The sensitive sex
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Chapter 2

General Methods

A gold standard is most golden
in the eye of its inventors.
DESCRIPTION OF THE TRAILS SAMPLE

TRacking Adolescents’ Individual Lives Survey is a large prospective population study of Dutch adolescents with bi- or triennial measurements from age 11 to at least age 25. The overall objective of the study is to contribute to the understanding of the determinants of adolescents’ mental (ill-)health and social development during adolescence and young adulthood, as well as the mechanisms underlying the associations between determinants and these outcomes. A particular aim is to focus on the interplay between individual characteristics and environmental factors. More information on the TRAILS study can be found in Huisman et al. (2008). So far, three assessments wave were completed which ran from, respectively, March 2001 to July 2002, September 2003 to December 2004 and September 2005 to December 2007. At the first wave, 2230 children were enrolled in the study; the response rate was 76.0% at T2 and 81.4% at T3. Numbers of participants mean ages and percentages of girls are presented in Table 1.

<table>
<thead>
<tr>
<th>Wave</th>
<th>n</th>
<th>mean age (SD)</th>
<th>% girls</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>2230</td>
<td>11.09 (0.56)</td>
<td>50.8</td>
</tr>
<tr>
<td>T2</td>
<td>2149</td>
<td>13.65 (0.53)</td>
<td>51.0</td>
</tr>
<tr>
<td>T3</td>
<td>1816</td>
<td>16.28 (0.71)</td>
<td>52.3</td>
</tr>
</tbody>
</table>

Selection of the TRAILS sample
The sampling procedure consisted of two stages. First, five municipalities in the North of The Netherlands (including urban and rural areas) were requested to provide information from the community registers (i.e. name, date of birth, gender, address) of all inhabitants that were born between 1 October 1989 and 30 September 1990 or between 1 October 1990 and 30 September 1991. Subsequently, all primary schools (including schools for special education) received a letter accompanied by detailed information about the goals, design and practical procedures of TRAILS. School participation was a prerequisite for eligible children and their parents to be approached. A total of 135 primary schools were identified, encompassing 3483 eligible children. Of the 135 schools 13 refused to participate, resulting in the exclusion of 338 children. Secondly, parents/guardians were informed through information brochures (one for themselves and one for their children) about the study goals, selection procedure, confidentiality and administered measures of the study. Shortly thereafter an interviewer contacted the parents by telephone to invite the parents and the child to participate. Of the 3145
remaining eligible children 210 were excluded because they were either unable to participate or incapable to participate due to severe mental retardation or due to a serious physical illness or handicap, or if no Dutch-speaking parent or parent surrogate was available (Turkish and Moroccan parents who were unable to speak Dutch were interviewed in their own language). After intensive recruitment efforts (including telephone calls, reminder letters and home visits), a total of 2230 children (76.0%) were included in the study at baseline (T1).

Selection of the TRAILS focus sample
At the third assessment wave a group of adolescents were invited to perform a series of laboratory tasks (hereafter referred to as the experimental session) in order to study a diversity of research questions within the TRAILS study. Of the 744 adolescents that were invited 715 (96.1%) agreed to participate. This sample consisted of 473 (66.2%) adolescents with an increased risk to develop mental health problems in order to increase the power to detect mental health-related differences in response patterns. High risk was defined based on baseline temperament (high scores on frustration and fearfulness, low scores on effortful control), parental psychopathology (depression, anxiety, addiction, psychoses, or antisocial behaviour), and environmental risk (living in a single-parent family). The other 33.8% (n = 242) were randomly selected from the total TRAILS sample. In Appendix I at the end of this chapter, more information is provide on the selection procedure and the numbers of participants in each stratum. Please note that, although high-risk adolescents were oversampled, the sample included the total range of mental health problems present in a community population of adolescents, only in a different distribution. Selection of a sample that is likely to be enriched for genetic susceptibility for stress-related disorders is useful in associating genetic variation with stress-related endophenotypes (Caspi and Moffit, 2006). However, with regard to the candidate genes examined in this dissertation, no differences in allele frequencies were found between the total TRAILS sample and the TRAILS focus sample (see Table 3, Appendix II).

PROCEDURES OF THE DATA COLLECTION
In this dissertation, data is used from the parent interview at T1, self-report and parent report questionnaires at all three assessment waves, DNA genotyping at T3, and a social stress test which was part of the experimental session at T3. Specific measures used in the studies are described in more detail in the research chapters.
Parent interview at T1
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, including the child’s developmental history and somatic health and parental psychopathology and care utilization.

Questionnaires at T1, T2, T3
At each assessment wave, participants filled out questionnaires in groups at the schools under the supervision of one or more TRAILS assistants. Additionally, parents were asked to fill out a questionnaire covering health, behaviour and development of their child. At T3, both biological parents were asked to report about own lifetime psychopathological problems.

Event History Calendar at T3
At T3 stressful life events (SLEs) were assessed by means of an Event History Calendar (EHC), a data collection method for obtaining retrospective data about life events and activities (Caspi et al., 1996). Participants were asked about events that had occurred since the first assessment wave (age 11). The SLEs that were used in the studies are listed in Appendix III.

Experimental session and cortisol collection at T3
At T3, the aforementioned focus sample participated in an experimental session in which behavioural and psycho-physiological responses (autonomic, cortisol, subjective arousal) to a variety of challenging conditions were recorded. These conditions included orthostatic stress (from supine to standing), a spatial orienting task, a gambling task, an EMG startle reflex task, and a social stress test. The experimental protocol was approved by the Central Committee on Research Involving Human subjects (CCMO). The test assistants, 16 in total, received extensive training in order to optimize standardisation of the experimental session.

Setting and instructions
The experimental sessions took place on weekdays, in sound-proof rooms with blinded windows at selected locations in the participants’ residence town. The sessions lasted about three hours and 15 minutes, and started between 08:00h and 09:30h (morning sessions, 43.8%) or between 12:30h and 02:30h (afternoon sessions). Although free salivary cortisol levels may be higher in the morning due to the circadian rhythm of cortisol production, morning and afternoon cortisol responses to social stress have been reported to be comparable at different times of the day (Kudielka et al., 2004). The participants were asked to collect two morning saliva samples on the day of the experimental session, one directly after waking up (Co1) (mean time of awakening = 07:39h, SD = 1:10h) and one 30
minutes later (Co2). They were instructed not to eat, brush their teeth, or engage in heavy exercise during this half hour, and to bring the saliva samples with them to the test location. In addition, we asked the participants to refrain from smoking and from using coffee, milk, chocolate, and other sugar-containing foods in the two hours before the session. At the start of the session, the test assistant, blind to the participants’ risk status, explained the procedure and administered a short checklist on current medication use (including oral contraceptive (OC) use), quality of sleep, and physical activity in the last 24 hours, and attached the equipment for heart rate and blood pressure measurements. Next, participants filled out four computerized questionnaires, assessing life events in the past week, state and trait anxiety, mood states, and feelings and thoughts in the last month. The participants were asked to relax until 35 minutes after the start of the session. After this period of rest, heart rate and blood pressure were recorded for a period of five minutes, in which the participants had to sit still and were not allowed to speak. Afterwards, the first cortisol sample was collected (Ce1). Subsequently, the challenges (i.e., laboratory tasks) were administered in the before-mentioned order. Every task was followed by a short break, during which participants reported subjectively experienced arousal by means of the Manikin task (Bradley and Lang, 1994). The social stress test was the last challenge of the experimental session. Detailed information about this test is presented in the next paragraph. Following the social stress test, the participants were debriefed about the experiment and could relax for about 15 minutes, after which heart rate and blood pressure were recorded once more and anxiety and mood were assessed again.

The Groningen Social Stress Test (GSST)
The Groningen social stress tests protocol was inspired by the Trier Social stress test (TSST; Kirschbaum et al., 1993) and the child version of the TSST (Buske-Kirschbaum 1997). Two main differences between these tests and ours concern the number of test-assistants (one in the GSST, three and two in the TSST and TSST-child, respectively), and the content of the speech task. Similar to the GSST, both the TSST and the TSST-child version end with a mental arithmetic task. Despite these differences, the GSST encompasses the three most important triggers of HPA axis: uncontrollability, threat of failure, and fear of negative social evaluation (Dickerson and Kemeny, 2002), and participants rated the GSST as the most stressful test of the experimental session (Oldehinkel, unpublished data). Furthermore, the GSST has shown to elicit significant changes in the cardiovascular and HPA system in various samples, including ours (Benschop et al., 1998; Van der Pompe et al., 1998; Bouma et al., 2009). During the GSST, heart rate was recorded continuously. Blood pressure was not recorded continuously so that the participants could move their hands freely to express themselves during the task. Participants were, on the spot, instructed to prepare a six-minute speech
about themselves and their lives and deliver this speech in front of the experimenter and a recording video camera. They were told that their videotaped performance would be judged on content of speech as well as on use of voice and posture and rank-ordered by a panel of peers after the experiment. The risk if being judged negatively by peers was included to induce threat of social rejection. Participants had to speak continuously for the whole period of six minutes. The experimenter watched the performance critically, without showing empathy or encouragement. After six minutes of speech, the participants were told that there was a problem with the computer and they had to sit still and be quiet. This interlude lasted three minutes, and was meant to asses cardiovascular recordings without the disturbance of speech on respiration recordings. After the interlude, participants were instructed to repeatedly subtract 17, starting at 13278. This difficult task was meant to induce a sense of uncontrollability. Uncontrollability was further provoked by negative feedback by the test assistant, including remarks such as, “No, wrong again, begin at 13278”, “Stop wiggling your hands” or “You are too slow, be as quick as you can, we are running out of schedule”. After six minutes of mental arithmetic, heart rate was recorded again during a three-minute period in which the participant was not allowed to speak. Directly after the GSST participants were ask to report their subjective arousal. Figure 2 shows a schematic representation of the GSST.

**Cortisol collection**

Cortisol was assessed from saliva by the Salivette sampling device (Sarstedt, Numbrecht, Germany) containing a small swab in a plastic tube on which the participants had to chew for 60 seconds, until the swab was soaked with saliva. This manner of collecting cortisol is relatively stress-free and avoids confounding by stress responses e.g. as induced by venipuncture (Schmidt, 1997). Correlations between saliva cortisol levels and serum cortisol concentrations are high (Kirschbaum and Hellhammer, 1994; Goodyer et al., 1996). Salivary cortisol levels rise about 15 to 20 minutes after a psychological stressor (Sharpley and McLean, 1992). The first sample, Ce2, was taken just before the start of the GSST and reflects HPA axis activity when participants filled out a rating scale while sitting quietly. Ce3 was collected directly after the end of the GSST and reflects response of the HPA axis during speech. Ce4 and Ce5, collected 20 respectively 40 minutes after the end of the GSST, are considered measures of post-stress activity. Timing of cortisol sampling is schematically presented in Figure 1.

After the test, salivettes were stored at -20°C until analysis. After the experimental session, the samples were placed in a refrigerator at 4°C, and within half a week brought to the laboratory of the University Medical Centre in Groningen, where they were stored at -20°C until analysis. The intra-assay coefficient of variation was
8.2% for concentrations of 1.5 nM, 4.1% for concentrations of 15 nM, and 5.4% for concentrations of 30 nM. The inter-assay coefficients of variation were, respectively, 12.6%, 5.6%, and 6.0%. The detection border was 0.9 nM.

**Figure 1.** Schematic overview of the Groningen Social Stress Test within the timeframe of the experimental session.

**DNA collection at T3 and genotyping**
At T3, DNA was collected of 1460 adolescents of whom 99.6% was successfully genotyped. Only adolescents from Caucasian descent were included in the analyses presented in the studies. DNA was extracted from blood samples or (in a few cases) buccal swabs (Cytobrush®) using a manual salting out procedure as described by Miller and colleagues (Miller et al., 1988).

**Genotyping SNPs in the MR, GR and BDNF gene**
SNPs were genotyping at the Genome Analysis Facility, Department of Genetics, University Medical Centre Groningen, University of Groningen, the Netherlands. Genotyping was done on the Golden Gate Illumina BeadStation 500 platform (Illumina Inc., San Diego, CA, USA) by laboratory personnel blinded to the identity of the individual samples. The used assay was designed within the framework of various research questions of the TRAILS study. Genotyping of all samples was performed following the manufacturers protocol. Genotyping data and clustering was performed in BeadStudio 3.0 (Illumina Inc., San Diego, CA, USA). Clustering clouds were manually investigated and adjusted if necessary.
Genotyping serotonin transporter polymorphic region

Genotyping of the 5-HTTLPR polymorphism was done at the Research lab for Multifactorial Diseases within the Human Genetics department of the Radboud University Nijmegen Medical Centre in Nijmegen, The Netherlands. Genotyping the promoter region of SLC6A4 (5-HTT, SERT) gene was performed by simple sequence length analysis. PCR was on 50 ng genomic DNA using 0.5 µM fluorescently labeled forward primer (FAM-5'-GGCGTTCGCCGCTGCTGAATGC-3') and reverse primer (5'-GAGGGACT-GAGCTGGACAACCAC-3'), 0.25 mM dNTPs, 1x PCR optimization buffer A (30 mM Tris-HCl pH 8.5, 7.5mM (NH4)2SO4, 0.75 mM MgCl2), 10% DMSO and 0.4 U AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The cycling conditions for the polymerase chain reaction started with 12 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at the optimized annealing temperature (57.5°C), and 2 min. 72°C, then followed by an extra 10 min 72°C. Determination of the length of the alleles was performed by direct analysis on an automated capillary sequencer (ABI3730, Applied Biosystems) using standard conditions. The single nucleotide substitution (A>G) present in the SLC6A4 long ‘l’ allele (rs25531) (Zalsman et al., 2006) was genotyped using a custom-made Taqman assay (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Genotyping of the SNP was performed on a 7500 Fast Real-Time PCR System and genotypes were scored using the algorithm and software supplied by the manufacturer (Applied Biosystems). This assay consisted of 2 primers (forward: CCCTCGCGGATCCC, reverse: ATGCTGAGGAGCTGCA) and 2 fluorescently labeled probes (VIC-CTGCACCCCCAGCAT, FAM-CTGCACCCCCGCGCAT). Genotyping was carried out in a volume of 10 µl containing 10 ng of genomic DNA, 5 µl of Taqman Mastermix (2x; Applied Biosystems), 0.25 µl of the Taqman assay (40x) and 3.75 µl of water. Genotyping was performed on a 7500 Fast Real-Time PCR System and genotypes were scored using the algorithm and software supplied by the manufacturer (Applied Biosystems). The assay was validated by digesting the SLC6A4 PCR product with MspI (New England Biolabs, Ipswich, USA) and separating the restriction fragments on a 2% agarose gel. This resulted in restriction fragments of 340 bp, 130 bp and 60 bp for the L allele, fragments of 175 bp, 165 bp, 130 bp and 60 bp for the Lg allele, and fragments of 300 bp, 130 bp and 60 bp for the S allele. The genotyping assay was carried out in a CCKL quality-certified laboratory. The HTTLPR assay has been validated earlier. Generally, 3% blanks as well as duplicates between plates were taken along as quality controls during genotyping.

Allele frequencies

Allele frequencies of the polymorphisms in the candidate genes used in this dissertation (MR, GR, BNDF, 5-HTTLPR) were comparable between the total TRAILS sample and the TRAILS focus sample (See Appendix II).
APPENDIX I. SELECTION PROCEDURE FOCUS SAMPLE

Selection procedure
At the third assessment wave a group of adolescents were invited to participate in an experimental session. In order to increase the power to detect mental health-related differences in response patterns a large part of this focus sample would be selected from adolescents with an increased risk to develop mental health problems. Initially, we planned to invite 810 adolescents to participate in the behavioural experiment at T3: 600 (300 boys, 300 girls) from high risk strata (75%) and the remaining 210 (105 boys, 105 girls) randomly from the total sample without any of the risk criteria (25%). Selection numbers are based on n = 2223 of which stratum data were available at T1.

Selection criteria high risk strata
High risk was defined based on the following three indicators. A) Temperament, as measured with the Early Adolescent Temperament Questionnaire (EATQ) at T1. High scores on frustration ≥ 90th percentile, or Fear ≥ 90th percentile or Effortful Control ≤ 10th percentile were indicated as high risk (NA = 597 (27.8%), 273 girls; 324 boys). B) Parental psychopathology: at least one parent with severe psychopathology (depression, anxiety, addiction, psychoses, or antisocial behaviour), based on information of the parent interview at T1. C) Environmental risk (at least one of the biological parents is not living with the participant) NC = 509 (23.7%), 264 girls, 245 boys. On the basis of these risk indicators (A,B,C) we constructed eight strata as indicated in Table 1.

Table 1. Strata formation percentages

<table>
<thead>
<tr>
<th>Stratum</th>
<th>n total (%)</th>
<th>n girls (%)</th>
<th>n boys (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No A, B, C</td>
<td>939 (42.2)</td>
<td>477 (42.2)</td>
<td>462 (42.2)</td>
</tr>
<tr>
<td>A</td>
<td>303 (13.6)</td>
<td>138 (12.2)</td>
<td>165 (15.1)</td>
</tr>
<tr>
<td>B</td>
<td>317 (14.3)</td>
<td>175 (15.5)</td>
<td>142 (13.0)</td>
</tr>
<tr>
<td>C</td>
<td>175 (7.9)</td>
<td>96 (8.5)</td>
<td>79 (7.2)</td>
</tr>
<tr>
<td>A &amp; B</td>
<td>138 (6.2)</td>
<td>66 (5.8)</td>
<td>72 (6.6)</td>
</tr>
<tr>
<td>A &amp; C</td>
<td>66 (3.0)</td>
<td>25 (2.2)</td>
<td>41 (3.7)</td>
</tr>
<tr>
<td>B &amp; C</td>
<td>175 (7.9)</td>
<td>99 (8.8)</td>
<td>76 (6.9)</td>
</tr>
<tr>
<td>A, B &amp; C</td>
<td>110 (4.9)</td>
<td>53 (4.7)</td>
<td>57 (5.2)</td>
</tr>
<tr>
<td>Total</td>
<td>2223 (100)</td>
<td>1129 (100)</td>
<td>1094 (100)</td>
</tr>
</tbody>
</table>
Exclusion criteria
Participants were excluded from selection for the TRAILS focus sample if their physical condition made participation impossible and/or would interfere with collection of the measures (for example use of corticosteroids, eye or ear impairment). Participants were also excluded when they lived more than 100 km away from a test location since travelling long distances could interfere with the behaviour during the experiment\(^1\).

Final numbers
The final numbers in each stratum are presented in Table 2. Because of non-response in certain strata, the goal of measuring 810 participants could not be reached. For example the combination of A&C is less prevalent in girls than in boys. The numbers and percentages in Table 1 and Table 2 might therefore differ slightly. In total, 744 adolescents were invited and 715 agreed to participate. In the research chapters we divided participants in low risk group (no A,B & C) and in the high risk groups (all other strata).

<table>
<thead>
<tr>
<th>Stratum</th>
<th>n total (%)</th>
<th>n girls (%)</th>
<th>n boys (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No A, B, C</td>
<td>242 (33.8)</td>
<td>123 (33.8)</td>
<td>119 (33.9)</td>
</tr>
<tr>
<td>A</td>
<td>109 (15.2)</td>
<td>56 (15.4)</td>
<td>53 (15.1)</td>
</tr>
<tr>
<td>B</td>
<td>103 (14.4)</td>
<td>52 (14.3)</td>
<td>51 (14.5)</td>
</tr>
<tr>
<td>C</td>
<td>66 (9.2)</td>
<td>38 (10.4)</td>
<td>28 (8.0)</td>
</tr>
<tr>
<td>A &amp; B</td>
<td>65 (9.1)</td>
<td>32 (8.8)</td>
<td>33 (9.4)</td>
</tr>
<tr>
<td>A &amp; C</td>
<td>23 (3.2)</td>
<td>10 (2.7)</td>
<td>13 (3.7)</td>
</tr>
<tr>
<td>B &amp; C</td>
<td>64 (9.0)</td>
<td>33 (9.1)</td>
<td>31 (8.8)</td>
</tr>
<tr>
<td>A, B &amp; C</td>
<td>43 (6.0)</td>
<td>20 (5.5)</td>
<td>23 (6.6)</td>
</tr>
<tr>
<td>Total</td>
<td>715 (100)</td>
<td>364 (100)</td>
<td>351 (100)</td>
</tr>
</tbody>
</table>

\(^1\) It is not plausible that moving to a city further away would be associated with specific risk factors.
## APPENDIX II. ALLELE FREQUENCIES OF POLYMORPHIC GENES

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>SNP</th>
<th>Total Sample</th>
<th>MAF</th>
<th>Focus Sample</th>
<th>MAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR-BclI</td>
<td>C/G</td>
<td>1454</td>
<td>G = 0.37</td>
<td>649</td>
<td>G = 0.38</td>
</tr>
<tr>
<td>GR-9beta</td>
<td>A/G</td>
<td>1454</td>
<td>G = 0.16</td>
<td>649</td>
<td>G = 0.16</td>
</tr>
<tr>
<td>MR-I180V</td>
<td>A/G</td>
<td>1453</td>
<td>G = 0.14</td>
<td>648</td>
<td>G = 0.13</td>
</tr>
<tr>
<td>MR-2G/C</td>
<td>G/C</td>
<td>1454</td>
<td>C = 0.49</td>
<td>649</td>
<td>C = 0.50</td>
</tr>
<tr>
<td>BDNF val/met</td>
<td>G/A</td>
<td>1452</td>
<td>A = 0.20</td>
<td>647</td>
<td>A = 0.19</td>
</tr>
<tr>
<td>5-HTTLPR</td>
<td>La/SLg</td>
<td>1414</td>
<td>S/Lg = 0.49</td>
<td>633</td>
<td>S/Lg = 0.49</td>
</tr>
</tbody>
</table>

*Note: MAF = minor allele frequency. The frequency differences between the total sample and the focus sample were not significant.*
APPENDIX III. STRESSFUL LIFE EVENTS

The following stressful life events were included in the SLE measure\(^2\) described in Chapter 4\(^3\) and Chapter 7\(^4\):

- I have had an illness or accident
- Somebody in my family had an illness or accident
- A good friend had an illness or accident
- My mother died
- My father died
- My brother or sister died
- Somebody else I cared about died
- I had to repeat a class
- I was expelled from school
- My parents lost their job
- I lost a good friend because of a fight
- My boyfriend/girl friend broke up with me
- My parents got divorced or broke up
- I ran away from home
- Somebody used violence against me
- I was bullied
- Our family moved to another city
- I moved to another family
- Somebody from my family moved away
- Somebody moved in with my family
- I had a fight with friends
- I had a fight with somebody in the family
- There were fights between my family members
- I lost contact with somebody
- I lost contact with a friend because he/she moved away

\(^2\) Every event could be scored on severity on a scale from 0 (= no severe at all) to 3 (= very severe). Only events with a minimal score of 1 were included in the final sum score.

\(^3\) Based on Stressful Life Events Questionnaire T3.

\(^4\) Based on Event History Calendar T3.