rol speelt in het ontstaan van angstklachten. De stress systemen van sommige mensen zouden gevoeliger zijn voor veranderingen in het lichaam of in de omgeving ('stressoren') en zouden dus eerder of anders reageren. Er zijn minder 'prikkels' nodig om de stress systemen te activeren. Deze mensen zijn dus eerder 'geprikkeld', of in het Engels 'aroused'. Dit kenmerk wordt in de literatuur 'hyperarousal' genoemd (denk aan de termen 'prikkelbaar', 'hypersensitief', of 'overgevoelig'). Omdat bepaalde situaties (denk aan het houden van een presentatie!) eerder tot 'hyperarousal' leiden bij deze mensen,

worden deze situaties eerder als beangstigend ervaren, waardoor deze mensen zich eerder zullen terugtrekken of mogelijk angstklachten zullen ontwikkelen. 'Hyperarousal' wordt gekenmerkt door hogere cortisol concentraties en door enerzijds lagere parasympathische (re)activiteit en anderzijds hogere sympathische (re)activiteit. Deze 'afwijkende' (re)activiteit van het autonome zenuwstelsel en/of de HPA-as zou het risico vergroten voor het ontwikkelen van angstklachten.

Aangezien angstklachten vaak toenemen in de puberteit besloten wij de rol van de (re)activiteit van het autonome zenuwstelsel en/of de HPA-as in het ontstaan van angstklachten te onderzoeken bij jongeren in deze leeftijdscategorie. Een grote groep jongeren uit Noord-Nederland werd benaderd voor deelname aan een grootschalig onderzoek, het TRAILS-onderzoek. Het onderzoek dat beschreven wordt in dit proefschrift is een onderdeel van het TRAILS-onderzoek.

In hoofdstuk 1 wordt de theoretische achtergrond van dit onderzoek beschreven en worden de onderzoeksvragen gepresenteerd. De belangrijkste onderzoeksvragen zijn:

1. Is 'hyperarousal' een kenmerk dat specifiek samenhangt met angstklachten of hangt het ook samen met depressie?
2. a: Hangen hoge cortisol concentraties samen met angstklachten?
   b: Worden jongeren met aanhoudende angstklachten gekenmerkt door hoge cortisol concentraties?
3. a: Voorspellen metingen van de (re)activiteit van het autonome zenuwstelsel de hoeveelheid angstklachten twee jaar later?
   b: Zijn er verschillen tussen jongens en meisjes?
   c: Gaan deze verbanden op voor angstklachten in het bijzonder of voor emotionele problemen in het algemeen (waaronder depressie)?
4. a: Voorspellen cortisol concentraties de hoeveelheid angstklachten twee jaar later?
   b: Is er een verschil in cortisol concentraties tussen jongeren met een verschillend beloop van hun angstklachten?
5. a: Voorspellen de werking van het autonome zenuwstelsel en de werking van de HPA-as samen de hoeveelheid angstklachten twee jaar later?
   b: Is dit verband duidelijker bij jongeren met ouders met emotionele problemen (een mogelijk extra kwetsbare groep)?
   c: Zijn er verschillen tussen jongens en meisjes?

Om deze vragen te kunnen beantwoorden zijn tijdens het eerste meetmoment van het TRAILS-onderzoek (leeftijd 10-12 jaar) de (re)activiteit van het autonome zenuwstelsel (cardiovasculaire regulatie; hartslag en bloeddruk), de cortisol concentraties in het speeksel en de hoeveelheid angstklachten en depressieve klachten gemeten. Twee jaar later zijn tijdens het tweede meetmoment (leeftijd

12-14 jaar) de hoeveelheid angstklachten en depressieve klachten nogmaals gemeten.

In hoofdstuk 2 wordt onderzocht of 'hyperarousal' een kenmerk is dat specifiek samenhangt met angstklachten of dat het ook samenhangt met depressie. Volgens de literatuur zou 'hyperarousal' namelijk specifiek zijn voor angst en niet opgaan voor depressie. In het TRAILS-onderzoek blijken angstklachten met name bij jongens samen te hangen met een lagere activiteit van het parasympatische systeem. Verlaagde activiteit van dit deelsysteem (de rem) kan leiden tot een hogere hartslag, wat gezien wordt als een teken van 'hyperarousal'. Echter, dit verband is niet specifiek voor angst. Er is namelijk ook een verband tussen een hogere hartslag en depressie. Bij meisjes is er tevens een verband tussen lagere parasympatische activiteit en depressie. Deze bevindingen duiden er op dat 'hyperarousal' ook samengaat met depressie. Daarentegen is er bij jongens een verband tussen een hogere parasympatische activiteit en depressie te zien, wat zou kunnen duiden op een verlaagde 'arousal' in relatie tot depressie bij jongens. Blijkbaar is het idee dat 'hyperarousal' specifiek is voor angst en niet opgaat voor depressie te simplistisch en kan een dergelijk model de complexere werkelijkheid niet goed beschrijven. Er zijn interessante verschillen tussen jongens en meisjes die in toekomstig onderzoek beter in kaart gebracht moeten worden.

In hoofdstuk 3 wordt onderzocht of angstklachten samenhangen met hoge cortisol concentraties en of jongeren met aanhoudende angstklachten gekenmerkt worden door hoge cortisol concentraties. Binnen het TRAILS-onderzoek is er geen samenhang gevonden tussen cortisol concentraties en de hoeveelheid angstklachten die in dezelfde periode gemeten werden (het eerste meetmoment, 10-12 jaar). Echter, cortisol concentraties die vlak na het wakker worden gemeten werden, zijn hoger bij jongeren die al sinds hun kleutertijd angstklachten hebben, dan bij jongeren die alleen nu angstklachten hebben of die nog nooit angstklachten hebben gehad. Het is mogelijk dat onderliggende factoren die een rol spelen in het ontstaan van angstklachten (zoals de werking van de HPA-as) alleen op te sporen zijn bij jongeren met aanhoudende angstklachten, omdat er bij deze jongeren sprake zou kunnen zijn van een aangeboren kwetsbaarheid voor angstklachten. Het is echter ook mogelijk dat de hogere cortisol concentraties die gemeten werden bij deze groep het gevolg zijn van de stress die vaak gepaard gaat met langdurige angstklachten. Het blijft daarom moeilijk om oorzaak en gevolg uit elkaar te houden bij het onderzoeken van de complexe samenhang tussen HPA-as activiteit en angstklachten.

In hoofdstuk 4 wordt onderzocht of de (re)activiteit van het autonome zenuwstelsel de hoeveelheid angstklachten twee jaar later voorspelt, of er verschillen tussen jongens en meisjes zijn en of eventuele verbanden specifiek zijn voor angst of dat ze opgaan voor emotionele problemen in het algemeen (dus ook depressie). Het blijkt dat binnen TRAILS de reactiviteit van het autonome zenuwstelsel de hoeveelheid angstklachten twee jaar later voorspelt.

Dit verband gaat echter alleen op voor meisjes en is niet specifiek voor angst, maar gaat op voor emotionele problemen in het algemeen. Het verband is niet heel sterk: de reactiviteit van het autonome zenuwstelsel speelt bij meisjes wel een rol in de ontwikkeling van angstproblemen, maar er lijkt een grotere rol weggelegd te zijn voor andere factoren. Deze factoren zouden bijvoorbeeld de opvoeding, belangrijke gebeurtenissen in het leven, of de erfelijke aanleg kunnen zijn. Dit zal toekomstig onderzoek echter moeten uitwijzen.

In hoofdstuk 5 wordt nagegaan of cortisol concentraties de hoeveelheid angstklachten twee jaar later voorspellen en of er een verschil is in cortisol concentraties tussen jongeren met een verschillend beloop van hun angstklachten. Binnen TRAILS voorspellen cortisol concentraties gemeten op 10-12 jarige leeftijd niet de hoeveelheid angstklachten twee jaar later. De cortisol concentraties die gemeten werden na het wakker worden zijn echter wel hoger bij jongeren waarbij de angstklachten gedurende deze twee jaar toegenomen zijn dan bij jongeren waarbij dit niet het geval is. Cortisol concentraties die 's avonds gemeten werden, zijn lager bij jongeren die gedurende deze twee jaar aanhoudend angstklachten hadden. Kennelijk hangen angstproblemen niet samen met alleen hoge of alleen lage cortisol concentraties, maar hangen verschillende aspecten van de werking van de HPA-as ('s ochtend/'s avonds gemeten; genetisch/omgevings bepaald) op een verschillende manier samen met angstklachten.

In hoofdstuk 6 wordt er getoetst of de werking van het autonome zenuwstelsel en de werking van de HPA-as samen de hoeveelheid angstklachten twee jaar later voorspellen, of dit verband duidelijker is voor jongeren met ouders met emotionele problemen (een mogelijk extra kwetsbare groep) en of er verschillen zijn tussen jongens en meisjes. Uit onze gegevens blijkt dat een hogere activiteit van de HPA-as in combinatie met een hogere activiteit van het parasympatische systeem de hoeveelheid angstklachten twee jaar later voorspelt. Dit gaat echter alleen op voor jongens met ouders met emotionele problemen. Een mogelijke verklaring voor deze bevinding is dat deze groep jongens erfelijk belast is of dat er bij deze jongens minder factoren zijn die hen beschermen tegen dit verhoogde risico (denk bijvoorbeeld aan sociale steun of opvoeding). Ook in dit hoofdstuk worden er verschillen tussen jongens en meisjes gevonden. Dit geeft nogmaals aan dat het belangrijk is om binnen toekomstig onderzoek naar factoren die een rol spelen in het ontstaan van angstklachten rekening te houden met geslachtverschillen en deze beter in kaart te brengen.

Tot slot worden in hoofdstuk 7 de belangrijkste bevindingen en conclusies gepresenteerd. De bevindingen worden besproken in het kader van bestaande theorieën en vergeleken met andere recente bevindingen. Er wordt geconcludeerd dat het onderzoek dat in dit proefschrift gepresenteerd wordt geen duidelijk bewijs geeft voor veel van de heersende theorieën. Deze theorieën geven wellicht een te simplistisch en beperkt beeld van de veel complexere werkelijkheid. Wij concluderen dat het huidige onderzoek heeft geleid tot

interessante bevindingen die de kennis over de onderliggende factoren van angstklachten vergroten. De bevindingen kunnen van dienst zijn bij het vormen van nieuwe theoretische modellen en bij het vormen van ideeën voor toekomstig onderzoek. De bevindingen leiden op dit moment nog niet tot belangrijke verbeteringen op het gebied van het voorkomen en behandelen van angstklachten. Metingen van de (re)activiteit van het autonome zenuwstelsel of cortisol concentraties kunnen (nog) niet gebruikt worden om te voorspellen welke jongeren uit de algemene bevolking op latere leeftijd angstklachten zullen ontwikkelen en welke niet. Het is de vraag in hoeverre verbanden tussen dit soort metingen en angstklachten specifiek zijn voor angst of dat deze wellicht ook opgaan voor andere emotionele en gedragsproblemen. Er wordt geopperd dat in de toekomst meer jongeren met klinische angststoornissen onderzocht zullen moeten worden, omdat in deze groep de angstklachten ernstiger zijn en daarom de resultaten mogelijkerwijs anders zullen zijn. Wij bevelen aan om in vervolgonderzoek een combinatie van biologische, psychologische, en sociale factoren te onderzoeken, zodat op die manier meer inzicht verkregen kan worden in de complexe mechanismen die een rol spelen in het ontstaan van angstklachten. We veronderstellen dat metingen van reactiviteit van de stress systemen wellicht betere voorspellers van toekomstige angstklachten zijn dan metingen van activiteit in rust. Nogmaals wordt het belang van het onderzoeken van geslachtsverschillen in toekomstig onderzoek benadrukt. Tezamen vormen onze bevindingen een goed vertrekpunt voor dergelijk toekomstig onderzoek.

Bedankt! & Curriculum Vitae

Bedankt!

De afgelopen vier jaar heb ik met veel plezier aan mijn promotietraject gewerkt binnen TRAILS; de TRacking Adolescents' Individual Lives Survey. Om iedereen te bedanken voor alle hulp en steun tijdens dit traject, nodig ik jullie uit voor een track over míjn adolescente levenpad!

Laten we beginnen bij mijn roots: Toen ikzelf een jaar of tien à elf was -de leeftijd waarop alle deelnemers aan TRAILS begonnen- woonde ik samen met mijn twee grote zussen Wanda en Stefanie en mijn pappa en mamma in Friesland. Mijn halfzussen Gaynor en Wendi en hun gezinnen en mijn andere familieleden woonden vrij ver weg. Ook binnen ons gezin ging ieder al snel zijns weegs, maar daardoor kwamen wel mijn leuke zwagers Gunter en Johan, en natuurlijk de lieve vriend van mijn moeder David, er bij op mijn levenspad. Lieve familie, ondanks dat we allemaal wijd en verspreid wonen, vormden jullie samen de veilige basis die elke jongere nodig heeft om fijn op te groeien. Hiervoor ben ik jullie allemaal ontzettend dankbaar! Ik ben blij met alle steun en aandacht die ik altijd krijg van jullie. Vooral mijn trotse ouders, die altijd interesse tonen, achter mij staan en mij helpen waar nodig, wil ik zeggen hoezeer ik dat waardeer: Jullie zorgen ervoor dat ik me gesteund en zeker voel en jullie hebben mij geleerd nieuwsgierig naar het leven te zijn en door te zetten! Heel erg bedankt!

Toen ik net begon te puberen droomde ik van een glansrijke carrière als artiest: acteren, dansen, zingen, schrijven, cabaret maken; creatief en slim zijn én gevat! Natuurlijk zou in dit alles mijn grote liefde achter mij staan. Mijn held, mijn inspirator, hard van buiten, zacht van binnen, zou mij steunen op ieder vlak. Op de middelbare school werd ik echter behoorlijk op de proef gesteld. Zoals we ook binnen TRAILS zien zijn pubers nou eenmaal niet altijd even makkelijk voor zichzelf en voor elkaar. Gelukkig vond ik gelijkgestemde vriendinnen. Lieve Anita, lieve Hennie, jullie waren er voor mij tijdens mijn middelbare schooltijd en samen zijn we opgegroeid van onzekere, wat teruggetrokken pubermeisjes, naar jonge vrouwen die volop in het leven staan! Deze ontwikkeling heb ik mede aan jullie te danken! Ik wil jullie dan ook allebei heel erg bedanken voor al jullie interesse en steun en jullie trouwe vriendschap! Samantha, ook jou heb ik in deze tijd leren kennen. Onze vriendschap is bijzonder omdat hij met name op papier plaats vindt, maar ik kon en kan altijd mijn verhaal bij jou kwijt en ik kan nog altijd rekenen op jouw leuke, lange, geïnteresseerde brieven en e-mails en daar ben ik heel erg blij mee! Dankjewel!!!

Mijn studietijd was voor mij een verademing! Ik leerde veel leuke mensen kennen en al mijn interesses werden gevoed. Brechtje, jij was mijn gezellige huisgenootje en ik vind het ontzettend leuk dat we altijd contact hebben gehouden! Het was fijn dat we juist toen we niet meer bij elkaar woonden door alle omstandigheden extra naar elkaar toe groeiden en steun aan elkaar hadden. Ik geniet altijd erg van onze wekelijkse bijpraat momenten! Bedankt voor onze vriendschap! Lieve Minous, wat later kwam ook jij bij mij wonen, maar sindsdien

zijn wij onafscheidelijk. Jij knuffelt mij als ik verdrietig ben en geeft mij een snauw als je chagrijnig bent; jij bent mijn beste vriendin ;-) Dankjewel!! Ik kreeg ook een hele leuke vriendinnengroep bij mijn studie. Esmé, Kirsten, Karin, Marcelle, Marike, Christina, Emke, wat hebben we altijd een lol! Met jullie kan ik altijd gezellig samen eten, lachen, kletsen, nóg meer kletsen! Bedankt voor al jullie gezelligheid en steun; ik vind het heerlijk om onderdeel uit te maken van zo'n vriendinnenclub! Marike, jij bedankt dat jij als vertegenwoordiger van deze vriendinnengroep tijdens mijn promotie naast mij wil staan als paranimf. Jouw positieve, pittige, gevatte manier van doen geeft mij altijd een goed gevoel en zal mij vast steunen op deze belangrijke dag.

Na mijn studie moest ik beslissen welk pad ik nu in zou slaan. Mijn droom om artiest te worden sluimerde nog steeds in mijn hoofd, maar mijn docenten hadden tijdens mijn studie ook mijn interesse voor de wetenschap gewekt (bedankt hiervoor!). Ik koos daarom voor nog een studie met daarnaast allerlei creatieve baantjes en clubjes. Toen bracht mijn voormalig scriptiebegeleidster Dr. Natasja van Lang het TRAILS-onderzoek op mijn pad. Natasja, je hebt mij ontzettend geholpen; met mijn scriptie, aan veel leuke bijbaantjes en uiteindelijk aan mijn baan bij TRAILS: heel erg bedankt!!! Ik ging bij TRAILS aan de slag. Op het eerste gezicht heeft een wetenschapsbaan misschien niet helemaal de 'glamour' die ik in gedachten had, maar het werk is erg afwisselend en uitdagend. Bovendien kun je best wat creativiteit en 'performer skills' gebruiken! Je krijgt met veel verschillende dingen en mensen te maken, dus je kunt er veel kanten in kwijt. Van de beslissing om dit pad in te slaan heb ik dan ook nooit spijt gekregen! TRAILS is een boeiend en veelzijdig onderzoek waar veel mensen zich ontzettend hard voor inzetten. De medewerkers, maar natuurlijk ook alle jongeren, ouders en leerkrachten die deelnemen aan het onderzoek. Hen wil ik dan ook graag op deze plaats bedanken voor hun inzet!

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Samen gingen we op een roadtrip door Noord Nederland om de laatste deelnemers persoonlijk te benaderen. Deze gezellige toertjes hebben denk ik de basis gevormd voor een geweldige verdere samenwerking en vriendschap. Lieve Hanneke, ik wil zeggen dat je een belangrijke vriendin voor mij geworden bent, bedankt voor je altijd zo sprankelende aanwezigheid in mijn leven! Ook bedankt voor alle gezellige logeerpartijtjes, ik heb me altijd welkom en thuis gevoeld! Ik ben ontzettend blij dat jij bij deze bijzondere gebeurtenis mijn paranimf wilt zijn!

En toen was het zover... Ik was vierentwintig, de leeftijd waarop wij de TRAILS jongeren voor het laatst zullen zien. Daarna behoren ze blijkbaar volwassen worden... en dat moest ik ook! Ik moest mijn dataverzamelingsstokje doorgeven en bezig met serieuze, volwassen- grote-mensen-werkzaamheden: Ik moest mijn proefschrift schrijven! Gelukkig kon ik mijn stokje goed overdragen: Rianne kwam bij ons werken en zij ging gelijk hard en gedegen aan de slag! Gelukkig had ze daarnaast ook nog wel eens tijd om gezellig met mij een praatje te maken, want alleen maar serieus hard werken dat kon ik niet meteen altijd. Lieve Rianne, als ik het allemaal even te spannend vond, kon ik altijd mijn hart bij je luchten en ook al werk je nu in Nijmegen, ik ben heel blij dat we bevriend zijn geraakt en gebleven!!! Bedankt voor alles!

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In mijn schrijfperiode ging ik meer in Rotterdam in het Sophia Kinderziekenhuis werken. Dr. Robert Ferdinand was daar mijn dagelijks begeleider. Beste Robert, jij introduceerde bij mij de afdeling en alle achtergrond bij mijn onderzoek. Ik moet eerlijk zeggen dat ik door mijn gewenning aan 'Noorderlingen' eerst even moest wennen aan jouw directe persoonlijkheid en je snelle, doelgerichte manier van werken, maar al snel leerde ik dit te waarderen

en ik heb er erg veel van geleerd. Ik leerde van jou duidelijker, meer 'to the point', en wat zakelijker te zijn. Bovendien daagde jij mij uit om nóg beter over dingen na te denken, ze nóg beter te verwoorden en stimuleerde je mij om zoveel mogelijk leerzame taken op mij te nemen, waardoor je ervoor zorgde dat ik het onderste uit de kan haalde. Heel erg bedankt voor alles!

Naast Dr. Robert Ferdinand, speelde Dr. Joke Tulen van de afdeling Psychiatrie ook een rol in mijn begeleiding. Beste Joke, jij hebt mij veel uitleg gegeven en belangrijke stof toegereikt. Ik heb daar veel van geleerd en vond ons contact altijd erg prettig. Bedankt voor alles!

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Professor dr. Frank Verhulst en Professor dr. Hans Ormel, mijn beide promotoren. Jullie waren natuurlijk al vanaf het allereerste begin bij mijn promotietraject betrokken en jullie rol werd steeds groter naarmate het project

vorderde. Beste Frank, bedankt voor je altijd positieve, opbouwende houding en alle interesse. Ik werk met veel plezier op de afdeling en wil je graag bedanken voor alle geboden mogelijkheden! Beste Hans, bedankt voor alle snelle, goede commentaren en je betrokkenheid. Professor dr. Tineke Oldehinkel, beste Tineke, ook jij was vanaf het eerste begin bij mijn promotietraject betrokken. Van je opmerkingen en vragen tijdens allerlei overleggen en je altijd snelle, zorgvuldige commentaar op mijn werk heb ik ontzettend veel geleerd! Ik stel het zeer op prijs dat je altijd de tijd voor mij nam en dat je de secretaris wilde zijn van mijn promotiecommissie. Bedankt! Professor dr. Minderaa en Professor dr. Muris, mijn hartelijke dank voor het zitting nemen in mijn leescommissie en de promotiecommissie tijdens mijn verdediging. Professor dr. van Doornen, Professor dr. Vermeiren en Professor dr. Bögels ook u hartelijk bedankt voor het zitting nemen in mijn promotiecommissie.

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Een levenspad is het mooist, als je het met bijzondere mensen mag bewandelen. Heel erg bedankt allemaal!!!

Kirstin

Curriculum Vitae

Kirstin Greaves-Lord werd op 29 augustus geboren te Den Helder. In 1998 behaalde zij haar VWO-diploma aan het Stellingwerf College te Oosterwolde, Friesland. Vanaf september 1998 studeerde zij Nederlandse Taal- en Letterkunde aan de Rijks Universiteit Groningen. Na het behalen van haar propedeuse in 1999 vervolgde zij haar studie binnen de doctoraalopleiding Algemene Taalwetenschappen. Zij koos voor de afstudeerrichting Neurolinguistiek en richtte zich hierbinnen op het vakgebied Taalontwikkelingsstoornissen. Tijdens haar studie werkte zij als studentassistent binnen de afdeling Experimentele Taalkunde en deed ERP onderzoek naar de taalverwerking van gezonde proefpersonen (begeleiding Dr. Laurie A. Stowe). Verder liep zij stage bij het Universitair Centrum Kinder- en Jeugdpsychiatrie binnen het promotieonderzoek van Natasja van Lang naar pervasieve ontwikkelingsstoornissen. Binnen deze stage schreef zij ook haar scriptie over de taalverwerking van kinderen met een pervasieve ontwikkelingsstoornis (begeleiding Dr. Natasja J.D. van Lang en Dr. Gerard Bol). In het najaar van 2002 studeerde zij cum laude af. Aansluitend werkte zij als onderzoeksassistent binnen de afdeling Sociale Psychiatrie van de Rijks Universiteit Groningen waar zij onderzoek deed naar pervasieve ontwikkelingsstoornissen en ADHD bij volwassenen. Hiernaast volgde zij de propedeuse Psychologie aan de Rijks Universiteit Groningen en was zij werkzaam als (trainings)actrice.

Vanaf september 2003 werkte zij als assistent in opleiding verbonden aan de Erasmus Universiteit Rotterdam en deed zij promotieonderzoek binnen de afdeling Kinder- en Jeugd Psychiatrie van het Erasmus MC-Sophia (Hoofd: Prof. Dr. Frank C. Verhulst). Zij begon haar promotieonderzoek met de dataverzameling binnen TRAILS (Tracking Adolescents' Individual Lives Survey, hoofdonderzoekers: Prof.dr. Frank C. Verhulst en Prof. dr. Johan Ormel) en beschreef vervolgens de onderzoeksresultaten over de relatie tussen stress fysiologie en angstproblematiek onder dagelijkse begeleiding van Dr. Robert F. Ferdinand en Dr. Anja C. Huizink. De resultaten van het promotieonderzoek staan beschreven in dit proefschrift. Ten tijde van het promotieonderzoek was Kirstin onder andere ook werkzaam als trainingsactrice en schreef zij mee aan lesmaterialen voor uitgeverij Wolters-Noordhoff. Vanaf september 2007 zal Kirstin werkzaam zijn als post doc onderzoeker binnen de afdeling Kinder- en Jeugd Psychiatrie van het Erasmus MC-Sophia. Zij zal binnen deze functie onderzoek doen op het gebied van de psychofysiologie bij jongeren uit de algemene bevolking en zij zal onderzoek doen naar de etiologie van pervasieve ontwikkelingsstoornissen.