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# Psychoneuroendocrinology

# Prenatal maternal stress is associated with lower cortisol and cortisone levels in the first morning urine of 45-month-old children

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## ARTICLE INFO

## ABSTRACT

Prenatal stress (PS) has been related to altered hypothalamic-pituitary-adrenal (HPA) axis activity later in life. So far, studies in children assessing HPA axis functioning have focused on salivary cortisol, reflecting daytime activity. The present work is part of a prospective study and aims to extend knowledge about the association between PS and HPA axis regulation in children.

To do so, we investigated cortisol, cortisone, and the ratio cortisone/(cortisone + cortisol) in the first morning urine of 45-month-old children in relation to several measures of maternal stress during pregnancy. Urinary cortisol and cortisone were measured by online turbulent flow chromatography coupled with high performance liquid chromatography-tandem mass spectrometry. PS was defined as: perceived stress for aim 1 (Perceived Stress Scale; n = 280); presence of self-reported (n = 371) and expert-rated psychopathology for aim 2 (Mini International Neuropsychiatric Interview; n = 281); continuous measures of anxiety and depression for exploratory aim 3 (State-Trait Anxiety Inventory and Edinburgh Postnatal Depression Scale; n = 280).

Aim 1: Perceived maternal PS showed negative associations with cortisol and cortisone levels. Aim 2: The presence of expert-rated maternal psychopathology was associated with reduced morning cortisone. Aim 3: Continuous measures of anxiety and depression showed negative associations with cortisol and cortisone levels. After correcting for multiple testing, perceived maternal PS (aim 1) and prenatal level of anxiety (aim 3) were significant predictors of children's urinary cortisol and cortisone in the morning (and, in the case of cortisone, also prenatal level of depression).

The ratio cortisone/(cortisone + cortisol) as a global marker for the balance between the enzymes metabolizing cortisol to cortisone and vice versa (11β-hydroxysteroid dehydrogenases type 1 and 2; 11β-HSD1 and 2) was not associated with any measure of maternal PS (aims 1–3).

The present study provides insight into possible programming effects of PS on nocturnal HPA axis activity and a proxy of 11β-HSD in a large sample. The results suggest that the nocturnal rate of cortisol production is lower in children exposed to PS, but do not support the hypothesis of divergent 11β-HSD activity.

## 1. Introduction

Early life stress can shape an individual's physiology. The hypothalamic-pituitary-adrenal (HPA) axis is crucial for the adaptation to stress and has been identified as a core mediator of early environmental influences. Its end product, cortisol, can be converted into its physiologically inactive form, cortisone, (and vice versa) by enzymatic processes involving 11β-hydroxysteroid dehydrogenases (11β-HSD), with type 1 (liver) acting as an oxo-reductase and type 2 (kidney) acting as a dehydrogenase. Stress occurring as early as during pregnancy has been

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linked to altered HPA axis activity in children. However, findings in children exposed to prenatal stress (PS) are heterogeneous: In some studies PS was associated with lower cortisol levels in the morning, evening and during the day (Laurent et al., 2013; Stonawski et al., 2018; Yehuda et al., 2005). In other studies PS was associated with higher cortisol levels in the morning and during the day (O'Connor et al., 2005; Simons et al., 2015). The exact relation between PS and HPA axis activity is not yet well understood, and the direction and strength of the effects might depend on the type of stressor and the children's age (Van den Bergh et al., 2017).

Most work on PS has exclusively focused on cortisol as an HPA axis marker. Expanding the studied metabolites can provide further insight into HPA axis functioning in relation to PS since the interplay of hormones and enzymes is complex. Thus, altered cortisol levels may originate from altered cortisol production or altered enzymatic conversion from cortisol to cortisone. Moreover, most studies in children have measured salivary cortisol during the day, therefore reflecting daytime HPA axis activity which may differ from nocturnal HPA axis activity. The first urine in the morning contains steroid hormones accumulated during the night and can thus inform about nocturnal HPA axis activity (as discussed in Sarkar et al., 2013). Urinary cortisol is a more timely integral measure in contrast to single time point determinations of cortisol in blood or saliva (Rosmalen et al., 2014).

In the present study, cortisol and cortisone in 45-month-old children's first morning urine were measured with a highly specific mass spectrometric method. The aim of this study was to evaluate the relationship between nocturnal HPA axis activity and different measures of maternal PS: perceived stress (aim 1), presence of psychopathology during pregnancy (aim 2) and continuous measures of anxiety and depression (exploratory aim 3).

## 2. Materials and methods

### 2.1. Sample

This work is part of the longitudinal study POSEIDON (Pre-, Peri-, and Postnatal Stress: Epigenetic Impact on Depression) which investigates PS and children's development and health. Between 2010 and 2013, 410 pregnant women were recruited about four to eight weeks before delivery. Data assessment was conducted in four waves: during third trimester of pregnancy (T1), within a few days of childbirth (T2), and six months (T3) and 45 months (T4) postpartum. For details see Send et al. (2017); Wolf et al. (2017), and Send et al. (2018). 302 children of the original POSEIDON cohort participated at T4. To compensate for dropouts, 101 age-matched children were recruited at T4. For inclusion and exclusion criteria see Table 1. In contrast to the original POSEIDON cohort, multiple birth was not an exclusion criterion in the new subsample and four twin pairs were included at T4. Children were excluded from the following analyses if their urine samples were either taken late (after 11am) or the parents did not recall the time. For the present work, data from the waves T1 and T4 were used.

The study was approved by the Ethics Committee of the Medical Faculty Mannheim (University of Heidelberg), and all families provided written consent.

### 2.2. Definition of prenatal stress

At T1, "perceived stress" was assessed using the Perceived Stress Scale (PSS). Mothers-to-be were also asked whether they were suffering from a psychiatric disorder ("self-reported psychopathology"). The structured Mini International Neuropsychiatric Interview (MINI) was conducted to assess "expert-rated psychopathology". Furthermore, the mothers-to-be completed the State-Trait Anxiety Inventory (STAI-S; STAI-T) and the Edinburgh Postnatal Depression Scale (EPDS). For details see Supplementary Table 1.

For the subsample recruited at T4 to replace dropouts, only one measure of maternal PS could be included in the analyses: Mothers reported retrospectively whether they had been suffering from a psychiatric disorder during pregnancy ("self-reported psychopathology").

### 2.3. Urine and data collection procedure

At T4, on the day of or prior to the appointment, parents were instructed to collect their child's first morning urine in a provided container and store it in a refrigerator until the appointment. Assessment at T4 comprised a stress test for preschool-aged children as well as a test of their cognitive development and has been described in more detail elsewhere (Send et al., 2018). If families did not participate in the in-person assessment at T4 or were unable to collect the urine beforehand, they were provided with special equipment to send a sample by postal service ($n = 37$).

### 2.4. Laboratory analyses

Urinary cortisol and cortisone were measured by online turbulent flow chromatography coupled with high performance liquid chromatography-tandem mass spectrometry (TFC-HPLC-MS/MS) operated in positive ion mode (described in Sanchez-Guijo et al., 2014). Urinary creatinine concentration was measured by Jaffe method.

### 2.5. Statistical analyses

Cortisol and cortisone were corrected for creatinine (cortisol/creatinine; cortisone/creatinine) and $\log_{10}$-transformed. The cortisone/(cortisone + cortisol) ratio was calculated as an indicator of 11β-HSD activity. For cortisol, one outlier > 3 standard deviations (SD) was winsorized to the nearest value < 3SD. Likewise, five outliers were winsorized for cortisone level and the ratio, respectively. Linear regression models were used to analyze the association between measures of nocturnal HPA axis activity and PS defined as (1) perceived stress and (2) self-reported and expert-rated psychopathology. Two exploratory analyses were performed to evaluate the association between nocturnal HPA axis activity and continuous measures of psychopathology (STAI, EPDS). All models were controlled for sex, the time of urine sample collection and whether the child was using a night diaper. The hypotheses were tested two-sided. The significance level was set to $p < .05$. Additionally, Bonferroni-corrected results (5 predictors; $p < .01$) are indicated. To investigate the influence of potential confounding variables, additional models were computed including the corresponding measure of postnatal stress at T4. Furthermore, several

**Table 1**
Inclusion and exclusion criteria for pregnant women and children.

| Inclusion criteria for pregnant women | Exclusion criteria for pregnant women | Exclusion criteria for children |
|---|---|---|
| • age 16–45 years<br>• German-speaking<br>• presumably one of the child's main caregivers | • diagnosis of hepatitis B, C or human immunodeficiency virus<br>• current psychiatric disorder requiring inpatient treatment<br>• history or current diagnosis of schizophrenia, psychotic disorder or substance dependency during pregnancy (except nicotine) | • preterm (< 30th week of pregnancy)<br>• underweight (< 1500 grams)<br>• congenital disease, malformation, deformation or chromosomal abnormality<br>• multiple birth (only original cohort)<br>• non-German-speaking (only new cohort) |

**Table 2**
Descriptive statistics for variables of interest and control variables.

| Variable | Mean ± SD or percentage |
|---|---|
| Cortisol values (ng/ml)[a,b] | 30.4 ± 26.4 |
| Cortisone values (ng/ml)[a,b] | 56.1 ± 31.6 |
| Creatinine values (mg/dl)[a,b] | 73.9 ± 30.9 |
| Cortisol/creatinine[a,b] | .50 ± .51 |
| Cortisone/creatinine[a,b] | .93 ± .77 |
| Ratio cortisone/(cortisone + cortisol)[a,b] | .67 ± .09 |
| Score on the Perceived Stress Scale (PSS)[c] | 21.2 ± 8.3 |
| Maternal self-reported psychiatric disorder during pregnancy | 12.1% |
| Maternal expert-rated psychiatric disorder during third trimester of pregnancy[d] | 12.5% |
| Score on the State-Trait Anxiety Inventory (STAI-T)[c,e] | 37.1 ± 9.3 |
| Score on the Edinburgh Postnatal Depression Scale (EPDS)[c] | 6.0 ± 5.4 |
| Children's sex (girls)[b] | 53.4% |
| Sampling time[b] | 7:44 ± 1:00 |
| Children using a night diaper[b] | 4.6% |

SD = standard deviation.
[a] Untransformed raw values.
[b] With respect to the complete sample (*n* = 371 children).
[c] With respect to the POSEIDON subsample (*n* = 280 children).
[d] With respect to the POSEIDON subsample (*n* = 281 children); Psychiatric disorder assessed with the Mini International Neuropsychiatric Interview.
[e] STAI-T and STAI-S are highly correlated, for results on STAI-S see Supplementary Table 3.

subsample analyses were carried out excluding twins, excluding children using night diapers, excluding the newly recruited children at T4 and excluding the children of the original cohort. Statistical analyses were carried out with IBM SPSS Statistics (Version 24).

## 3. Results

### 3.1. Descriptive statistics

At T4, 403 children (age: 45.0 months ± 1.0) and their parents participated. 32 children were excluded from the analyses: 21 parents did not deliver a sample; 11 samples were either taken late (after 11am) or the parents did not recall the time. Thus, 371 children were eligible for the analyses (descriptive statistics see Table 2).

Cortisol and cortisone were strongly correlated (*r* = 0.84, *p* < .001), and both negatively correlated with the cortisone/(cortisone + cortisol) ratio (*r* = -.51, *p* < .001; *r* = -.17, *p* = .001 respectively). For correlations between measures of PS see Supplementary Table 2.

### 3.2. Aims 1–3: PS and nocturnal HPA axis activity at T4

#### 3.2.1. Cortisol levels in morning urine

Perceived stress during pregnancy was negatively associated with the children's cortisol levels (aim 1: β = -.19, p = .002, n = 280). However, neither self-reported nor expert-rated psychopathology was related to cortisol (aim 2: β = -.026, p = .62, n = 371 for self-reported; β = -.086, p = .14, n = 281 for expert-rated psychopathology). To investigate possible effects of continuous measures of anxiety and depression on children's nocturnal HPA axis activity, exploratory analyses were performed: STAI-T and EPDS scores at T1 were negatively associated with the children's cortisol (exploratory aim 3: β = -0.20, p = .001, n = 280 for STAI-T; β = -.13, p = .028, n = 280 for EPDS; see Table 3). The results for perceived stress and anxiety were significant after Bonferroni-correction. Fig. 1 displays the conversion between cortisol and cortisone and the associations observed in the present study.

#### 3.2.2. Cortisone levels in morning urine

Cortisone showed associations with PS highly similar to cortisol (see Table 3). Moreover, expert-rated psychopathology was associated with reduced cortisone. The results for perceived stress and continuous measures of anxiety and depression were significant after Bonferroni-correction (see also Fig. 1).

The sample sizes are identical to the ones reported for cortisol. In all models for cortisol and cortisone, male sex, sampling time and wearing a night diaper were positively associated with cortisol and cortisone levels.

#### 3.2.3. Ratio cortisone/(cortisone + cortisol)

In the regression models for the ratio cortisone/(cortisone + cortisol), all predictors were non-significant (see Table 3 and Fig. 1). The sample sizes are identical to the ones reported for cortisol.

#### 3.2.4. Effects of potential confounders on observed associations

No substantial changes in the associations between measures of nocturnal HPA axis activity and PS were observed in the models additionally assessing the corresponding measure of postnatal stress (see Supplementary Table 4). However, for continuous measures of depression a small increase of the p-values was noted for cortisol (p = .057) and cortisone (p = .027), which in the case of cortisol surpassed the threshold of significance. The corresponding effect sizes remained unchanged for cortisol (β = -0.13) and slightly decreased for cortisone (β = -.14). Furthermore, no substantial changes in the reported associations were observed in the subsample analyses excluding twins, excluding children using night diapers, excluding the newly recruited children at T4 and excluding the children of the original cohort (see Supplementary Table 5, 6, 7, and 8).

## 4. Discussion

The results of this study indicate an association of PS with reduced cortisol production in 45-month-old children, extending evidence of the effects of the prenatal environment on HPA axis function: Maternal PS defined as perceived stress was associated with reduced cortisol and cortisone levels in the first morning urine (aim 1), expert-rated psychopathology was related to reduced cortisone in the first morning urine (aim 2), and exploratory analyses showed that continuous measures of anxiety and depression were associated with reduced cortisol and cortisone in the first morning urine (exploratory aim 3). After correcting for multiple testing, perceived maternal PS and prenatal level of anxiety were significant predictors of cortisol and cortisone and, in the case of cortisone, also prenatal level of depression. Interestingly, no associations were found with the ratio cortisone/(cortisone + cortisol). These results extend the findings of a previous study with the same sample at the same age in which PS was negatively associated with cortisol in response to a psychosocial stressor (Send et al., 2018).

Concentrating on cortisol as the most extensively studied measure of HPA axis activity limits our understanding of this system. E.g., low levels of cortisol in the first morning urine may originate either from lower nocturnal production rates of cortisol or from higher enzymatic inactivation of cortisol to cortisone. The highly specific mass spectrometric method used in the present work clearly distinguishes between cortisol and cortisone. No associations were found between PS and the ratio cortisone/(cortisone + cortisol), which has been used as a marker for the balance between 11β-HSD1 and 11β-HSD2. Since in our data cortisol and cortisone are highly correlated, and the results for cortisone largely mirror the results for cortisol, this suggests that nocturnal production rates of cortisol are lower in children exposed to PS. Although some studies provide evidence for 11β-HSD as target for prenatal programming (see Chapman et al., 2013), the present results support the hypothesis of lower cortisol production rates, but not of early environmental programming effects on 11β-HSD. However, cortisol and

**Table 3**
Predictors of cortisol (corrected for creatinine), cortisone (corrected for creatinine) and the ratio cortisone/(cortisone + cortisol) at T4.

| Aim | Predictor | $\frac{Cortisol}{Creatinine}$ | | $\frac{Cortisone}{Creatinine}$ | | $\frac{Cortisone}{(Cortisone + Cortisol)}$ | |
|---|---|---|---|---|---|---|---|
| | | Effect size β | p value | Effect size β | p value | Effect size β | p value |
| 1[a] | a) Perceived Stress Scale (PSS) | −.19 | .002** | −.20 | .001** | .029 | .63 |
| | b) Sex | .10 | .090 | .11 | .074 | −.029 | .63 |
| | c) Sampling time | .15 | .010* | .18 | .002** | .008 | .89 |
| | d) Night diaper | .17 | .005** | .15 | .012* | −.071 | .25 |
| 2[b] | a) Self-reported psychopathology | −.026 | .62 | −.051 | .32 | −.026 | .62 |
| | b) Sex | .083 | .11 | .093 | .070 | −.034 | .51 |
| | c) Sampling time | .091 | .080 | .11 | .037* | −.026 | .62 |
| | d) Night diaper | .14 | .007** | .13 | .014* | −.036 | .49 |
| 2[c] | a) Expert-rated psychopathology | −.086 | .14 | −.13 | .029* | −.040 | .50 |
| | b) Sex | .075 | .20 | .078 | .18 | −.026 | .66 |
| | c) Sampling time | .14 | .019* | .17 | .004** | .012 | .84 |
| | d) Night diaper | .15 | .012* | .13 | .034* | −.076 | .22 |
| 3[a] | a) State-Trait Anxiety Inventory (STAI-T) | −.20 | .001** | −.23 | < .001** | −.011 | .85 |
| | b) Sex | .096 | .10 | .10 | .079 | −.025 | .69 |
| | c) Sampling time | .15 | .009** | .18 | .002** | .011 | .86 |
| | d) Night diaper | .17 | .005** | .15 | .012* | −.069 | .26 |
| 3[a] | a) Edinburgh Postnatal Depression Scale (EPDS) | −.13 | .028* | −.16 | .005** | −.019 | .75 |
| | b) Sex | .090 | .13 | .097 | .10 | −.024 | .70 |
| | c) Sampling time | .15 | .011* | .18 | .002** | .012 | .84 |
| | d) Night diaper | .15 | .010* | .13 | .024* | −.070 | .26 |

Significant p-values are indicated by:

* for p < .05.

** for p < .01, corresponding to significance after Bonferroni-correction for the five predictors of interest (α = .05/5).

[a] With respect to the POSEIDON subsample ($n = 280$ children).

[b] With respect to the complete sample ($n = 371$ children).

[c] With respect to the POSEIDON subsample ($n = 281$ children); Psychiatric disorder assessed with the Mini International Neuropsychiatric Interview.

cortisone are also metabolized by other enzymes and 11β-HSD was not measured directly. As cortisol and cortisone were the only measured HPA metabolites, the conclusions regarding the production rates of cortisol and cortisol-cortisone conversion rate can only be drawn with caution.

The results from this prospective study support the growing evidence that the intrauterine period is an important developmental window for HPA axis functioning later in life. Overall, we find PS is associated with reduced cortisol and cortisone levels, which is in line with previously reported results (Laurent et al., 2013; Stonawski et al., 2018; Yehuda et al., 2005). However, other authors have found opposite effects, with higher cortisol levels in relation to PS (O'Connor et al., 2005; Simons et al., 2015). Several explanations may account for this: The assessed outcomes represent different aspects of HPA axis activity. Cortisol in saliva or blood is a marker for HPA axis activity at a certain time of the day; morning urine contains cortisol accumulated during the

night and is thus a measure of nocturnal HPA axis activity. Additionally, confounders might influence diurnal and nocturnal HPA axis activity differently, which might contribute to divergent results. Furthermore, PS-induced alterations of HPA axis activity can vary over the lifespan. For example, PS was associated with a higher cortisol awakening response (CAR) and higher cortisol at awakening in 10-year-olds, but with a lower CAR and a flatter diurnal curve when the same cohort was 15 years old (O'Connor et al., 2005; O'Donnell et al., 2013). To date, the impact of prenatal stressor type and timing and the different pathways of prenatal programming have not been investigated sufficiently to draw compelling conclusions.

This study has several limitations: First, sampling time was not fully held constant and children wearing a night diaper were not excluded, but both factors were controlled for statistically. The positive association of sampling time with cortisol and cortisone levels is consistent with the morning surge which is captured in later samples. In the main
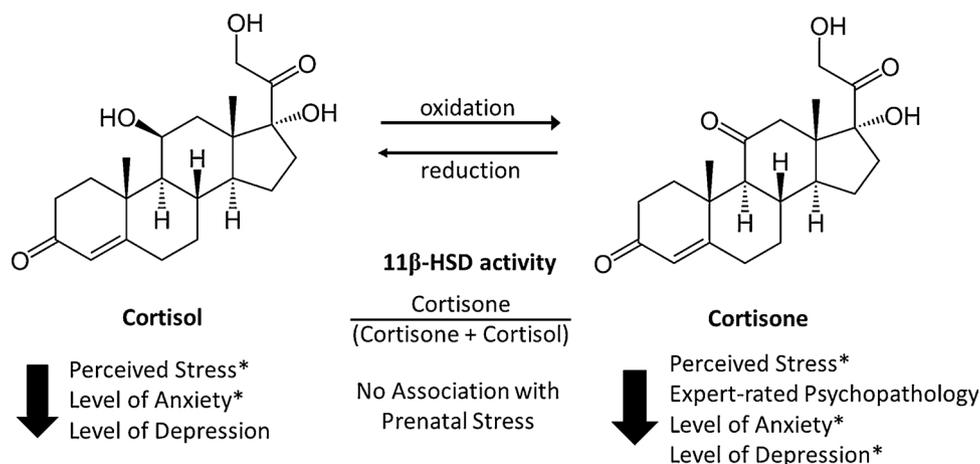


**Fig. 1.** Conversion between cortisol and cortisone: Schematic depiction of 11β-HSD driven conversion of cortisol to cortisone and vice versa. Associations observed in the present study between measures of prenatal stress and hormonal measures are depicted. *Significant after correction for multiple testing.

analyses, we did not exclude children still wearing a diaper at night, since these children can be considered slightly delayed in their physical development and are thus especially interesting for our research questions. If children used their diaper at night, morning secretion might be overrepresented in those samples, i.e. the morning rise in cortisol may cause the observed higher hormonal levels. However, the additional analysis excluding the subgroup wearing a night diaper indicated no influence of this variable. Second, measures of HPA axis activity at T4 were only measured on one day. Assessing morning urine after several nights or hair cortisol as a long-term measure would have been advantageous. Furthermore, the comparison of additional saliva samples as a measure of basal HPA axis activity during the day and measures of nocturnal HPA axis activation would have provided interesting information. However, the children and their families provided various samples besides those measuring HPA axis activity and it was not feasible to assess more samples of basal HPA axis function. Third, our additional analyses indicate that the observed effects of PS are independent of postnatal stress. However, they did not include genetic factors known to account for differences in HPA axis activity and further postnatal influences such as attachment, parenting style or parental psychopathology at an earlier age of the children (Laurent et al., 2013; Marceau et al., 2013). Postnatally observed interindividual differences may be connected to many confounding and potentially interrelated factors and our analyses do not allow drawing conclusions regarding the causality of the described effects. Thus, the possible influences of postnatal factors cannot be ruled out completely.

To our knowledge, this is the first study analyzing children's cortisol and cortisone levels measured in the first morning urine in relation to PS. The results indicate an association of PS with reduced cortisol production in 45-month-old children, likely unrelated to altered 11β-HSD activity. Both hyper- and hypoactivity of the HPA axis have been observed in psychiatric disorders (Handwerger, 2009) and it is unclear how alterations in basal cortisol levels during childhood may predispose the individual to develop disorders later in life. Future research is needed to address the effects of PS and mediating effects of HPA axis regulation on mental and somatic health, preferably in a longitudinal follow-up of prospective cohorts.

## Declarations of interest

None.

## Author contribution

All authors discussed the results and contributed to the final manuscript.

## CRediT authorship contribution statement

**T.S. Send:** Data curation, Formal analysis, Investigation, Project administration, Writing - original draft, Writing - review & editing. **S. Bardtke:** Data curation, Investigation, Project administration, Writing - review & editing. **M. Gilles:** Conceptualization, Data curation, Investigation, Project administration, Writing - review & editing. **I.A.C. Wolf:** Data curation, Investigation, Writing - review & editing. **M.W. Sütterlin:** Investigation, Writing - review & editing. **S.A. Wudy:** Investigation, Methodology, Supervision, Writing - original draft, Writing - review & editing. **R. Wang:** Investigation, Methodology, Writing - original draft, Writing - review & editing. **M. Laucht:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **S.H. Witt:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **M. Rietschel:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing. **F. Streit:** Formal analysis, Investigation, Supervision, Writing - original draft, Writing - review & editing. **M. Deuschle:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing - original draft, Writing - review & editing.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen0.2019.01.017.

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