ORIGINAL ARTICLE

Early life adversity and serotonin transporter gene variation interact at the level of the adrenal gland to affect the adult hypothalamo-pituitary-adrenal axis

RHA van der Doelen[1,2], W Deschamps[3], C D'Annibale[3], D Peeters[2,3], RA Wevers[4], D Zelena[5], JR Homberg[2,6] and T Kozicz[1,6]

The short allelic variant of the serotonin transporter (5-HTT) promoter-linked polymorphic region (5-HTTLPR) has been associated with the etiology of major depression by interaction with early life stress (ELS). Furthermore, 5-HTTLPR has been associated with abnormal functioning of the stress-responsive hypothalamo-pituitary-adrenal (HPA) axis. Here, we examined if, and at what level, the HPA-axis is affected in an animal model for ELS × 5-HTTLPR interactions. Heterozygous and homozygous 5-HTT knockout rats and their wild-type littermates were exposed daily at postnatal days 2–14 to 3 h of maternal separation. When grown to adulthood, plasma levels of adrenocorticotropic hormone (ACTH), and the major rat glucocorticoid, corticosterone (CORT), were measured. Furthermore, the gene expression of key HPA-axis players at the level of the hypothalamus, pituitary and adrenal glands was assessed. No 5-HTT genotype × ELS interaction effects on gene expression were observed at the level of the hypothalamus or pituitary. However, we found significant 5-HTT genotype × ELS interaction effects for plasma CORT levels and adrenal mRNA levels of the ACTH receptor, such that 5-HTT deficiency was associated under control conditions with increased, but after ELS with decreased basal HPA-axis activity. With the use of an *in vitro* adrenal assay, naïve 5-HTT knockout rats were furthermore shown to display increased adrenal ACTH sensitivity. Therefore, we conclude that basal HPA-axis activity is affected by the interaction of 5-HTT genotype and ELS, and is programmed, within the axis itself, predominantly at the level of the adrenal gland. This study therefore emphasizes the importance of the adrenal gland for HPA-related psychiatric disorders.

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INTRODUCTION

The risk to develop depression is largely determined by both genetic and environmental factors, and understanding the precise mechanisms is essential to design personalized treatments. Although severe adverse events such as childhood abuse and neglect have been convincingly associated with depression,[1] a discrepancy exists between the high heritability estimates of depression and the replicability of genetic association studies.[2,3] It has become apparent that the effects of genetic and environmental factors should not merely be regarded as independent, but should be considered to have an interactive nature. For instance, the effects of stressful life events on the individual risk to develop depression have been shown to be dependent on serotonin transporter (5-HTT) promoter-linked polymorphic region (5-HTTLPR) genotype.[4] Although some meta-analyses could not confirm this gene × environment (G × E) interaction,[5,6] others have shown that it is especially significant after a history of early life stress (ELS).[7] Specifically, individuals with the short (S) allele of the 5-HTTLPR polymorphism were found to be more sensitive to the depressogenic effects of stress.[7–9]

One biological system through which the 5-HTTLPR may interact with stress is the stressor-responsive hypothalamo-pituitary-adrenal (HPA) axis.[10] A stress response of the HPA-axis is initiated by parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus, by secreting corticotropin-releasing factor (CRF) at the median eminence to stimulate the synthesis and release of adrenocorticotropic hormone (ACTH), which itself stimulates the synthesis and release of glucocorticoids from the adrenal cortex.[11] The major glucocorticoid in humans is cortisol, whereas in rodents it is corticosterone (both referred to as CORT).

For 5-HTTLPR as an independent factor, it has been reported that S-allele carriers display increased basal activity of the HPA-axis,[12–16] and that S/S homozygotes show increased CORT stress reactivity compared with individuals carrying a long (L) allele of the 5-HTTLPR.[17–19] In macaques, however, 5-HTTLPR genotype has not been shown to affect basal and stress-induced CORT levels.[20,21] In mice, 5-HTT knockout (5-HTT$^{-/-}$) leads to increased adrenomedullary but not CORT responses to stress, and basal plasma CORT levels have been reported to be unaltered or lower in 5-HTT$^{-/-}$ mice.[22–30] In the case of ELS × 5-HTTLPR genotype interaction, only a history of severe stress has been shown to trigger increased CORT responses in human S-allele carriers.[31,32] In contrast, in macaques the combination of the S-allele with adverse

[1]Department of Anatomy, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, The Netherlands; [2]Department of Cognitive Neuroscience, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, The Netherlands; [3]Department of Cellular Animal Physiology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, The Netherlands; [4]Department of Laboratory Medicine, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, The Netherlands and [5]Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary. Correspondence: RHA van der Doelen, Department of Anatomy, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Geert Grooteplein 21 (route 126), 6525 EZ Nijmegen, The Netherlands.
E-mail: rick.vanderdoelen@radboudumc.nl
[6]These authors contributed equally to this work.
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rearing conditions results in increased ACTH but unaffected CORT responses to social separation.[20,21]

Despite the relatively large body of literature it is yet unclear at what level of the HPA-axis and through what mechanisms the activity of the HPA-axis is programmed by 5-HTTLPR × ELS interactions. Therefore, we assessed the HPA-axis at both central and peripheral levels in 5-HTT knockout rats, which model the 5-HTTLPR S-allele and display depression-related behavior.[9,33] Specifically, we tested the effect of ELS—that is, maternal separation—on plasma stress hormone, PVN, pituitary and adrenal gene expression levels, and we measured ACTH sensitivity of the adrenal gland as a function of 5-HTT genotype. The outcome of this study is potentially important, because whether to target central or peripheral components of the HPA-axis is essential for future drug design, due to the constraints of the blood–brain barrier.

MATERIALS AND METHODS

Animals
All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands, and all efforts were made to minimize animal suffering and to reduce the number of animals used. Serotonin transporter knockout rats (Slc6a4[1Hubr]) were generated by N-ethyl-N-nitrosourea-induced mutagenesis.[34] Experimental animals (5-HTT homozygous knockout (5-HTT^{−/−}), 5-HTT heterozygous knockout (5-HTT^{+/−}) and wild-type (5-HTT^{+/+})) were derived from crossing 3-month-old 5-HTT^{+/−} rats that were outcrossed for at least 12 generations with commercial (Harlan, Ter Horst, The Netherlands) wild-type Wistar rats. The pregnant dams were housed in standard polypropylene cages (40 × 20 × 18 cm) with sawdust bedding and *ad libitum* access to water and rodent chow (Sniff Spezialdiäten, Soest, Germany) in a temperature (21 ± 1 °C) and humidity-controlled room (45–60% relative humidity), with a 12:12 h light:dark cycle (lights on at 0700 hours). The dams were inspected daily for delivery at 1700 hours and day of birth was designated as postnatal day (PND) 0. At PND1, two paper towels (22.5 × 24.5 cm) were supplied to the mother for nest construction. Further, the litters were culled to a maximum of 10 pups, with gender ratios in favor of a male majority to maximally 7:3.

Early life stress
We used repeated and prolonged maternal separation as a model for ELS, as this paradigm has previously been shown to affect adult HPA-axis functioning.[35,36] Litters were randomly allocated to one of two rearing conditions (from PND 2 to 14): maternal separation for 180 min (MS180) or a control treatment with immediate reunion of mother and pups (MS0). A detailed description of the procedure can be found in the Supplementary Material. From PND 2 to 8, the mothers were observed to score their maternal care behavior outside the maternal separation period. The scoring of maternal care was performed daily at 0700, 1300, 1700 and 2000 hours. The distribution of the observation periods was based on the finding that nursing in rats occurs more frequently during the light period.[37] The observation periods lasted 1 h, with 20 single, focal observations spaced by 3 min. The observations were scored within 5–10 s by a trained observer in front of the cages. The following behaviors were scored (not mutually exclusive): (1) mother away from the pups, (2) mother in any type of contact with the pups, (3) mother licking/grooming any pup, (4) arched-back nursing (ABN), (5) blanket-posture nursing and (6) passive-posture nursing. The scoring of the nursing postures was based on the descriptions by Myers *et al*.[38] The frequency of the (combinations of) behaviors across each observation period was calculated by dividing the number of times the specific behavior was observed (0–20) by the total number of observations in that period.[20]

At PND 14, ear punches were taken of the pups for identification and genotyping, which was performed by Kbiosciences (Hoddesdon, UK). The procedure of genotyping has been described previously.[39] At PND 22, the pups were weaned, weighed and housed in groups of 2–3 littermates of the same sex, under the same conditions as mentioned above. From weaning until adulthood, the rats were regularly weighed (PND 30, 38, 46, 58, 65, 72, 79).

Tissue collection
For the collection of tissues only adult (PND85–95) male rats were used. Of every litter, where possible, a single rat was selected of all three genotypes. The rats were sacrificed between 0900 and 1400 hours by either acute decapitation or by transcardial perfusion. Across this time period, the rats were randomized for their genotype and early life treatment.

For decapitations, the rats were taken from their home cage into a separate room and decapitated within 10 s. Immediately, the trunk blood was collected in EDTA-coated vials and the brain and pituitary were dissected. The blood samples were put on ice and subsequently centrifuged (3400 r.p.m., 15 min) to obtain plasma samples, which were then stored at − 80 °C until measurements. The brains and pituitaries were frozen in aluminum foil on dry ice and also stored at − 80 °C.

Before transcardial perfusion, rats received an intraperitoneal injection of sodium pentobarbital (50 mg kg^{−1} body weight). With anesthetization commencing within 3–5 min, the transcardial perfusion was performed with a clamp on the abdominal aorta to limit the perfusion to the upper body parts. The perfusion was performed with phosphate-buffered saline and followed by fixation with 4% paraformaldehyde in phosphate-buffered saline. Directly after the start of the perfusion (5 min), the adrenal glands were dissected, weighed and stored at − 80 °C.

Plasma measurements
All plasma measurements were performed on samples derived from acutely decapitated rats. Plasma CORT was measured in duplicates using a colorimetric enzyme-linked immunosorbent assay kit (Demeditec Diagnostics GmbH, Kiel, Germany), ACTH with a luminescent enzyme-linked immunosorbent assay kit (Calbiotech, Spring Valley, CA, USA) and plasma adrenalin by analyzing 2,3-diphenyl quinoxalin derivatives using isocratic high-pressure liquid chromatography with fluorimetric detection after extraction from the plasma as described elsewhere.[40]

RNA isolation & cDNA synthesis
Frozen brains were cut in 420 μm-thick coronal slices in a cryostat (−15 °C). From two of these slices (cut at Bregma − 1.30 and − 1.72 mm) the PVN was bilaterally punched out with a Miltex 1.0 mm biopsy puncher (Integra Miltex, York, PA, USA). The punched samples were collected in sterile vials, immediately placed on dry ice and stored at − 80 °C. After punching was completed for all samples, PVN RNA was isolated with the NucleoSpin RNA II kit (Macherey-Nagel GmbH, Düren, Germany). For RNA isolation from the pituitary and adrenal glands, 800 μl of ice-cold TRIzol (Life Technologies, Carlsbad, CA, USA) was added to the samples, which were thereafter homogenized by sonication. After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in 30 μl of DEPC-treated, Rnase-free water. All RNA samples were stored at − 80 °C. RNA concentrations were measured and RNA purity checked (A260/280 ratio between 1.8 and 2.0) with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). First strand cDNA synthesis was performed using 40 ng of PVN RNA and 100 ng of pituitary and adrenal gland RNA. The RNA was dissolved in 12 μl of Rnase-free DEPC containing 0.25 mU random hexamer primers (Roche Applied Science, Penzberg, Germany) and then incubated at 70 °C for 10 min, followed by double-strand synthesis in first strand buffer with 10 mM DTT, 100 U Superscript II (Life Technologies), 0.5 mM dNTPs (Roche Applied Science) and 20 U of rRNasin (Promega, Fitchburg, WI, USA) at 37 °C for 75 min. The cDNA samples were stored at − 20 °C.

Quantitative real-time PCR
Quantitative real-time PCR (qRT–PCR) was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). For the reactions a total volume of 25 μl buffer solution was used containing 5 μl template cDNA, 12.5 μl Power SYBR Green Master mix (Applied Biosystems, Foster City, CA, USA), 1.5 μl Rnase-free DEPC and 0.6 μM of each primer. The sequences of the primers are available in Supplementary Table 1. Before analysis of the relative expression of the genes of interest, for each tissue it was evaluated whether Rn18S, Gapdh or Hprt1 would be the best internal control gene. The cycling protocol started with 10 min at 95 °C, followed by 39 reaction cycles with 15 s at 95 °C and 1 min at 60 °C. For each reaction, the Ct (cycle threshold) was determined, that is, the number of cycles needed to detect fluorescence above the arbitrary threshold. Relative expression of the genes of interest was calculated by the $2^{-\Delta Ct}$ method.[41] The procedure was concluded with a melting curve protocol,

from 65 °C to 95 °C, measuring fluorescence every 0.5 °C, to control for product specificity. All qRT–PCR analyses were carried out in triplicate, with newly synthesized cDNA.

Adrenal in vitro assay

Adult male 5-HTT$^{+/+}$, 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ rats without any ELS were acutely decapitated, and trunk blood and adrenal glands were collected. After dissection, the adrenals were immediately placed in 1 ml of chilled (4 °C) Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) containing 3.7 g l^{-1} NaHCO$_3$ and 2.5% BSA (one gland per tube). The adrenals were then processed into four pieces of equivalent size by two perpendicular cuts right through the medial axes of the adrenals, with the use of forceps, a sterile razor blade and a cutting mat. Then, the adrenal pieces were incubated at 37 °C in a 95% O$_2$-5% CO$_2$ atmosphere in 1 ml DMEM. After 2 × 60 min pre-incubation and refreshment of medium, 15-min samples were collected six times with ACTH-containing medium in the second fraction. The selected concentration of ACTH (10^{-12} M) was based upon experience with previous experiments.[42,43] After every 15-min incubation, medium was aspired, centrifuged and the supernatant was used for measurement of CORT concentrations by the use of a radioimmunoassay, as described elsewhere.[43]

Statistical analysis

All statistical tests have been carried out using SPSS (version 20, IBM corporation, Armonk, NY, USA)). The results are presented as the mean with the standard error of the mean (s.e.m.). For the qRT–PCR results, the 2$^{-\Delta Ct}$ data have been expressed as a ratio compared with the average of the MS0 wild-type group. For the adrenal assay, CORT levels were expressed as percentage of the basal secretion measured in the first two 15-min samples. For the maternal care scores, body weight data and the adrenal assay, analysis of variance (ANOVA) with repeated measures was performed. In case of violation of the assumption of sphericity, Greenhouse–Geisser correction was applied to determine the F-ratio. Factorial ANOVA was applied for data from the plasma hormone, adrenal weight and qRT–PCR measurements, and if a significant main effect ('genotype', 'early life stress') or interaction ('genotype × early life stress') was found, appropriate a posteriori tests were performed (one-way ANOVA and independent samples t-test). For the adrenal assay, we examined the a priori hypothesis of greater CORT response in 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats with one-sided t-tests. If doubt about the normality of the sample distributions existed, logarithmic transformation and bootstrapping were applied to test the robustness of the parametric tests (see also Supplementary Material). Statistical significance was set at $P < 0.05$.

RESULTS

Maternal Care and Body Weight

Significant effects for both ELS ($F_{1,20} = 23.49$, $P < 0.001$) and time ($F_{11.14,222.85} = 8.51$, $P < 0.001$) on the percentage of time that the pups received ABN were found (MS0: $31.3 \pm 2.0\%$ versus MS180: $41.5 \pm 0.9\%$). The main effect of ELS on ABN was found to be significant from PND 3–7 ($P < 0.05$, Figure 1). No interaction effects were found, nor main effects of ELS, on the other measures of maternal care including the licking/grooming of pups and its combination with ABN.

For the post-weaning body weight development, significant main effects of time ($F_{1.73,153.65} = 14380.56$, $P < 0.001$), ELS ($F_{1,89} = 14.76$, $P < 0.001$) and genotype ($F_{2,89} = 17.57$, $P < 0.001$) were obtained. Further, significant interactions were present for time × ELS ($F_{1.73,153.65} = 9.56$, $P < 0.001$) and time × genotype ($F_{3.45,153.65} = 12.07$, $P < 0.001$), but not for ELS × genotype or time × ELS × genotype. The MS180 male offspring developed a significantly lower body weight than MS0 animals from PND30 onwards, whereas 5-HTT$^{-/-}$ rats had a significantly lower body weight compared with 5-HTT$^{+/-}$ and 5-HTT$^{+/+}$ rats across all measurements ($P < 0.05$, Supplementary Figure 1).

Plasma measurements

Plasma ACTH levels of the adult offspring (PND85-95) were not significantly affected in our experimental design, which was also
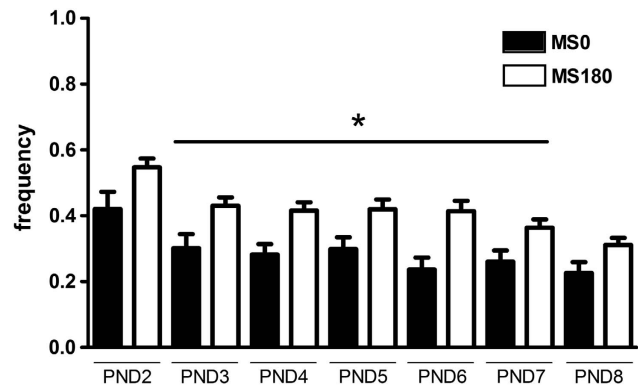

Figure 1. The frequency of arched-back nursing (ABN) of the pups as displayed by the mother rats across postnatal days (PND) 2–8. The frequency of ABN by the mother was observed daily during four observation periods (0700, 1300, 1700 and 2000 hours) in litters subjected daily to 3 h maternal separation (MS180, $n = 13$) or a control treatment (MS0, $n = 12$). Data show mean ± s.e.m. percentage of time. *$P < 0.05$ for MS180 versus MS0.

the case for plasma adrenalin (Figures 2a and c), the major output of sympathetic-adrenal medulla activation. It should be noted that it is difficult to reliably obtain basal plasma adrenalin levels from non-catheterized animals, as adrenalin levels rise within seconds when handling animals.

In contrast, plasma CORT levels were significantly affected by an interaction of ELS with genotype ($F_{2,34} = 3.51$, $P < 0.05$), and not by either factor independently. Specifically, the G × E interaction comprised an opposite effect of 5-HTT genotype depending on ELS; 5-HTT$^{-/-}$ rats displayed the highest plasma CORT levels in the control group (MS0), which was absent after MS180 exposure. In contrast, the 5-HTT$^{+/+}$ rats showed an upregulation of plasma CORT levels due to MS180, such that the 5-HTT$^{+/+}$ rats showed the highest plasma CORT levels after MS180 treatment (Figure 2b).

PVN mRNA levels

In the PVN we measured the mRNA levels of CRF, glucocorticoid receptor (GR), mineralocorticoid receptor and the GR chaperone FK506-binding protein 51 (FKBP5). Factorial ANOVA revealed a significant effect of 5-HTT genotype on GR mRNA levels ($F_{2,36} = 3.51$, $P < 0.05$). It followed that 5-HTT$^{-/-}$ rats exhibited a significantly lower GR mRNA expression than 5-HTT$^{+/+}$ rats, independent of ELS ($P < 0.05$, Supplementary Figure 2). The PVN CRF, mineralocorticoid receptor and FKBP5 mRNA levels were not affected (Supplementary Figure 3).

Pituitary mRNA levels

In the pituitary gland no significant effects on the expression of pro-opiomelanocortin (precursor protein of ACTH), GR and mineralocorticoid receptor mRNA were found (Supplementary Figure 4). For the mRNA levels of CRF receptor 1 (CRF$_1$R) there was a trend towards a 5-HTT genotype effect (Supplementary Figure 5A) ($F_{2,34} = 2.63$, $P = 0.087$), whereas pituitary FKBP5 mRNA levels were affected by a main effect of ELS ($F_{1,34} = 6.42$, $P < 0.05$). The exposure of ELS led to a decrease of FKPB5 expression in the pituitary of both 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats (Supplementary Figure 5B).

Adrenal mRNA levels

Interestingly, the qRT–PCR analysis of adrenal mRNA levels revealed gene expression patterns that resemble the plasma CORT levels as a function of ELS × 5-HTT interaction. Although we did not find independent effects of either factor on adrenal mRNA levels, the G × E interaction significantly affected the expression of
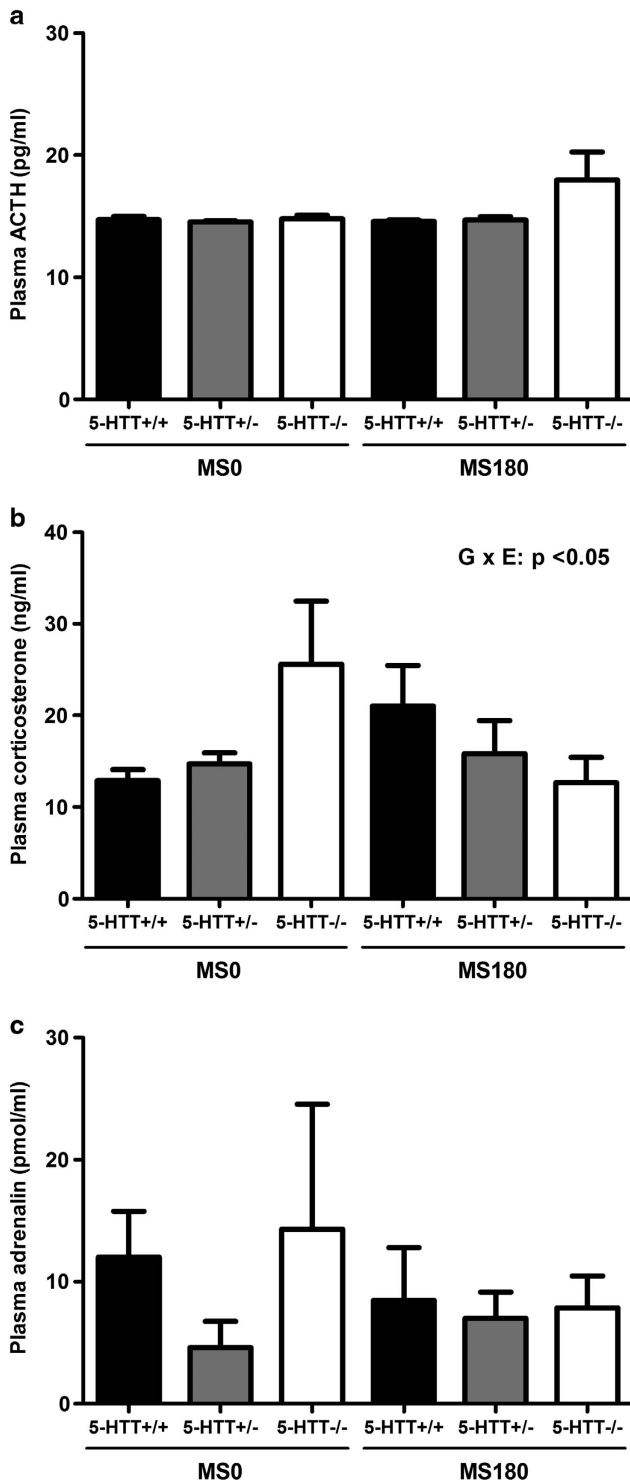
Figure 2. Plasma levels ($\pm$ s.e.m.) of adrenocorticotropic hormone (**a**, ACTH), corticosterone (**b**, CORT) and adrenalin (**c**) of serotonin transporter (5-HTT) homozygous knockout (5-HTT$^{-/-}$), heterozygous knockout (5-HTT$^{+/-}$) and wild-type (5-HTT$^{+/+}$) rats ($n = 5$–8) exposed to daily 3 h separations (MS180) or a control treatment (MS0). The plasma CORT concentrations were found to be significantly affected by the interaction of 5-HTT genotype and early life treatment (G $\times$ E, $P < 0.05$).
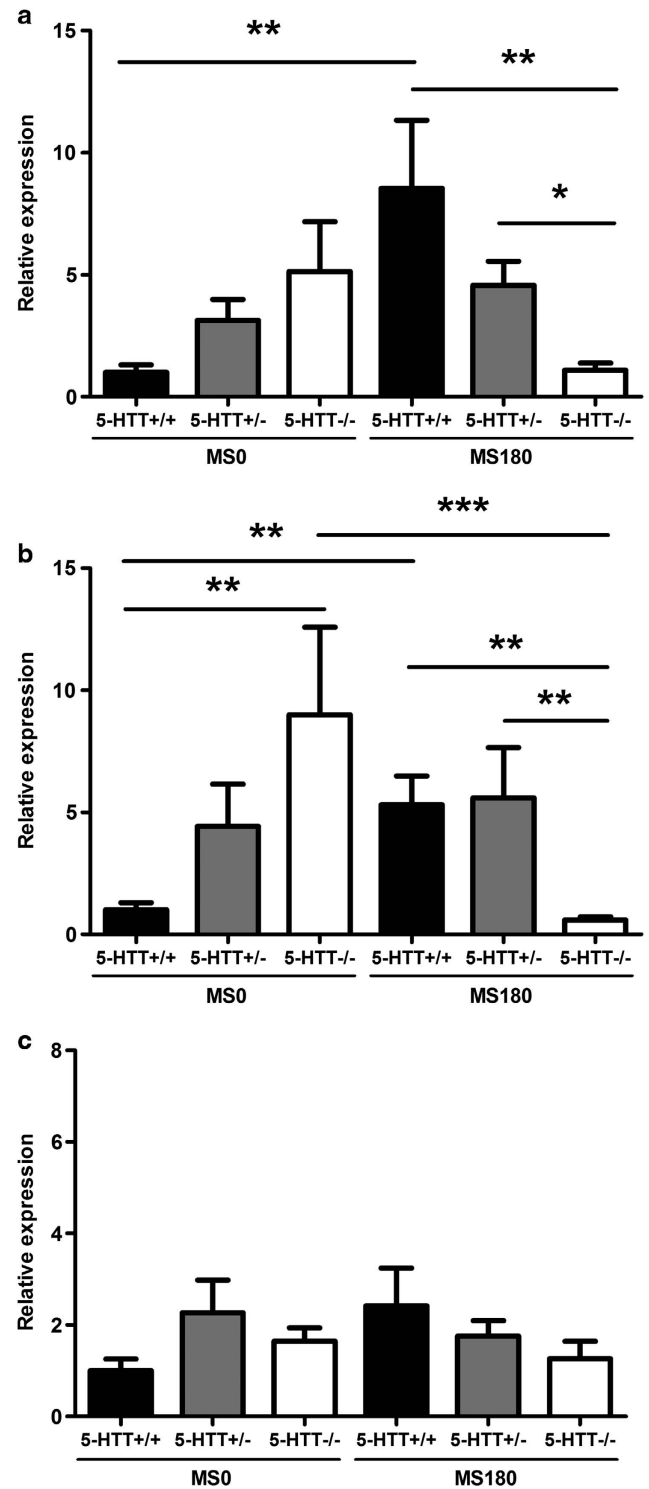
Figure 3. Adrenocorticotropic hormone receptor (**a**, ACTH-R), 11β-hydroxylase (**b**, cytochrome P450 11B1/3, CYP11B1/3) and tyrosine hydroxylase (**c**) mRNA levels in the adrenal glands of serotonin transporter (5-HTT) homozygous knockout (5-HTT$^{-/-}$), heterozygous knockout (5-HTT$^{+/-}$) and wild-type (5-HTT$^{+/+}$) rats ($n = 7$) exposed to daily 3 h separations (MS180) or a control treatment (MS0). The mRNA levels of both ACTH-R and CYP11B1/3 were found to be significantly affected by the interaction of 5-HTT genotype and early life treatment ($P < 0.01$, $P < 0.001$, respectively). Data were normalized to the average of the MS0-5-HTT$^{+/+}$ group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the ACTH-receptor ($F_{2,36} = 7.91$, $P < 0.01$) and the mitochondrial enzyme 11β-hydroxylase ($F_{2,36} = 15.38$, $P < 0.001$) (Figures 3a and b), which is responsible for the last step in glucocorticoid biosynthesis.[44] Furthermore, ELS × 5-HTT genotype interaction significantly affected the mRNA levels of steroidogenic acute regulatory protein ($F_{2,36} = 3.61$, $P < 0.05$) and 3β-hydroxysteroid dehydrogenase (3βHSD1, $F_{2,36} = 12.17$, $P < 0.001$; Supplementary Figure 6). Steroidogenic acute regulatory protein transports the steroid precursor cholesterol into the mitochondria, a process regarded as the rate-limiting step in corticosteroid synthesis,[45] whereas 3βHSD1 participates in the CORT synthesis pathway.[46] In contrast, we found no effect of ELS, 5-HTT gene variation or their interaction on the adrenal mRNA levels of tyrosine hydroxylase (Figure 3c), the rate-limiting enzyme in (nor)adrenalin biosynthesis.[47] Furthermore, adrenal weight (percentage of body weight) was not found to be affected by ELS, 5-HTT genotype or their interaction (Supplementary Figure 7).

Adrenal *in vitro* assay

As we found that the interaction of ELS and 5-HTT gene variation affected plasma CORT but not ACTH levels, as well as adrenal gene expression of the ACTH receptor, we hypothesized that the basal CORT levels were effectuated by programming of adrenal ACTH sensitivity. To provide a proof of concept, we performed an *in vitro* experiment with adrenal glands derived from adult male 5-HTT$^{+/+}$, 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ rats without any early life environmental manipulation (standard animal facility rearing, AFR). As the AFR and MS0 groups are both considered to be control groups, we expected that, upon administration of ACTH, adrenal tissue derived from AFR 5-HTT$^{-/-}$ rats would show a greater *in vitro* release of CORT than adrenal tissue of AFR 5-HTT$^{+/+}$ rats. The basal plasma CORT levels of AFR 5-HTT$^{-/-}$ rats were indeed, just as for the MS0 group, found to be higher than that of 5-HTT$^{+/+}$ rats ($P < 0.05$, Supplementary Figure 8).

For the *in vitro* adrenal assay, ANOVA-RM revealed that the adrenal explants showed a significant CORT response after the application of 10^{-12} M ACTH to the medium (time, $F_{5,75} = 11.92$, $P < 0.001$), with furthermore no main effect of 5-HTT genotype, but a significant interaction of time × 5-HTT genotype ($F_{10,75} = 1.96$, $P < 0.05$). Unfortunately, Mauchly's test indicated a violation of sphericity and we therefore had to apply Greenhouse--Geisser correction, after which the effect of time remained significant ($F_{2.09,\ 31.36} = 11.92$, $P < 0.001$) but the interaction of time × 5-HTT genotype did not ($F_{4.18,\ 31.36} = 1.96$, $P > 0.05$). However, as we had an *a priori* hypothesis, we used one-sided *t*-tests to confirm that immediately after application of ACTH (fractions 3 and 4) 5-HTT$^{-/-}$ adrenal tissue shows a significantly higher CORT response than 5-HTT$^{+/+}$ adrenal tissue ($P < 0.05$) (Figure 4). An area under the curve analysis was used to quantify the total CORT release upon ACTH stimulation, which also showed a higher CORT response of adrenal tissue derived from 5-HTT$^{-/-}$ compared with 5-HTT$^{+/+}$ rats (Supplementary Figure 9).

DISCUSSION

In this study, we show for the first time that ELS and 5-HTT genotype interact to program basal CORT levels and that this is paralleled by an equivalent G × E programming of adrenal mRNA levels of the ACTH-receptor, steroidogenic acute regulatory protein, 3β-HSD1 and 11β-hydroxylase, which regulate the sensitivity of the adrenal glands to synthesize and release CORT upon stimulation by ACTH. In contrast to the adrenal gland, gene expression in the PVN and pituitary gland were not affected by ELS × 5-HTT genotype interaction. It therefore seems that the ELS × 5-HTT genotype programming of basal HPA-axis activity is, within the axis itself, predominantly effectuated at the level of the adrenal gland. It remains, however, to be investigated how the
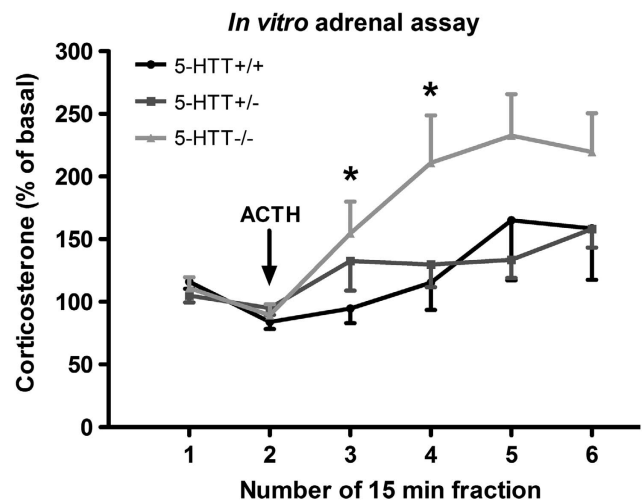

Figure 4. Corticosterone (CORT) response to 10^{-12} M adrenocorticotropic hormone (ACTH), of adrenal tissue derived from serotonin transporter (5-HTT), homozygous knockout (5-HTT$^{-/-}$), heterozygous knockout (5-HTT$^{+/-}$) and wild-type (5-HTT$^{+/+}$) rats ($n = 6$), which were reared in standard animal facility conditions. The CORT response was measured *in vitro*, in a static incubation system from which samples were collected every 15 min. *, 5-HTT$^{-/-}$ significantly different from 5-HTT$^{+/+}$ ($P < 0.05$, one-sided *t*-test).

interaction of ELS and 5-HTT genotype can actually program adrenal ACTH sensitivity. Interestingly, there are numerous intraadrenal, paracrine pathways that are involved in the regulation of adrenocortical steroidogenesis, including the intraadrenal CRF–ACTH and renin–angiotensin systems.[48,49] Moreover, the chromaffin cells of the rat adrenal medulla are known to contain 5-HT, which potently stimulates CORT release by the adrenal cortex.[50–52] In humans and frogs, this stimulation is mediated by activation of 5-HT$_4$ receptors, but for the rat the responsible 5-HT receptor subtype remains elusive.[52] In this study, we found no effect of ELS × 5-HTT genotype on the expression of the 5-HT$_4$ receptor in the adrenals (data not shown), but so far we have not further explored the possibility of ELS × 5-HTT genotype programming of the intraadrenal 5-HT system.

In human 5-HTTLPR S-allele carriers basal CORT levels are increased,[12–16] just as we found for 5-HTT$^{-/-}$ rats in control conditions. We expand this finding by showing that after exposure to ELS, the effect of 5-HTT deficiency is abolished, whereas 5-HTT$^{+/+}$ rats show an upregulation of their basal HPA-axis activity. Therefore, without a history of ELS, 5-HTT$^{-/-}$ rats show the highest CORT levels, but after ELS 5-HTT$^{-/-}$ rats display decreased and 5-HTT$^{+/+}$ rats show increased levels of basal CORT. These results resonate with the finding that 5-HTTLPR S/S individuals displayed the highest basal CORT levels within a low-risk for depression group, whereas in the high-risk group the S/S subjects showed the lowest and the L/L subjects the highest baseline CORT levels.[53] Accordingly, CORT levels could mediate the combined effects of (early life) stress and 5-HTTLPR on later life risk for psychopathology.[54] However, although CORT is expected to have a significant role in the onset and course of depression, it is not exactly clear what this role is.[55] For instance, some studies have, whereas others have not, found a relation between basal CORT levels and the recurrence of depression in remitted patients.[12,56,57] Nevertheless, altered basal HPA activity seems to be an endophenotype that cuts across disorders, with lower CORT levels consistently observed for post-traumatic stress disorder,[58] whereas elevated plasma CORT levels are found in a subset of depressive patients,[59] which possibly reflect the melancholic clinical subtype of depression.[60]

From animal studies, the perspective arises that the adaptive- or maladaptiveness of the early life programming of HPA activity is highly dependent upon the match or mismatch with the later life environment,[61–63] likely due to the specific demands of a given stressful context.[64] Indeed, ELS has been reported to lead to both hypo- and hyperactivity of the human HPA-axis,[60,65–67] and it seems that these divergent effects can be explained by distinguishing between different types of ELS and by including their possible interaction with later life adversity.[68–70] Therefore, the life history of stressful life events, in addition to the environmental demands of the specific stressful life events that triggers a current depressive episode, may influence the relation between HPA-axis measures and psychiatric variables. Furthermore, in the case of perinatal stress, the maternal HPA-axis may be an important mediator of the consequences for the offspring, which are also predicted to depend on 5-HTT genotype.[71,72]

Interestingly, in our study the interaction between ELS and 5-HTT gene variation determines basal HPA-axis output and matches an identical G×E programming of gene expression in the adrenal glands. Given that these genes include the ACTH receptor as well as several key enzymes involved in the biosynthesis of CORT and that plasma ACTH levels are simultaneously unaltered, we propose that the interaction between ELS and 5-HTT genotype programs the sensitivity of the adrenals to translate a stimulation of ACTH into the synthesis and release of CORT. In support, as 5-HTT$^{-/-}$ rats without a history of ELS (MS0/AFR) show increased basal CORT and adrenal ACTH-R mRNA compared with 5-HTT$^{+/+}$ rats, their adrenals also show an increased CORT release upon in vitro stimulation with ACTH. These findings strongly suggest that the ELS×5-HTT genotype effect on basal CORT levels would influence stress-induced HPA-axis output activity as well. In addition, the limited adaptations within the HPA-axis (pituitary, PVN) to the programming of the adrenal glands found in this study predict that CORT would influence extra-hypothalamic sites (for example, hippocampus, amygdala and prefrontal cortex) involved in HPA regulation/programming.

As no previous studies have assessed the rodent HPA-axis after combining ELS exposure and 5-HTT knockout, our results have to be considered independently too for these factors to compare them to the literature. In our 5-HTT$^{-/-}$ rats, we confirm previous findings in 5-HTT$^{-/-}$ mice showing decreased GR mRNA levels in the PVN and unaltered pituitary CRF$_1$R mRNA, adrenal tyrosine hydroxylase mRNA, plasma ACTH and adrenalin levels. We however could not replicate the finding that 5-HTT$^{-/-}$ mice show decreased CRF mRNA in the PVN and GR mRNA in the pituitary gland.[26,27,73] Regarding basal plasma CORT, both lower and unaltered levels have been reported in 5-HTT$^{-/-}$ mice,[22–29] complicating a comparison with the present data. For ELS exposure, we replicate here previous studies that showed that maternal separation leads to higher baseline plasma CORT levels in Wistar and Sprague–Dawley rats,[74–76] with unaltered CRF mRNA levels in the PVN of Sprague–Dawley rats.[77] In Long–Evans rats, however, maternal separation leads to an increase in PVN CRF gene expression with unaltered basal CORT levels.[36,78,79] These strain differences, in addition to 5-HTT gene variation, show that the effects of ELS are highly dependent on genetic variation.

Our G×E interaction findings on plasma CORT and adrenal mRNA levels consist of a strong and opposite regulation of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats by the exposure to ELS. In contrast, 5-HTT$^{+/-}$ rats seem to be unaffected. Yet, it should be noted that 5-HTT$^{+/-}$ rats do consistently display an intermediate phenotype on these measures consistent with a gene dosage effect. Although 5-HTT$^{+/-}$ rodents have been proposed as the foremost model for human 5-HTTLPR S-allele carriers, 5-HTT$^{-/-}$ rodents are regarded as a robust model for the S-allele plus a history of stress exposure.[80] Indeed, we have shown previously that when 5-HTT$^{+/-}$ rats are exposed in adulthood to an additional stressor the experience of early life adversity directs the stress coping behavior of 5-HTT$^{+/-}$ rats towards that as displayed by 5-HTT$^{-/-}$ rats.[62] Therefore, the effects of ELS on the HPA-axis of 5-HTT$^{+/-}$ rats might only become apparent with exposure to additional stressors in later life.

For the interpretation of the effects of ELS on HPA-axis programming, we have considered the role of alterations in the care that the mother rats provide to their pups. The group of Michael Meaney and others have namely shown that a very specific part of maternal care, the licking and grooming of pups, can influence the programming of the HPA-axis into adulthood.[81–83] However, the exposure of ELS was not found to affect the frequency of licking and grooming displayed by the mothers. In contrast, we found that ELS increased the frequency of ABN, but this maternal behavior is not known to affect HPA-axis programming.[81–83] Therefore, we conclude that the ELS-induced programming of the HPA-axis is not mediated by alterations in maternal care. The increased ABN due to maternal separation could be considered as an expression of nutritional compensation, although it did not prevent a decreased body weight from PND 30 onwards. The isolated, negative effects of ELS and 5-HTT deficiency on body weight development have both been documented before.[74,84]

In conclusion, we report here that early life programming of basal HPA-axis activity is moderated by 5-HTT genotype and that this interaction seems to be effectuated predominantly by the regulation of adrenocortical gene expression. Altered HPA activity is an endophenotype that is widely relevant across the spectrum of psychiatric disorders, therefore, this study emphasizes the importance of the adrenal gland in stress-related psychopathology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)