Neurobiological and behavioral stress reactivity in children prenatally exposed to tobacco

Stephan C.J. Huijbregts a,b,*, Sheila R. van Berkel a, Hanna Swaab-Barneveld a,b, Stephanie H.M. van Goozen a,c

a Leiden University, Faculty of Social Sciences, Department of Clinical Child and Adolescent Studies, Wassenaarseweg 52, P.O. Box 9555, 2300 RB Leiden, The Netherlands
b Leiden Institute for Brain and Cognition, Leiden University Medical Center, Albinusdreef 2, P.O. Box 9600, 2300 RC Leiden, The Netherlands
c School of Psychology, Cardiff University, Tower Building, Park Place, Cardiff CF10 3 AT, United Kingdom

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Summary This study examined neurobiological and behavioral stress reactivity in children who had been prenatally exposed to tobacco. Neurobiological stress reactivity was measured using salivary cortisol and alpha-amylase levels at five different time points throughout a stressful neuropsychological test session, which involved a competition against a videotaped opponent. Participants (mean age: 10.6 years, SD 1.3) were 14 prenatally exposed (PE) children, 9 children with disruptive behavior problems (DBD), and 15 normal controls (NC). For cortisol responses, no significant differences between the three groups were observed. Normal controls, however, had significantly higher alpha-amylase levels than PE-children throughout the test session, and their alpha-amylase levels also increased throughout the session, whereas these remained low and stable for PE-children. Alpha-amylase levels and trajectory of PE-children were similar to those observed for DBD-children. PE-children also showed significantly increased behavioral stress reactivity compared to NC-children, and neurobiological and behavioral stress reactivity were inversely related in PE-children, again similar to what was observed for DBD-children. These results support the hypothesis that prenatal smoking may lead to long-lasting neurobiological and behavioral changes in exposed offspring.

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1. Introduction

Prenatal tobacco exposure has been related, in both and animal and human studies, to structural and neurobiological changes in the offspring brain. These changes include cortical thinning (e.g., of the orbitofrontal cortex) (Toro et al., 2008), smaller volume of specific brain structures (e.g., the corpus callosum (Paus et al., 2008)), disruptions of white matter...
Acetylcholine and norepinephrine are neurotransmitters that play a role in the nervous system. Nicotine exposure experienced prenatally may lead to under-stimulation after conception because the direct effects of tobacco exposure are likely to have further (indirect) effects on neurotransmitter availability (Oncken et al., 2003). Both structural damage and increases in receptor density are likely to have functional consequences. Whereas structural damage and reductions in receptor density may be relatively easy to associate with systemic or functional down-regulation, an important hypothesis in the context of prenatal tobacco exposure is that decreases in receptor density are likely to have functional consequences. Acetylcholine and norepinephrine are two of the most important neurotransmitters for functioning of the hypothalamic–pituitary–adrenal (HPA) axis and the Sympathetic Nervous System (SNS), which are both involved in stress regulation. The SNS is part of the autonomic nervous system and is involved in quick (fight-or-flight type) reactions to stress; at the hormonal level its activity is represented by the enzyme alpha-amylase, for which increases can be observed immediately after a stressor. Compared to the SNS, the HPA-axis has more reciprocal connections with cortical structures involved in cognitive-emotional control and memory, such as the orbitofrontal cortex and the hippocampus: in humans, its activity is generally represented by changes in the hormone cortisol, which reaches its peak level approximately 20–30 min after a stressor.

Based on the evidence for PE-related changes to brain microstructure (Jacobsen et al., 2007), reduction of binding sites for serotonin transporter (Xu et al., 2001), and both increases and decreases in specific brain regions in serotonin and acetylcholine receptor density (Falk et al., 2005; Slotkin et al., 2006), stimulation of nicotinic acetylcholine receptors (nAChRs) triggers catecholamine (epinephrine and norepinephrine) secretion, so prenatal tobacco exposure is likely to have further (indirect) effects on neurotransmitter availability (Oncken et al., 2003). Both structural damage and in-and decreases in receptor density are likely to have functional consequences. Whereas structural damage and reductions in receptor density may be relatively easy to associate with systemic or functional down-regulation, an important hypothesis in the context of prenatal tobacco exposure is that decreases in receptor density are likely to have functional consequences. Acetylcholine and norepinephrine are two of the most important neurotransmitters for functioning of the hypothalamic–pituitary–adrenal (HPA) axis and the Sympathetic Nervous System (SNS), which are both involved in stress regulation. The SNS is part of the autonomic nervous system and is involved in quick (fight-or-flight type) reactions to stress; at the hormonal level its activity is represented by the enzyme alpha-amylase, for which increases can be observed immediately after a stressor. Compared to the SNS, the HPA-axis has more reciprocal connections with cortical structures involved in cognitive-emotional control and memory, such as the orbitofrontal cortex and the hippocampus: in humans, its activity is generally represented by changes in the hormone cortisol, which reaches its peak level approximately 20–30 min after a stressor.

Based on the evidence for PE-related changes to brain microstructure, neurotransmitter systems important for stress regulation, it may be hypothesized that children of mothers who smoked during pregnancy will have altered stress reactivity. Studies to date have indeed provided indications that this is the case. Behaviorally, it was shown that PE-children had less frustration or stress tolerance than non-exposed controls during delays in a (simple) cognitive task (Huijbregts et al., 2008a). Findings regarding neurobiological stress reactivity are mixed, with reports of increased cortisol reactions in PE-children (Schuetze et al., 2008) and reports of the absence of any difference in cortisol and alpha-amylase reactivity with non-exposed children (Granger et al., 2007). There are also reports showing reduced physiological stress reactivity in exposed children, although these generally used non-hormonal measurements, such as several different heart rate indices (Fifer et al., 2009). An important consideration here might be that these studies almost exclusively focused on infants, whose stress systems work differently from those of older children and whose (neurobiological) stress reactivity may also change following (continuously) high exposure to stress hormones early in life (from hyper-reactive to hypo-reactive: Miller et al., 2007). This is the first study to investigate hormonal stress reactivity in combination with mood changes and behavioral stress reactivity in exposed children from an older age group. For this group we hypothesize lower neurobiological stress reactivity compared to controls, in part based on the evidence suggesting reduced functional activity of cholinergic and catecholaminergic systems. We further expect to replicate our earlier finding of behavioral hyper-reactivity in the exposed group (Huijbregts et al., 2008a), more negative moods and more extreme mood changes.

A similar discrepancy between neurobiological and behavioral stress reactivity has been reported for children with disruptive behavior disorders (DBDs) (Van Goozen et al., 2000, 2007; Ortiz and Raine, 2004). Prenatal tobacco exposure has repeatedly been related to externalizing behavior problems in children, even when controlling for other established risk factors, but the mechanisms underlying this association are still unclear (Wakschlag et al., 2006; Huijbregts et al., 2008b). Altered patterns of behavioral and neurobiological stress reactivity in exposed children might, in part, explain their elevated levels of externalizing behavior. Thus, our two main research questions were (1) do children prenatally exposed to tobacco have altered stress reactivity, both neurobiologically and behaviorally? and (2) does stress reactivity of children prenatally exposed to tobacco resemble that of children with DBDs?

2. Methods

2.1. Participants

One-hundred-and-fifty children aged 8–13 from three regular primary schools and their families received an invitation to take part in the study. Out of 90 potential participants (i.e., those who expressed their willingness to participate), all children were selected whose mother indicated she had smoked during pregnancy (PE: N = 14, mean age: 10.6, SD 1.6) and all children whose mother indicated relatively high levels of disruptive behavior (i.e., a score of 12 or higher on “aggressive behavior” plus “rule-breaking behavior” for all girls and for boys aged 11 or younger, or a score of 14 or higher for boys aged 12 or older) on the child behavior checklist (CBCL, Achenbach, 1991) (DBD: N = 9, mean age: 10.5, SD 1.3). Normal controls (N = 15, mean age: 10.6, SD: 1.2) were matched to the PE- and DBD-children on age and gender. Thus, in total thirty-eight children (22 boys (PE: 7; DBD: 7; NC: 8); 16 girls (PE: 7; DBD: 2; NC: 7) took part in this study. Written informed consent was obtained from the parents of all children. Ethical approval for this study was granted by Leiden University’s Education and Child Studies Ethics Committee.

2.2. Procedure

Participants performed 9 neuropsychological computer tests (De Sonneville, 1999) during a session lasting approximately 90 min. During the test session, a situation of competition was created between the subject and a videotaped opponent (i.e., an actor not really involved in the competition). The video-opponent criticized the performance of the participant in a competitive and derogatory way. The rationale behind choosing this stress paradigm (based on Van Goozen et al., 2000) was that it has been shown that neurobiological stress responses are most pronounced when tasks are characterized by ‘uncontrollability’ and social-evaluative threat (i.e., task performance can be judged negatively by others) (Dickerson and Kemeny, 2004). Uncontrollability was introduced in two of the tasks: the Delay Frustration task, a relatively simple work-
ing memory task with frequent built-in delays, which invoke stress/frustration (Huijbregts et al., 2008a), and the unsolvable mazes-task, which requires participants to manoeuvre the mouse cursor through 5 different mazes presented on the computer screen. When a level (i.e., a particular maze) was not completed successfully, the program started again with the first maze. The task was unsolvable, because at higher levels the set time limit to complete the maze was too tight. In contrast with the delay frustration task, where the level of stress or frustration is operationalized as the number of response button presses during delays, the unsolvable mazes task does not produce a measure for the level of stress/frustration. The 7 remaining tasks were relatively simple tasks measuring motor control and cognitive control (working memory and inhibitory control). These non-stressful tasks were introduced to the test session in order to increase the competition’s credibility as well as credibility of the announcement that was made at the end of the test session, where experimenters told participants that they had beaten their opponent and would receive a prize. Moreover, we were interested in changes in stress hormone levels. Thus, a longer test session would allow greater insight in patterns of hormonal responses.

At three moments during the test session, participants were asked to indicate how they felt, using the adapted Von Zerrens’s clinical self-rating scales (see Van Goorzen et al., 2000), which comprise 10 Likert-scales about the extent to which someone feels “distressed”, “upset”, “angry”, “disgusted”, “fearful”, “sad”, “revolted”, “happy”, “cheerful”, and “relaxed” (1: not at all; 5: a great deal). The first ratings were provided at the start of the test session, the second set of ratings after a series of stressful tasks and film clips and the third set of ratings at the end of the test session.

Five saliva samples were collected during the session, for determination of alpha-amylase (sAa) and cortisol levels (which were both measured in all five samples). The first sample was taken at the start of the session, in order to obtain baseline sAa- and cortisol levels. Further sampling was timed to co-occur with peaks in sAa (i.e., immediately after stressful events: #2 and #4), or to co-occur with peaks in cortisol (#3 and #5). Specifically, sample #2 was taken immediately after the unsolvable mazes-task; #4 was taken immediately after a film clip in which the video opponent states the participant “will never win the prize” and the delay frustration-task. Sample #3 was taken approximately 20 min after participants had to perform a task for a second time following feedback from the video opponent, and sample #5 was taken approximately 20 min after the “you will never win the prize” film clip and the delay frustration task. Saliva was sampled using Salivette oral devices (Sarstedt, Etten-leur, The Netherlands). Sampling time was exactly 1 min. The saliva samples were stored at –20°C. Biochemical analysis was performed by the Cortisollabor at Universität Trier (Germany). All samples were assayed in duplicate; duplicates that varied by more than 15% for cortisol or 12% for sAa were reassayed. Cortisol and sAa-values were square root transformed in order to normalize distributions.

2.3. Data analysis

Group differences regarding externalizing behavior and behavioral stress reactivity (i.e., number of button presses during delays in the delay-frustration task) were analyzed using general linear model (GLM) analyses of variance (ANO-VAs). Mood and neurobiological stress reactivity were analyzed using GLM repeated measures ANOVAs with group (PE v DBD v NC) as between-subjects variable. For mood, the three rating points constituted the within-subjects variable and participants’ mood ratings the dependent variables. Mood was expected to worsen from the first to the second set of ratings and then to improve. For neurobiological stress reactivity, sampling time (1–5) was the within-subjects variable and square root transformed cortisol- and sAa-levels were the dependent variables. Pairwise comparisons for differences in mean (transformed) cortisol- and sAa-levels were performed using Fisher’s least significant difference (LSD) post-hoc tests. Cortisol and sAa-levels were expected to increase more for NC- than for PE- and DBD-children.

In order to test dose-dependency regarding associations between prenatal tobacco exposure and neurobiological stress reactivity the PE-group was further subdivided into those exposed to <10 cigarettes/day during pregnancy (N = 6) and those exposed to ≥10 cigarettes/day (N = 8). Because of small sample sizes in these analyses, non-parametric Mann–Whitney tests were performed, in which the cortisol and sAa-slopes (i.e., the differences between levels at the fifth and the first sampling time) were compared between NC, PE < 10, and PE ≥ 10. Pearson correlations were used to examine associations between behavioral, mood, and neurobiological indices of stress reactivity.

3. Results

3.1. Externalizing behavior

Mean CBCL externalizing scores were 8.5 (SD = 7.0) for the PE-group, 21.3 (SD = 9.8) for the DBD-group, and 4.6 (SD = 3.8) for NC. The three groups differed significantly from each other [F(2,35) = 11.2, p < .001, ηp² = .39], with the DBD-group displaying significantly more externalizing behavior than both the NC [Fisher’s LSD: p < .001] and the PE-group [Fisher’s LSD: p = .001], but no significant difference between PE and NC-children.

3.2. Mood

Repeated measures ANOVAs showed significant overall group differences for reported anger [F(2,35) = 7.0, p = .003, ηp² = .29], unhappiness [F(2,35) = 6.4, p = .004, ηp² = .27], and lack of cheerfulness [F(2,35) = 4.3, p = .022, ηp² = .20]. DBD-children generally indicated feeling more angry and unhappy, and less cheerful than both PE- and NC-children. There were no significant differences between PE- and NC-children. There was one trend suggesting a group by rating time interaction. This was observed for reports of feeling stressed [F(4,70) = 2.4, p = .061, ηp² = .12]. Whereas generally the stress (and recovery) manipulations appeared to work [F(2,70) = 21.5, p < .001, ηp² = .38], with significantly higher reported stress at rating point 2 compared to rating point 1, and significantly lower reported stress at rating point 3 compared to rating point 2, DBD-children’s reports of feeling stressed remained stable between rating points 1 and 2, whereas PE and NC-children showed an increase in...
reported stress between rating points 1 and 2. Note, however, that there were significant group differences in reported stress at rating point 1 ($F(2,35) = 4.3, p = .022, \eta^2_p = .20$), with DBD-children feeling more stressed than both PE-children (Fisher’s LSD: $p = .036$) and NC-children (Fisher’s LSD: $p = .007$).

3.3. Behavioral stress reactivity

Univariate analysis of variance showed significant group differences regarding behavioral stress reactivity (i.e., the square root transformed number of response button presses during 20s-delays in the delay frustration task): $F(2,35) = 19.8, p < .001, \eta^2_p = .53$. Both the PE-group ($M = 23.8, SD = 4.3$) and the DBD-group ($M = 22.2, SD = 4.8$) pressed the response button significantly more often than controls ($M = 13.1, SD = 5.4$) (Fisher’s LSD: $p < .001$ in both instances). PE- and DBD-children did not differ from each other in behavioral stress reactivity.

3.4. Neurobiological stress reactivity

Repeated measures ANOVA showed a decline in cortisol levels throughout the test session [$F(4,136) = 14.8, p < .001, \eta^2_p = .30$]. There were no significant differences in cortisol levels between groups, either at particular measurement points or with respect to the pattern of responses (Fig. 1). Repeated measures ANOVA further showed a significant increase in sAa-levels during the test session [$F(4,140) = 8.2, p < .001, \eta^2_p = .19$]. A significant interaction between group and sampling time [$F(8,140) = 2.2, p = .032, \eta^2_p = .11$] indicated that the sAa-reactivity pattern differed between groups: whereas sAa-levels in both the DBD-group and the PE-group did not change, sAa levels of controls increased over the session (Fig. 2). Contrast analyses showed that the interaction was best captured by a cubic contrast [$F(2,35) = 4.5, p = .018, \eta^2_p = .21$], indicating that, whereas sAa-levels remained stable for PE and DBD, they increased at the beginning of the session for NC (between measurements 1 and 2), then became more stable, and then increased again (starting between measurements 3 and 4, with a further increase between measurements 4 and 5). The ANOVA further showed an almost significant overall group difference for mean sAa-level [$F(2,35) = 3.2, p = .055, \eta^2_p = .15$], which was determined by significantly higher sAa-levels for NC compared to PE-children ($p = .017$). This overall difference, in turn, was determined by PE–NC differences at assessments 2–5.

Further non-parametric analyses with PE children divided into those exposed to <10 cigarettes/day and those exposed to ≥10 cigarettes/day, showed that differences in both sAa-levels and sAa-slope were particularly evident when NC-children were compared to PE-children exposed to ≥10 cigarettes/day. Mann–Whitney tests comparing the sAa-slopes showed a significant difference between NC and PE ≥10 ($U = 19, z = -2.6, p = .007$) and a non-significant trend for

![Figure 1](https://via.placeholder.com/150)

Figure 1  Salivary cortisol levels for children prenatally exposed to tobacco (PE), children characterized by high levels of disruptive behavior (DBD), and normal controls (NC) during a stress-inducing test session.

![Figure 2](https://via.placeholder.com/150)

Figure 2  Salivary alpha-amylase levels for children prenatally exposed to tobacco (PE), children characterized by high levels of disruptive behavior (DBD), and normal controls (NC) during a stress-inducing test session.
the difference between NC and PE < 10 ($U = 23$, $z = -1.7$, $p = .087$). NC-children had significantly higher sAa-levels than PE ≥ 10-children at assessments 2–5 ($H2: p = .014$; $#3: p = .029$; $#4: p = .025$; $#5: p = .012$), whereas mean sAa-levels were only higher for NC than for PE < 10 at assessment 3 ($p = .042$), with a trend observed at assessment 5 ($p = .079$).

3.5. Associations between mood, behavioral stress reactivity and neurobiological stress reactivity

There were only few significant correlations between indices of neurobiological and behavioral stress reactivity on the one hand and (changes in) mood on the other. Overall, reported anger and stress at baseline were positively related to behavioral stress reactivity (i.e., number of button presses during delays in the DF-task), although these correlations just failed to reach significance ($r = .23$, $p = .087$ for baseline stress, and $r = .24$, $p = .072$ for baseline anger). These correlations (both in direction and magnitude) were similar for all groups. Furthermore, there were differential correlations between sAa and reported stress for PE-children and NC-children at rating point 2 (i.e., after a series of stress-inducing tasks and film clips). There was a negative association for PE-children ($r = -.48$, $p = .042$) and a positive association for NC-children ($r = .46$, $p = .043$). These results indicate that, for PE-children, lower sAa-levels were associated with more stress feelings, whereas for NC-children higher sAa-levels were associated with more stress feelings. Something similar was observed for associations between sAa and reported anger at rating point 2: PE: $r = -.66$, $p = .005$; NC: $r = .39$, $p = .077$.

Most importantly, there was an overall inverse association between sAa-reactivity and DF-reactivity (i.e., between neurobiological and behavioral stress reactivity): $r = -.49$, $p = .001$, indicating that less sAa-reactivity was associated with more behavioral reactivity. This association was accounted for by the PE-children ($r = -.73$, $p = .001$) and a trend for the DBD-children ($r = -.53$, $p = .070$), while there was no significant association for NC.

4. Discussion

The results of this study showed that prenatal tobacco exposure was associated with reduced neurobiological stress reactivity and increased behavioral stress reactivity compared to controls, a pattern similar to that observed in DBD-children. The different neurobiological reaction to stress was specifically found for sAa and not for cortisol. There are several possible explanations for why no cortisol response was found (cf. Gunnar et al., 2009). Children classified as disruptive in this study had not actually been diagnosed with disruptive behavior disorders, and it is in those that hypo-reactivity of the hypothalamic–pituitary–adrenal axis has been observed (Van Goozen et al., 2007). Possibly our stress paradigm was not strong enough to elicit a cortisol response: this would however imply that in order to elicit a cortisol response a stronger stressor is required than to elicit a mood response, as both PE- and NC-children reported significantly increased stress after some provocative clips and frustrating tasks, and a stronger stressor than is necessary to elicit an alpha-amylase response. It is also possible that children felt nervous or stressed in advance of participating (as was particularly evident for the DBD-children, who reported relatively high levels of unhappiness, stress, and anger at baseline), or that the time of day when tests were performed (halfway through the morning, when cortisol levels are generally declining) was not ideal to elicit a cortisol response.

Abnormal stress reactivity (as indicated by sAa-reactivity and a lack of frustration tolerance) in PE-children may not be uniquely caused by exposure to nicotine in utero. Our results show that DBD-children have similar neurobiological and behavioral stress reactivity patterns, and the mothers of these children reported not to have smoked during pregnancy. There are many correlates of prenatal smoking and externalizing behavior that may have played a part in the development of these stress response patterns, including adverse family circumstances (low socioeconomic status, maternal depression, maltreatment and/or abuse, and exposure to community violence) and exposure to other pre- and postnatal environmental factors (Gunnar and Quevedo, 2007; Herrmann et al., 2008). In order to disentangle the contributions of prenatal tobacco exposure and other factors that might influence the development of the stress systems large population studies are required. Dose-dependency, as observed in an inverse relation between number of cigarettes/day and sAa-reactivity in this study and a positive relation between number of cigarettes/day and behavioral stress reactivity in an earlier study (Huijbregts et al., 2008a), provides some support for potential direct effects of prenatal tobacco exposure, although this should be confirmed in larger studies as well.

Altered stress reactivity of PE-children could predispose them to development of externalizing problems. The combination of low or blunted neurobiological stress reactivity and increased behavioral stress reactivity was observed in both PE- and DBD-children. Chronic underarousal theories of disruptive or antisocial behavior state that hypoactivity of neurobiological stress systems may result in stimulation-seeking and fearlessness (cf. Van Goozen et al., 2007). This could be applicable to PE-children as well. Whereas there were relatively strong inverse associations between behavioral and neurobiological stress reactivity for PE- and DBD-children (which were not present for NC-children), there were no associations between neurobiological reactivity and mood reactivity. Moreover mood and behavioral responses were not very strongly related. There were several group differences in moods at different rating points, and more negative moods were associated with higher sAa at rating point 2 (i.e., shortly after a stress-inducing task and a provocative video-clip) for controls and with lower sAa for PE-children, but in order to find the differential reactivity relations for different groups, a more reliable instrument for measuring mood during a stressful test session may have to be incorporated. Repeated self-reports for mood in the presence of an experimenter do not seem ideal and standardized behavioral measures that might also capture some aspects of mood (such as the delay frustration task, or, as has also been done before, opponent ratings, see Van Goozen et al., 2000, 2007) may be preferable in this context.

A different question that remains to be answered is whether the abnormal (combination of) stress reactivity
patterns of PE-children can explain their often-replicated increased levels of externalizing behavior. This may best be investigated in studies including more PE-children in order to allow the creation of subgroups of PE-children with and without DBD. Based on the data available from the current study, however, it can be concluded that sAA- and behavioral responses to stress are different for children exposed to prenatal smoking compared to non-exposed children without behavior problems, and that these responses resemble those of children characterized by externalizing behavior.

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Conflict of interest

All authors declare that they do not have any conflicts of interest.

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