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Research paper

Offspring's own serotonin transporter genotype, independently from the maternal one, increases anxiety- and depression-like behavior and alters neuroplasticity markers in rats

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ABSTRACT

Introduction: Developmental changes due to early life variations in the serotonin system affect stress-related behavior and neuroplasticity in adulthood. These outcomes can be caused both by offspring's own and maternal serotonergic genotype. We aimed to dissociate the contribution of the own genotype from the influences of mother genotype.

Methods: Sixty-six male homozygous (5-HTT$^{-/-}$) and heterozygous (5-HTT$^{+/-}$) serotonin transporter knockout and wild-type rats from constant 5-HTT genotype mothers crossed with varying 5-HTT genotype fathers were subjected to tests assessing anxiety- and depression-like behaviors. Additionally, we measured plasma corticosterone levels and mRNA levels of BDNF, GABA system and HPA-axis components in the prelimbic and infralimbic cortex. Finally, we assessed the effect of paternal 5-HTT genotype on these measurements in 5-HTT$^{+/-}$ offspring receiving their knockout allele from their mother or father.

Results: 5-HTT$^{-/-}$ offspring exhibited increased anxiety- and depression-like behavior in the elevated plus maze and sucrose preference test. Furthermore, *Bdnf isoform VI* expression was reduced in the prelimbic cortex. *Bdnf isoform IV* and GABA related gene expression was also altered but did not survive false discovery rate (FDR) correction. Finally, 5-HTT$^{+/-}$ offspring from 5-HTT$^{-/-}$ fathers displayed higher levels of anxiety- and depression-like behavior and changes in GABA, BDNF and HPA-axis related gene expression not surviving FDR correction.

Limitations: Only male offspring was tested.

Conclusions: Offspring's own 5-HTT genotype influences stress-related behaviors and *Bdnf isoform VI* expression, independently of maternal 5-HTT genotype. Paternal 5-HTT genotype separately influenced these outcomes. These findings advance our understanding of the 5-HTT genotype dependent susceptibility to stress-related disorders.

1. Introduction

Neuropsychiatric disorders are one of the leading causes of human disability, resulting in a considerable social and economic burden (Collins et al., 2013; Gonda et al., 2019). The prevalence of most neuropsychiatric disorders has been increasing over the past few

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decades, particularly anxiety and depression. Among the multifactorial risk factors, it is widely accepted that serotonin (5-HT) plays a role in both disorders (Cipriani et al., 2018; Jakubovski et al., 2019).

The first-line treatment of depression consists of selective serotonin reuptake inhibitor (SSRI) treatment, which acts by increasing 5-HT levels thereby exerting anxiolytic and antidepressant effects. Independently, some studies have shown that the low activity short (s) allelic variant of the serotonin transporter-linked polymorphic region (5-HTTLPR) is, upon exposure to life stress, related to increased vulnerability to affective disorders, including depression (Bleys et al., 2018; Delli Colli et al., 2022). Yet, the 5-HTTLPR s-allele is theorized to be associated with reduced transcription of the 5-HTT gene (Lesch et al., 1997; Yohn et al., 2017), and thereby reduced 5-HT reuptake and increased synaptic 5-HT levels. Thus, it appears paradoxical that the 5-HTTLPR s-allele is associated with increased vulnerability to depression. 5-HT is primarily known as a neuromodulator and targeted as such by 5-HTergic pharmacotherapies like SSRIs. However, in early brain development, serotonin also influences various developmental processes such as cell differentiation and migration (Zhang, 2003). Accordingly, the 5-HTTLPR s-allele may increase risk of vulnerability to depression either through altered neuromodulation in adulthood, or brain developmental effects.

By offering the possibility to control environmental and genetic factors, animal studies have helped to clarify the serotonin 'paradox' in anxiety and depression research. For instance, it has been reported that 5-HTT knockout rodents exhibit increased extracellular 5-HT levels (Homberg et al., 2007) and anxiety- and depression-like behavior (Olivier et al., 2008; Pan et al., 2020; Shoji et al., 2023) as well as changes in the wiring of the prefrontal cortex (PFC) during embryonic development (Chaji et al., 2021; Witteveen et al., 2013). As the effects of 5-HTT knockout in rodents resemble the effects of prenatal (thus neurodevelopmental) selective SSRI exposure in terms of increasing anxiety- and depression-like symptoms in offspring (Gallo et al., 2023; Homberg et al., 2010; Kepser and Homberg, 2015), the behavioral profile of 5-HTT knockout rodents may be of neurodevelopmental origin. Given that 5-HTergic receptors are already expressed in areas of the forebrain such as the medial PFC (mPFC) before they are innervated by 5-HTergic fibers (Booij et al., 2015; Buznikov et al., 2001; Gaspar et al., 2003), a maternal/placental exogenous source has been hypothesized to supply the fetus with 5-HT. Some studies showed that maternal 5-HT stored in blood platelets can reach and supply the fetal brain when the blood-brain-barrier is not yet fully functional (Cote et al., 2007; Kliman et al., 2018), whereas other work has reported that the placenta is able to synthesize 5-HT (Bonnin et al., 2011; Hudon Thibeault et al., 2017; Mao et al., 2021). Regardless of the exact route, during this early stage of development 5-HT levels in the fetus are likely dependent on maternal factors influencing 5-HT levels, including *maternal 5-HTT genotype*. In support of this hypothesis, it has been demonstrated in mice that maternal 5-HTT genotype affects placenta and embryonic forebrain 5-HT levels and induces a broadening of 5-HT-sensitive thalamocortical axon projections in the embryo (Muller et al., 2017). In addition, we lately showed that maternal 5-HTT genotype in rats affected -through a combination of placental (affecting 5-HT supply to the embryo) and maternal care effects- anxiety-like behavior and mPFC 5-HT levels in same genotype offspring (Hanswijk et al. in preparation).

From halfway pregnancy, (embryonic day 11.5 in rats), 5-HT neurons are born in the dorsal raphe nucleus-allowing the fetal brain itself to synthesize 5-HT. The amount of 5-HT that is available for neurotransmission in the fetal brain is then influenced by the *offspring's own 5-HTT genotype*. Notably, 5-HTergic projection neurons only reach the forebrain at embryonic day 17.5 (Bonnin et al., 2011). There is thus a transition phase from maternal to offspring's genotype effects depending on the brain regions. This situation in development raises the question: Does the offspring's own 5-HTT genotype influence brain development and behavior in adulthood independently of maternal 5-HTT genotype?

The aim of this study was to address this question, specifically to dissociate the contribution of the own 5-HTT genotype from the influences of mother genotype on anxiety- and depression-like behavior and related neuroplastic changes. To this end we made use of the well-established 5-HTT knockout rat model mimicking the 5-HTTLPR s-allele in humans (Schipper et al., 2019). We compared homozygous 5-HTT knockout (5-HTT$^{-/-}$), heterozygous 5-HTT knockout (5-HTT$^{+/-}$) and wild-type (5-HTT$^{+/+}$) offspring from 5-HTT$^{+/-}$ mothers crossed with either 5-HTT$^{-/-}$ or 5-HTT$^{+/+}$ fathers. In this breeding design, offspring are exposed to similar prenatal placental-derived 5-HT levels and postnatal maternal care. Additionally, 5-HTT$^{+/-}$ offspring have either a 5-HTT$^{-/-}$ or a 5-HTT$^{+/+}$ father, which allowed us to investigate in 5-HTT$^{+/-}$ adult rats the possible effects of the paternal 5-HTT genotype (5-HTT$^{-/-}$ or 5-HTT$^{+/+}$) or of the parental origin of the 5-HTT^{-} allele (paternal or maternal), which is relevant for parent-of-origin effects reported for the 5-HTTLPR in humans (Kistner-Griffin et al., 2011; Laplante et al., 2019). To phenotype the offspring, the rats were exposed to a variety of depression- and anxiety-related behavioral paradigms in adulthood. In addition, we investigated the molecular mechanisms in the mPFC underlying the observed phenotypes and focused on the Brain-Derived-Neurotrophic Factor (BDNF), and GABAergic systems that are not only well known to be implicated in stress-related disorders (Armeanu et al., 2017; Dean and Keshavan, 2017; Liu et al., 2015; Lydiard, 2003; Naaijen et al., 2017; Songtachalert et al., 2018), but are also influenced by 5-HT's trophic actions (Ciranna, 2006; Dean and Keshavan, 2017; Guidotti et al., 2012; Homberg et al., 2014). Earlier studies have shown that 5-HTT$^{-/-}$ rats display reduced BDNF levels and GABAergic markers in the brain, from early development in a stable manner up to adulthood (Calabrese et al., 2013; Guidotti et al., 2011; Miceli et al., 2017). Because 5-HTT genotype also affects baseline and stress-induced plasma corticosterone levels in adulthood (Van der Doelen et al., 2014a), we additionally investigated the effects of offspring genotype on the hypothalamus-pituitary-adrenal (HPA) axis. This axis represents the core stress axis of the body, plays a key role in stress-related disorders, and is regulated by the mPFC (Carmichael and Price, 1995).

These analyses contribute to a better understanding of the offspring's own 5-HTT genotype effects, independently from maternal 5-HTT genotype influences, on molecular and behavioral changes in individuals characterized by inherited 5-HTT down-regulation and at risk for stress-related disorders.

2. Material and methods

2.1. Animals

All experiments were conducted in accordance with national guidelines and regulations of EU Council Directive 2010/63/EU, also approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre. Every effort is made to minimize the suffering of animals and to reduce the number of animals used. The experimental paradigm consisted of three groups of animals, male Wistar 5-HTT$^{-/-}$ (homozygous knockout), 5-HTT$^{+/-}$ (heterozygous knockout) and 5-HTT$^{+/+}$ (wild-type) rats. 5-HTT$^{-/-}$, 5-HTT$^{+/-}$ and 5-HTT$^{+/+}$ offspring were derived by crossings between 5-HTT$^{+/-}$ mothers (similar placental-derived 5-HT levels and maternal care) and either 5-HTT$^{-/-}$ or 5-HTT$^{+/+}$ fathers. The parents and offspring were socially housed in a standard Macrolon® type 3 cage, with free access to food and tap water, in a humidity (50 %–60 %) and temperature (21 °C) -controlled room. Dark/light conditions were adapted for each experiment as specified.

2.2. Adult offspring behavioral assessment

To address our research question, we subjected adult 5-HTT$^{+/+}$, 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ offspring to a series of affective behavioral tests during adulthood. The timeline of the experiments is shown in Fig. 1.

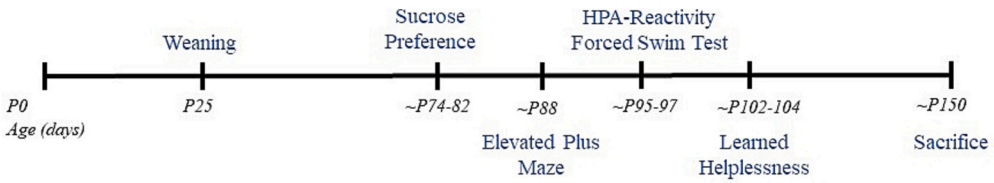

Fig. 1. Overview of experimental design.

2.3. Sucrose preference test

The rats were individually housed in transparent cages, to avoid any effects on sucrose consumption resulting from social interference (Liu et al., 2018). On top of the cage, there were two drinking bottles. The test consisted of a four-day habituation period and two-day testing period with one day of rest between the two test days (van der Kam, 2006). During the habituation and rest days both bottles were filled with water. During the two testing days, one bottle was filled with an 8 % sucrose solution and the other with water. The rats could freely choose between them. To prevent spatial bias, the sucrose solution was administered at a different position each test day. Fluid consumption (mg) and body weight (kg) were collected each day to calculate two indicators: the preference for sucrose over water ((sucrose solution intake in mL corrected for the voluminal weight of sucrose / (sucrose solution intake + water intake)) x 100 %), and the intake of sucrose in grams per kilogram bodyweight (intake in mL corrected for the voluminal weight of sucrose, recalculated towards a 100 % solution, and divided by the body weight in kg).

2.4. Elevated plus maze test

The elevated plus maze test was used to assess anxiety-like behavior (de Jong et al., 2006). The apparatus (made of black PVC) consisted of a "+" shaped maze elevated above the floor (50 cm) with two oppositely positioned closed arms (50 × 10 cm, light intensity 4.5 lx), two oppositely positioned open arms (50 × 10 cm, light intensity 12 lx), and a central area (10 × 10 cm). At the beginning of the test, each rat was placed in the center of the maze facing one of the open arms for a 5-min free exploration. During these 5 min, movements were recorded and automatically recorded using EthoVision XT10 Tracking System (Noldus, Wageningen, The Netherlands). We measured the total distance moved (cm), the time spent in open arms (s), the latency towards open arms (s), and the number of head-dips in open arms (s).

2.5. Forced swim test

The forced swim test (FST) was used to assess stress coping behavior. The FST was performed in a cylindrical glass tank (25 cm diameter, 50 cm height), and the water level was 30 cm of height (22 ± 1 °C). There were 2 sessions, 24 h apart. In the first session, the rats were individually placed in the cylinder filled with water for 15 min (pre-test). 24 h later, the rats were placed in the same tank for 5 min (test). We changed the water after every test session to avoid any influence between rats (pre-test and test). Movements were automatically recorded using DeepLabCut and categorized as immobility, diving time, and floating time (Mathis et al., 2018).

2.6. Learned helplessness test

The learned helplessness procedure was conducted according to a previous protocol with some adjustments (van der Doelen et al., 2013). In this test, we used a shuttle box (model ENV-010MD, Med Associates, St. Albans, VT, USA), which consisted of two chambers: One with a shock generator and a "Safe House" in which no shocks were delivered. During two consecutive pre-exposure sessions of 10 min, the rats were subjected to 50 inescapable and unpredictable 0.6 mA foot shocks (scrambled shock generator, model ENV -Model 412, Med Associates). The door separating the two chambers was raised in between the shocks, to provide them with the experience of the possibility to move between chambers. 24 h after the second pre-exposure, the rats were subjected to 30 trials during which they were initially placed in the shock chamber. The door was lifted 1 s after the start of each electric shock. The rats' position was detected by eight infrared beams to record the escape latencies from the shock chamber to the chamber without shock. By moving to the other compartment, the rats could escape from the electric shock. If rats failed to escape, we assigned an escape latency of 15 s.

2.7. Brain punching

Rats were decapitated and the brains were immediately collected and frozen in aluminum foil on dry ice before being stored at −80 °C. Using a cryostat at −20 °C, 80 μm thick coronal slices were prepared, and punches of the prelimbic and infralimbic mPFC were taken bilaterally using a Miltex 1.0 mm biopsy puncher (Integra Miltex, York, PA, USA) between Bregma +3.72 and + 2.52 mm. These punches were then stored in sterile vials on dry ice at −80 °C until being analyzed for quantitative Polymerase Chain Reaction (qPCR).

2.8. mRNA extraction and gene expression analyses

mRNA from the prelimbic and infralimbic cortex was extracted using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. All samples were treated with DNase to avoid DNA contamination and analyzed by a TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories, Italy) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories, Italy). Samples were run in 384 well formats in triplicate as multiplex reactions with a normalized internal control (β-actin). Primers and probes for the genes listed in Table 1 were purchased from Eurofins MWG-Operon (Germany) and Life Technologies (Italy). Samples were incubated for RNA reverse transcription (10 min at 50 °C) and TaqMan polymerase was activated (5 min at 95 °C). Subsequently, 39 PCR cycles were performed (10 s of melting process at 95 °C and 30 s of annealing and stretching at 60 °C). Relative target gene expression was calculated using the comparative cycle threshold method.

2.9. HPA-axis reactivity test

Blood samples for corticosterone analysis were collected by tail cut 24 h before FST habituation as well as 20 and 60 min after the FST test. Samples were collected in capillary blood collection tubes (Microvette® CB 300 Di-Kalium-EDTA, Sarstedt, Germany) and centrifuged at 3400 rpm for 15 min at 4 °C. Plasma was collected and stored at −20 °C until further analysis. Samples were analyzed in duplicates using a radioimmunoassay kit for corticosterone (7120103, MP Biomedals, USA) for rats.

2.10. Statistical analysis

Data were statistically analyzed using Statistical Package for the

Table 1
qPCR genes of interest' primer and probes sequences.

Genes of interest	Forward primer	Reverse primer	Probe
total Bdnf	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACAGTTTAT	TGTGGTTTGTTGCCGTTGCCAAG
Parvalbumin	CTGGACAAAGACAAAAGTGGC	GACAAGTCTCTGGCATCTGAG	CCTTCAGAATGGACCCCAGCTCA
Gad65	TGAGGGAAATCATTGGCTGG	TCCCCTTTTCCTTGACTTCTG	TGCCATCTCCAACATGTACGCCA
Gad67	ATACTTGGTGTGGCGTAGC	AGGAAAGCAGGTTCTTGGAG	AAAACTGGGCCTGAAGATCTGTGGT
$Gaba_Ag2$	ACTCATTGTGGTTCTGTCCTG	GCTGTGACATAGGAGACCTTG	ATGGTGCTGAGAGTGGTCATCGTC
Vgat	ACGACAAACCCAAGATCACG	GTAGACCCAGCACGAACATG	TTCCAGCCCGCTTCCCACG
Nr3c1	GAAAAGCCATCGTCAAAAGGG	TGGAAGCAGTAGGTAAGGAGA	AGCTTTGTCAGTTGGTAAAACCGTTGC
Nr3c2	GACAATTCCAAGCCTGACAC	ACTGGATGAGGGTAATTTGGTC	AAGTCGTGAAGTGGGCCAAGGT
β-actin (reference gene)	CACTTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG	TCTGGGTCATCTTTTCACGGTTGGC
Genes of interest	**Accession number**	**Assay ID**	
Bdnf long 3'UTR	EF125675	Rn02531967_s1	
Bdnf isoform IV	EF125679	Rn01484927_m1	
Bdnf isoform VI	EF125680	Rn01484928_m1	
Gadd45β	BC085337.1	Rn01452530_gI	

Social Sciences (SPSS) version 23.0 (IBM Corp, Armonk, NY, USA). Per group, outliers (data points further than 3 interquartile ranges from the nearer edge of the box plot) were excluded from the analyses. Univariate Analysis of Variance (ANOVA) was performed with offspring's own 5-HTT genotype (5-HTT$^{-/-}$, 5-HT$^{+/-}$, 5-HTT$^{+/+}$) as an independent factor. Significant main effects were further investigated using the Fisher LSD post hoc test comparing the three 5-HTT genotypes. In addition, for the 5-HTT$^{+/-}$ genotype group, we determined if the observed phenotypes were dependent on whether the 5-HTT^{-} allele was received from the mother or from the father, also using ANOVA testing. For all analyses, the Benjamini-Hochberg Procedure (significant effect: False Discovery Rate (FDR) 5 %) was used to correct for multiple testing. Correlations between outcomes were determined through Pearson correlation analyses. Outcomes from Pearson correlation analyses, and post hoc tests were considered statistically significant at $p \leq 0.05$. FDR corrected *p*-values are indicated as p_{corr}. Graphs were made using the ggplot2 package in R (4.1.1) and measured variables as means ± standard error of the mean (SEM).

3. Results

3.1. The effect of offspring's own 5-HTT genotype on anxiety-and depression-like behavior

To clarify to what extent the offspring's own 5-HTT genotype influences anxiety- and depression-like behavior independently from the maternal 5-HTT genotype, 5-HTT$^{+/+}$, 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ adult male offspring, all from 5-HTT$^{+/-}$ mothers, were subjected to the elevated plus maze, sucrose preference, learned helplessness and forced swim tests (Supplementary Table 1, Figs. 2, 3).

In the elevated plus maze test, there was a significant main effect of the offspring's 5-HTT genotype on all measured parameters. As shown in Fig. 2A, there was a significant genotype effect for the latency to enter the open arms ($F_{(2, 116)} = 22.89$, $p_{corr} = 1.68E-08$). Compared to 5-HTT$^{-/-}$ rats, 5-HTT$^{+/-}$ rats and 5-HTT$^{+/+}$ rats showed a decreased latency ($p < 0.001$, $p < 0.001$, respectively). No significant difference was found between 5-HTT$^{+/-}$ rats and 5-HTT$^{+/+}$ rats ($p > 0.050$). The time spent in the open arms was associated with a significant difference as well ($F_{(2, 118)} = 13.67$, $p_{corr} = 9.42E-06$). In comparison to 5-HTT$^{-/-}$ rats, 5-HTT$^{+/+}$ and 5-HTT$^{+/-}$ rats spent more time in the open arms ($p < 0.001$, $p = 0.001$, respectively) (Fig. 2B). Furthermore, the offspring's own 5-HTT genotype influenced the percentage of time spent in the open arms ($F_{(2,120)} = 13.30$, $p_{corr} = 6.07E-06$), with 5-HTT$^{+/+}$ and 5-HTT$^{+/-}$ rats exhibiting higher percentage in comparison to 5-HTT$^{-/-}$ ($p < 0.005$, $p < 0.050$, respectively) (Fig. 2C). Offspring of different genotypes varied also significantly in the time spent on head-dipping in the open arms ($F_{(2, 115)} = 8.57$, $p_{corr} = 4.54E-04$). In comparison to 5-HTT$^{-/-}$ rats, 5-HTT$^{+/+}$ and 5-HTT$^{+/-}$ rats spent more time ($p < 0.050$, $p < 0.050$, respectively) on head dipping, while no differences between 5-HTT$^{+/+}$ and 5-HTT$^{+/-}$ rats ($p > 0.500$) were found (Fig. 2D). We also found a significant effect of genotype for the total distance moved in the maze ($F_{(2, 108)} = 3.41$, $p_{corr} = 0.037$). Compared to 5-HTT$^{-/-}$ rats, 5-HTT$^{+/+}$ and 5-HTT$^{+/-}$ rats traveled less ($p < 0.050$, $p < 0.050$, respectively), while no difference was found between 5-HTT$^{+/+}$ and 5-HTT$^{+/-}$ rats ($p > 0.400$) (Fig. 2 E).

In the sucrose preference test, the rats' own 5-HTT genotype significantly affected sucrose preference and intake on both testing days (Fig. 3; day 1: $F_{(2, 119)} = 9.17$, $p_{corr} = 7.95E-04$; day 2: $F_{(2, 118)} = 7.81$, $p_{corr} = 0.002$). On day 1, compared to 5-HTT$^{-/-}$ and 5-HTT$^{+/-}$ rats, 5-HTT$^{+/+}$ rats displayed the greatest preference for sucrose ($p < 0.001$, $p < 0.050$). No significant difference was found between 5-HTT$^{-/-}$ rats and 5-HTT$^{+/-}$ rats ($p = 0.072$) (Fig. 3A). On day 2, compared with 5-HTT$^{-/-}$ rats, 5-HTT$^{+/+}$ rats displayed a higher preference for sucrose ($p < 0.010$) and no difference was found between 5-HTT$^{+/+}$ versus 5-HTT$^{+/-}$ rats ($p > 0.050$) and 5-HTT$^{-/-}$ versus 5-HTT$^{+/-}$ rats ($p > 0.050$) (Fig. 3B). Sucrose intake was not different on day one ($F_{(2,121)} = 0.495$, $p = 0.202$, Fig. 3C), and the genotype effect was not confirmed after FDR on day 2 ($F_{(2, 121)} = 3.19$, $p = 0.045$, $p_{corr} = 0.060$) (Fig. 3D).

Next, offspring were exposed to the learned helplessness test and the average escape latency over the 30 trials was measured. No genotype differences were found ($F_{(2,123)} = 0.461$, $p = 0.660$) (Supplementary Fig. 1A).

Finally, we tested the animals in the forced swim test. Diving time, floating time and immobility were measured. However, no significant genotype effect was found ($F_{(2, 60)} = 0.149$, $p = 0.660$; $F_{(2, 60)} = 0.022$, $p = 0.978$; $F_{(2, 60)} = 0.037$, $p = 0.964$, respectively) (Supplementary Fig. 1B, C, D).

In sum, 5-HTT$^{-/-}$ offspring showed increased anxiety- and depression-like behavior compared to 5-HTT$^{+/-}$ and 5-HTT$^{+/+}$ rats with mothers of the same genotype, indicating that their own 5-HTT genotype regulates these traits in adulthood.

3.2. The effect of offspring's own 5-HTT genotype on GABAergic, BDNF and HPA-axis-related gene expression in the mPFC

Next, we studied genotype differences in the mRNA expression levels of genes involved in GABA neurotransmission, neuroplasticity, and HPA-axis functioning, in the infralimbic (Supplementary Table 2) and prelimbic (Supplementary Table 3) parts of the mPFC.

Regarding the genes related to the GABAergic system, we measured *Gad65*, *Gad67*, *GabaAg2, Vgat* and *parvalbumin*. For *Gad67* in the infralimbic cortex, which catalyzes the production of GABA, after ANOVA, a main effect of genotype was found ($F_{(2,22)} = 4.113$, $p = 0.030$) (Fig. 4A), which was not maintained after FDR correction (FDR = 0.146). For *Gad67* in the prelimbic cortex, no significant main effect was found ($F_{(2,22)} = 0.117$, $p = 0.890$) (Fig. 4B). For *parvalbumin*, a marker for a

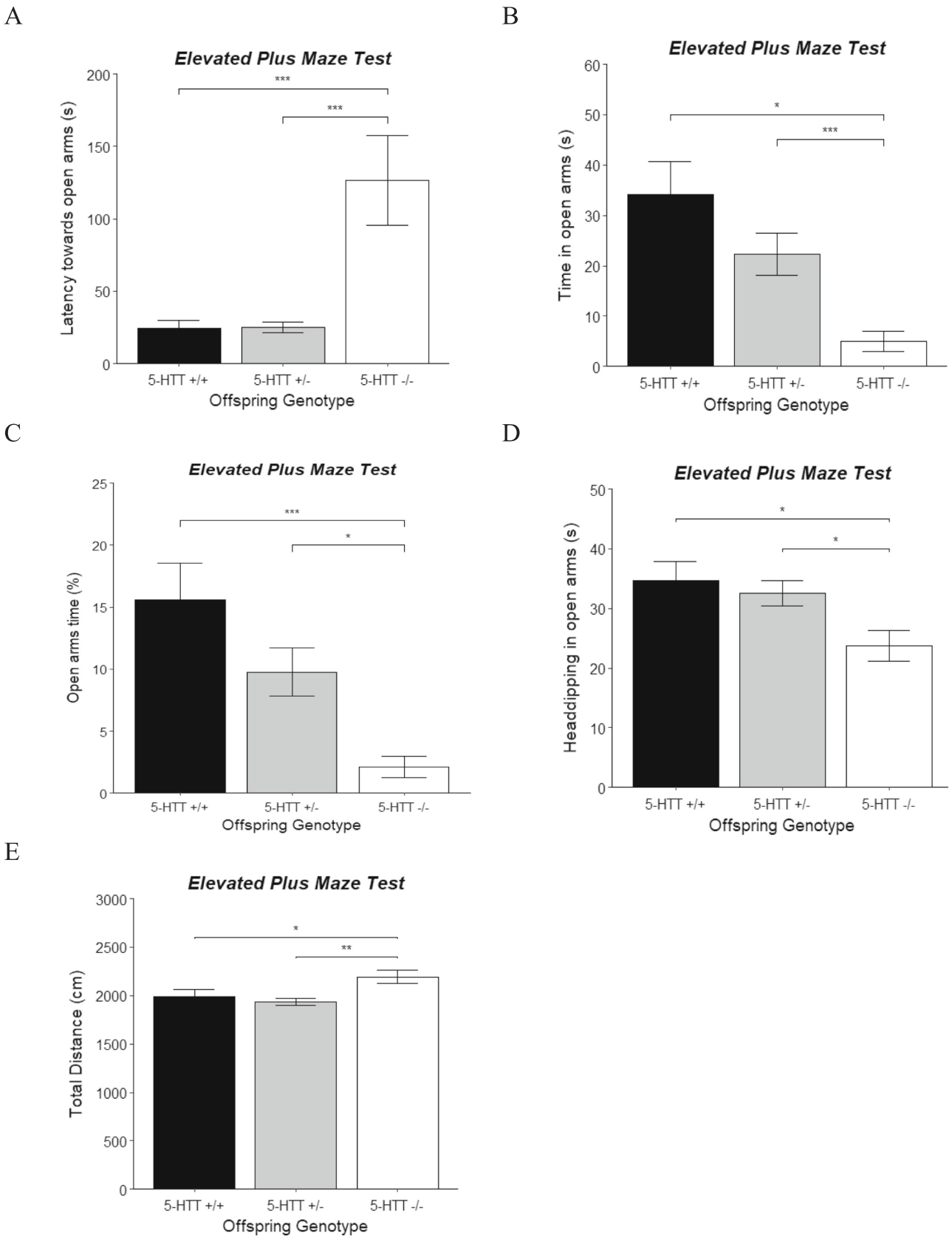

Fig. 2. Effect of own 5-HTT genotype in male offspring on anxiety-like behavior in the elevated plus maze (n = 27–30 (WT), 59–65 (HET), 27–30 (HOM)). A) latency times towards the open arms, B) time spent in open arms, C) time spent on open arms versus total open and closed arm time (%), and D) time spent in performing head-dips in open arms, E) traveled distance. Data are presented as mean ± S.E.M. One-way ANOVA with an FDR-correction of 5 % for 4 variables (excluding derived data expressed in %); post hoc LSD * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$.

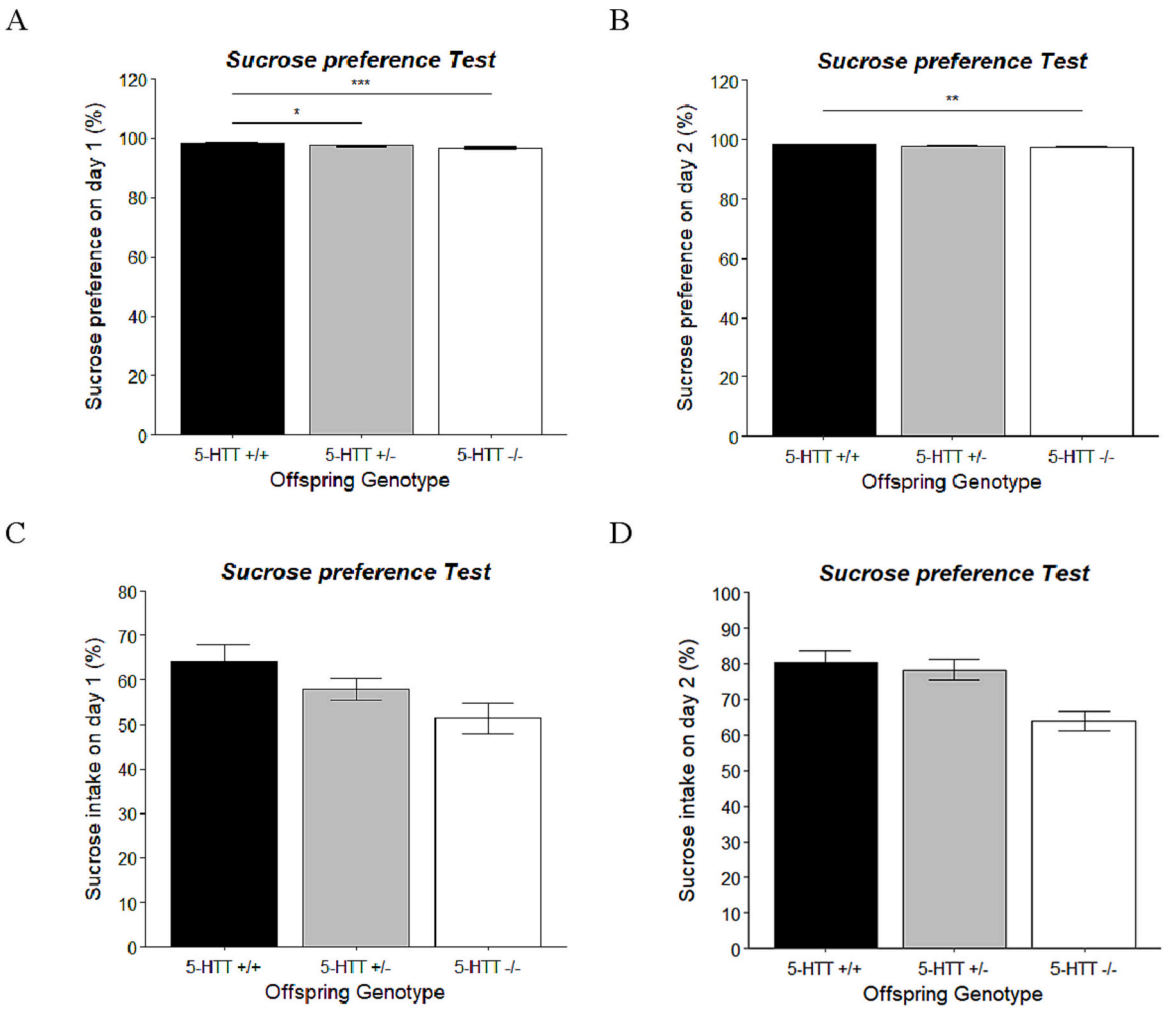

Fig. 3. Effect of own 5-HTT genotype in male offspring on anhedonia as measured using the sucrose preference test (n = 28–31 (WT), 62–65 (HET), 28–30 (HOM)). A) sucrose preference on day 1, B) sucrose preference on day 2, C) sucrose intake on day 1, D) sucrose intake on day 2. Data are presented as mean ± S.E.M. One-way ANOVA with an FDR-correction of 5 % for 4 variables; post hoc LSD * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$.

subset of GABAergic interneuron, in the infralimbic cortex, no significant genotype effect was found ($F_{(2,21)} = 0.701$, $p = 0.508$) (Fig. 4C). Conversely in the prelimbic cortex, as shown in Fig. 4D, there was a main effect of genotype for the mRNA expression levels of *parvalbumin* ($F_{(2,22)} = 4.566$, $p = 0.023$), that was not confirmed after FDR correction ($p_{corr} = 0.136$). For other genes, no significant genotype effects were found (Supplementary Fig. 2).

We focused our investigation of neuroplasticity-related genes on the BDNF system, specifically the mRNA expression levels of *Bdnf isoform IV, Bdnf isoform VI, long 3'UTR* and *Gadd45β* transcripts. Although no difference was found between genotype for *Bdnf isoform VI* in the infralimbic cortex ($F_{(2,21)} = 1.416$, $p = 0.264$) (Fig. 5A), a significant genotype effect was observed in the prelimbic cortex ($F_{(2, 21)} = 16.188$, $P_{corr} = 0.001$). Compared to 5-HTT$^{-/-}$ and 5-HTT$^{+/-}$ rats, 5-HTT$^{+/+}$ rats showed increased *Bdnf isoform VI* mRNA expression levels ($p < 0.050$, $p < 0.010$, respectively). Furthermore, in comparison to 5-HTT$^{-/-}$ rats, 5-HTT$^{+/-}$ rats showed lower expression levels ($p < 0.050$) (Fig. 5B). Some significant genotype differences on other genes' expression were detected before FDR correction, although they were not present after correction. A significant regulation was found for infralimbic *Bdnf isoform IV* ($F_{(2, 21)} = 6.266$, $p = 0.007$, $p_{corr} = 0.088$). Post hoc testing revealed a significant increase when comparing 5-HTT$^{+/-}$ rats to 5-HTT$^{-/-}$ rats ($p < 0.010$). For 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats, and 5-HTT$^{+/+}$ versus 5-HTT$^{+/-}$ rats, no significant difference was found ($p > 0.050$, $p > 0.050$, respectively), and no main effect of genotype was found for prelimbic *Bdnf isoform IV* ($F_{(2,21)} = 2.276$, $p = 0.129$) (Fig. 5C, D). Additionally, we found no significant effect in infralimbic *Bdnf long 3'UTR* or prelimbic *Bdnf long 3'UTR* ($F_{(2,19)} = 1.388$, $p = 0.272$, $F_{(2,19)} = 3.298$, $p = 0.059$, respectively) (Fig. 5E, F). *Gadd45β* (Growth arrest and DNA damage-inducible β) is associated with promoter demethylation of the BDNF gene (Gavin et al., 2012). For infralimbic *Gadd45β*, a significant main effect of genotype was found after FDR correction ($F_{(2, 21)} = 5.225$, $p = 0.014$, $p_{corr} = 0.115$). Compared with 5-HTT$^{-/-}$ rats, 5-HTT$^{+/+}$ rats showed lower expression levels ($p < 0.010$, $p < 0.050$, respectively), while no significant difference was found between 5-HTT$^{-/-}$ and 5-HTT$^{+/-}$ rats ($p > 0.050$) (Fig. 5G). For prelimbic *Gadd45β*, no significant genotype effect was found ($F_{(2, 21)} = 3.443$, $p = 0.052$, $p_{corr} = 0.178$) (Fig. 5H).

Finally, we measured *Nr3c1* and *Nr3c2* mRNA expression levels to evaluate the basal activity of HPA-axis. The glucocorticoid receptor encoded by the *Nr3c1* and the mineralocorticoid receptor encoded by *Nr3c2* both participate in the activation of the HPA- axis by glucocorticoids. For infralimbic *Nr3c2* we found a significant genotype effect that didn't survive after FDR correction ($F_{(2,22)} = 4.111$, $p = 0.030$, $p_{corr} = 0.122$) (Fig. 6A). In the prelimbic cortex, no genotype effect was found for *Nr3c2* ($F_{(2, 22)} = 0.309$, $p = 0.707$) (Fig. 6B). Regarding *Nr3c1*, no genotype effect in the infralimbic nor the prelimbic cortex was found ($F_{(2, 22)} = 1.537$, $p = 0.237$, $F_{(2, 22)} = 2122$, $p = 0.146$, respectively) (Fig. 6C, D).

Overall, 5-HTT genotype influences gene expression in mPFC

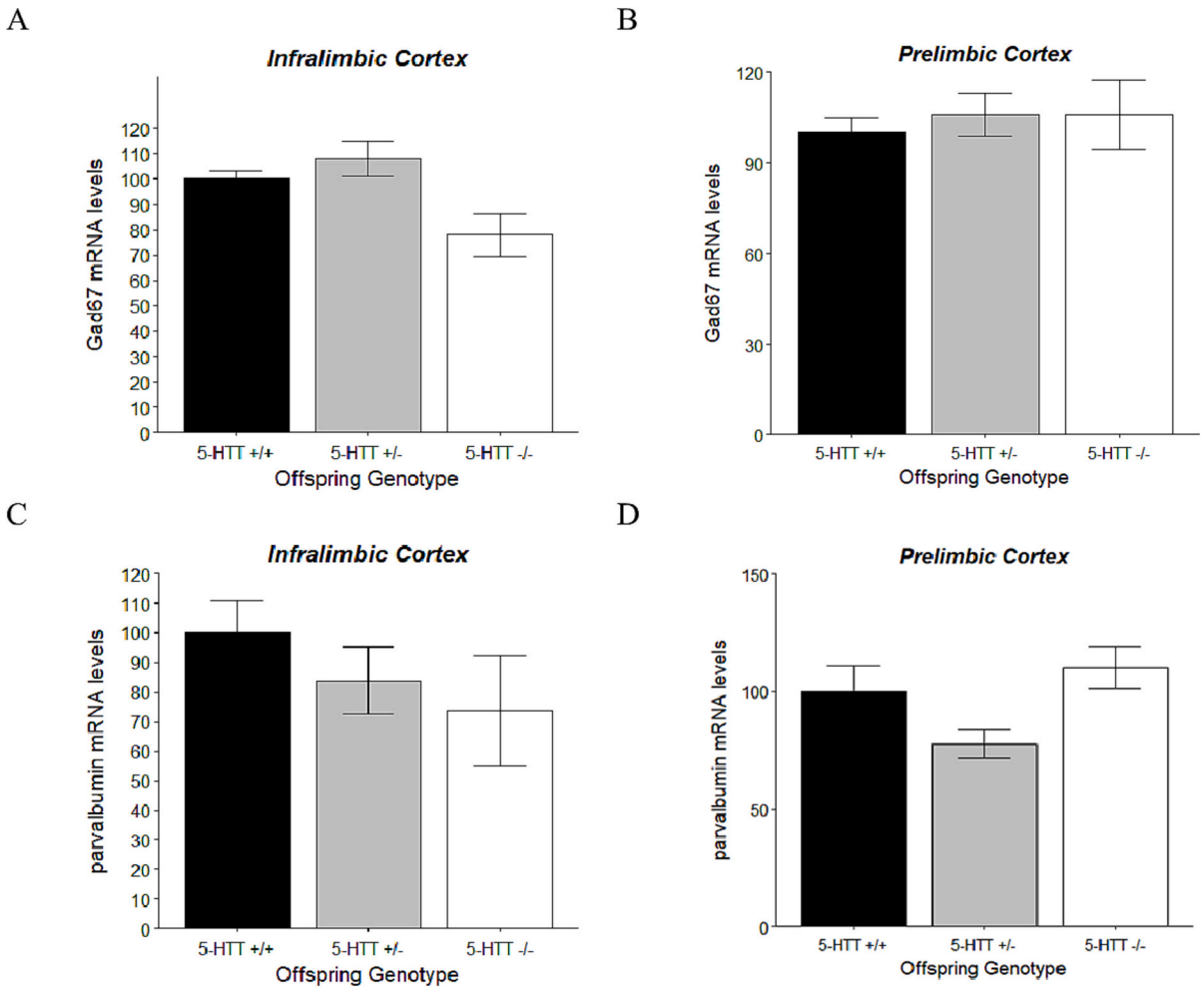

Fig. 4. Effect of own 5-HTT genotype in male offspring on GABA gene expression levels in mPFC subregions (n = 4 (WT), 12–13 (HET), 5–6 (HOM)). A) *Gad67* expression in infralimbic cortex, B) *Gad67* expression in prelimbic cortex, C) *parvalbumin* expression in infralimbic cortex, D) *parvalbumin* expression in prelimbic cortex. Data are presented as mean ± S.E.M. One-way ANOVA with an FDR-correction of 5 % for 24 variables; post hoc LSD * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$.

subregions. For 5-HTT$^{-/-}$ rats, *Gad67, Gadd45β, Bdnf isoform IV* and *Nr3c2* were down-regulated specifically in the infralimbic cortex. Furthermore, the expression of *Bdnf isoform VI* was also decreased in the prelimbic cortex, with the genotype effect only maintained after correction for multiple comparisons for this gene's expression.

3.3. The effect of offspring's own 5-HTT genotype on HPA-axis function

To determine if own 5-HTT genotype is associated with changes in HPA-axis function, the animals were subjected to the forced swim test. Plasma corticosterone (CORT) levels were measured at 1) baseline and 2) at 20 and 60 min after the stressor (forced swim test). The latter were then normalized to baseline CORT levels (Supplementary Table 4). The genotype did not affect CORT levels at baseline ($F_{(2, 40)} = 1.028$, $p = 0.367$), nor at 20 min ($F_{(2,31)} = 0.854$, $p = 0.435$) or at 60 min ($F_{(2, 35)} = 1.345$, $p = 0.274$) after stress exposure (Supplementary Fig. 3).

3.4. Correlations between molecular changes and behavior

To explore potential correlations between behavior and brain genes' expression findings, we performed Pearson correlation analyses (Supplementary Table 5–6, Fig. 7).

As shown in Fig. 2, there was a positive correlation between *Gadd45β* mRNA expression levels in the prelimbic cortex and the latency to enter the open arms ($r = 0.51$, $p = 0.013$, n = 23), and a negative correlation between *Gadd45β* mRNA expression levels in the prelimbic cortex and sucrose preference on day 1 ($r = -0.51$, $p = 0.015$, n = 22). Furthermore, *Nr3c2* expression levels in the infralimbic cortex were positively correlated with the total distance moved in the elevated plus maze test ($r = 0.41$, $p = 0.049$, n = 24), the percentage of time spent in the open arms ($r = 0.44$, $p = 0.029$, n = 25), and the sucrose preference on both test days (day 1: $r = 0.59$, $p = 0.003$, n = 24; day2: $r = 0.43$, $p = 0.041$, n = 23). *Bdnf exon IV* mRNA levels in the infralimbic cortex were negatively correlated with the latency to enter the open arms ($r = -0.47$, $p = 0.015$, n = 24). Finally, there was a positive correlation between sucrose intake on day 2 and *gad67* mRNA expression levels in the infralimbic cortex (r = 0.41, $p = 0.048$, n = 24) as well as 5-HT levels ($r = 0.57$, $p < 0.001$, n = 31).

3.5. The influence of 5-HTT paternal genotype on anxiety- and depression-like behavior in 5-HTT$^{+/-}$ offspring

Beside the offspring's own 5-HTT genotype, we examined the influence of the paternal 5-HTT genotype, i.e. 5-HTT$^{+/+}$ or 5-HTT$^{-/-}$, on anxiety- and depression-like behavior in 5-HTT$^{+/-}$ adult male offspring (Supplementary Table 8, Supplementary Fig. 4, 5).

In the elevated plus maze test, a significant paternal genotype effect was found for the latency to enter the open arms ($F_{(1, 60)} = 4.769$, $p_{corr} = 0.044$) (Supplementary Fig. 4 A). 5-HTT$^{+/-}$ adult male offspring with 5-HTT$^{-/-}$ fathers showed a higher latency to enter the open arms than 5-

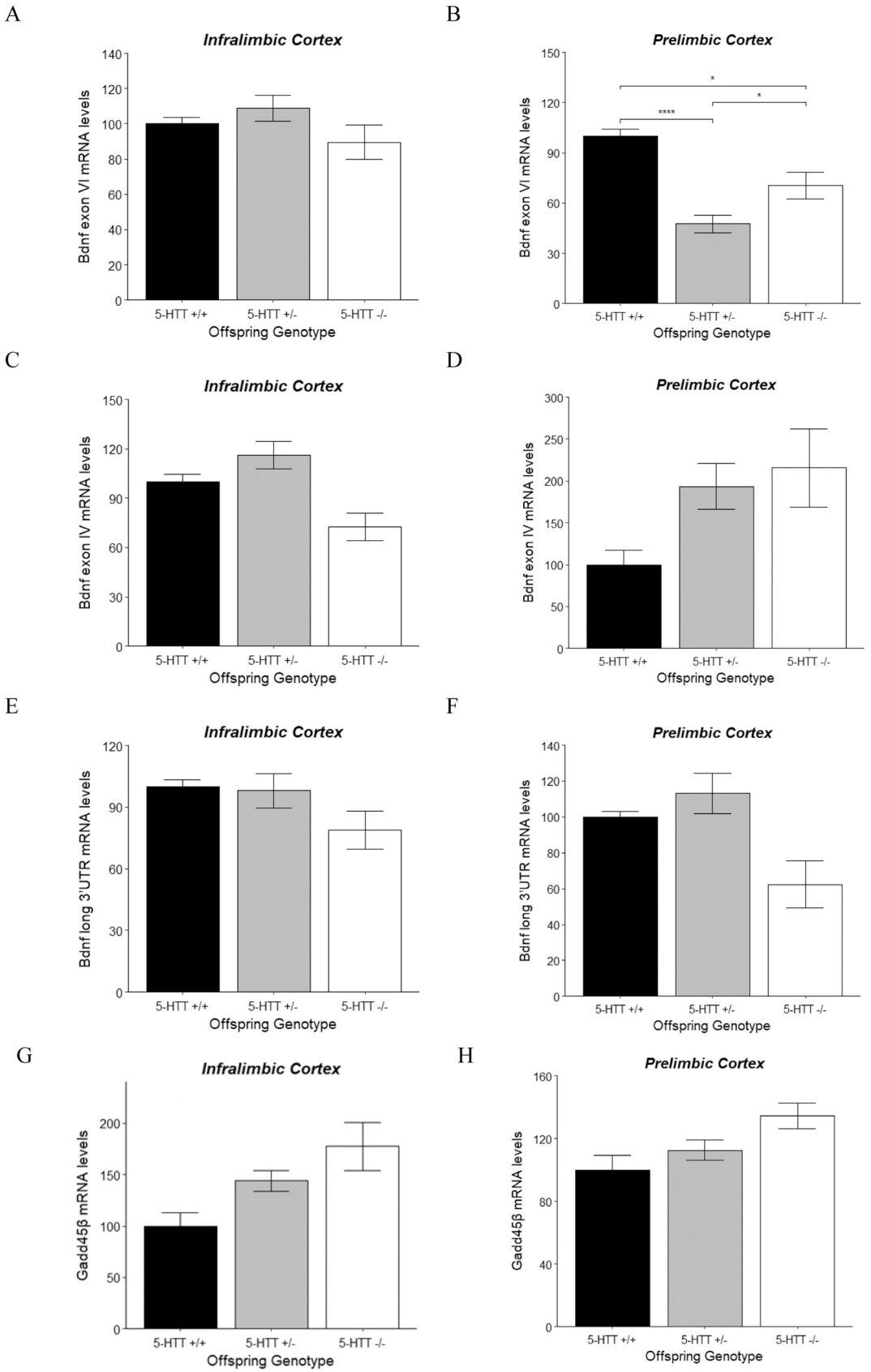

Fig. 5. Effect of own 5-HTT genotype in male offspring on BDNF-related gene expression levels in mPFC subregions (n = 4 (WT), 12–13 (HET), 5–6 (HOM)). A, B) *Bdnf isoform VI* expression in infralimbic and prelimbic cortex, C, D) *Bdnf isoform IV* expression in infralimbic and prelimbic cortex, E, F) *Bdnf long 3'UTR* expression in infralimbic and prelimbic cortex, G, H) *Gadd45β* expression in infralimbic and prelimbic cortex. Data are presented as mean ± S.E.M. One-way ANOVA with an FDR-correction of 5 % for 24 variables; post hoc LSD * = $p \leq 0.05$, and ** = $p \leq 0.01$.

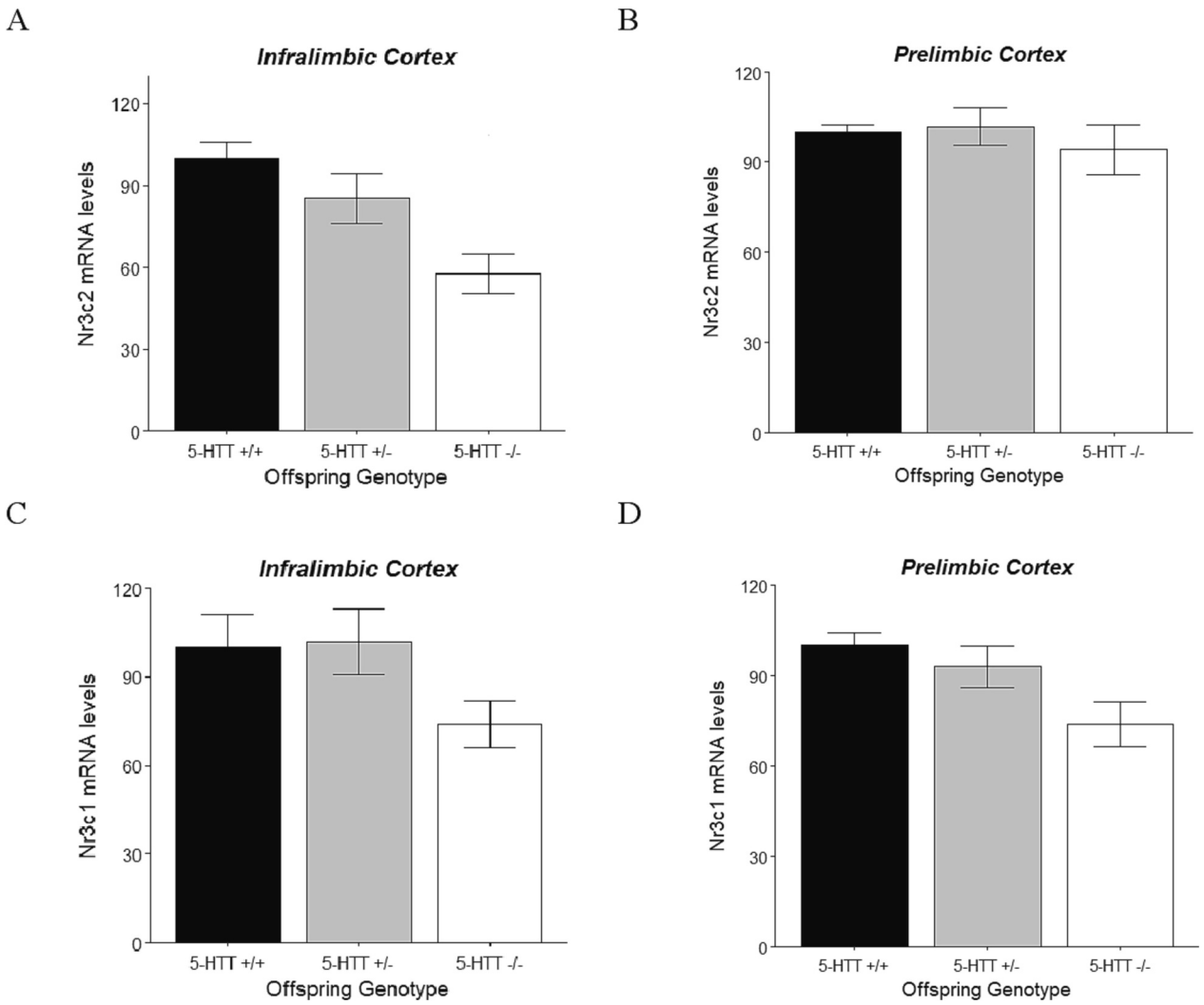

Fig. 6. Effect of own 5-HTT genotype in male offspring on HPA-related gene expression levels in mPFC subregions (n = 4 (WT), 12–13 (HET), 5–6 (HOM)). A, B) *Nr3c2* expression in infralimbic and prelimbic cortex, C, D) *Nr3c1* expression in the infralimbic and prelimbic cortex. Data are presented as mean ± S.E.M. One-way ANOVA with an FDR-correction of 5 % for 24 variables; post hoc LSD * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$.

$HTT^{+/-}$ rats with $5\text{-}HTT^{+/+}$ fathers. The time spent in the open arms was also significantly influenced by 5-HTT paternal genotype: $5\text{-}HTT^{+/-}$ rats with $5\text{-}HTT^{-/-}$ fathers spent less time in open arms ($F_{(1,59)} = 7.703$, $p_{corr} = 0.030$) (Supplementary Fig. 4B). Furthermore, the percentage time spent on open arms respective to the total time also varied significantly as function of the father's genotype. Specifically, $5\text{-}HTT^{+/-}$ offspring from $5\text{-}HTT^{-/-}$ fathers exhibited a lower percentage of time spent in the open arms compared to $5\text{-}HTT^{+/-}$ offspring with $5\text{-}HTT^{+/+}$ fathers ($F_{(1,63)} = 9.420$, $p = 0.003$) (Supplementary Fig. 4C). Finally, $5\text{-}HTT^{+/-}$ offspring from different fathers varied also significantly in the time spent on head-dipping in the open arms ($F_{(1,\ 58)} = 6.591$, $p_{corr} = 0.026$) (Supplementary Fig. 4D). $5\text{-}HTT^{+/-}$ rats from $5\text{-}HTT^{-/-}$ fathers spent less time on head-dipping in the open arms. However, there was no significant effect on total distance moved ($F_{(1,\ 60)} = 1.321$, $p = 0.255$) (Supplementary Fig. 4E).

In the sucrose preference test, 5-HTT paternal genotype significantly affected sucrose preference at testing day 1 (Supplementary Table 8). Offspring from $5\text{-}HTT^{-/-}$ fathers showed lower sucrose preference on testing day 1 compared to $5\text{-}HTT^{+/-}$ offspring from $5\text{-}HTT^{+/+}$ fathers ($F_{(1,60)} = 8.62$, $p_{corr} = 0.019$) (Supplementary Fig. 5A). We found no significant effect on the other parameters of the sucrose preference test (Fig. 5B, C, D). No differences between the two groups defined by the paternal genotype were found in the average of the 30-trials escape latencies in the learned helplessness test ($F_{(1,63)} = 0.005$, $p = 0.944$) (Fig. 5E). Finally, no significant paternal genotype effect was found on the behavior of $5\text{-}HTT^{+/-}$ offspring in the forced swimming test (Diving time: $F_{(1,\ 28)} = 0.909$, $p = 0.348$; floating time: $F_{(1,\ 28)} = 1.207$, $p = 0.281$; immobility: $F_{(1,\ 28)} = 0.931$, $p = 0.343$) (Supplementary Table 7).

Taken together, $5\text{-}HTT^{+/-}$ adult male offspring from $5\text{-}HTT^{-/-}$ fathers showed higher anxiety- and depression-like behavior compared to $5\text{-}HTT^{+/-}$ offspring from $5\text{-}HTT^{+/+}$ fathers.

3.6. The influence of 5-HTT paternal genotype on GABAergic, BDNF and HPA-axis-related gene expression in the prelimbic mPFC of $5\text{-}HTT^{+/-}$ offspring

We also analyzed the effect of father genotype on the mRNA expression levels of genes involved in GABA neurotransmission, neuroplasticity, and HPA-axis functioning, in the infralimbic and prelimbic parts of the mPFC of $5\text{-}HTT^{+/-}$ offspring. We did not find any significant paternal 5-HTT genotype difference in the infralimbic cortex, while in prelimbic parts of the mPFC, some genes were significantly modulated by paternal 5-HTT genotype, although none were maintained after FDR correction (Supplementary Table 8, 9) (Supplementary Fig. 6). More specifically, $5\text{-}HTT^{+/-}$ adult male offspring from $5\text{-}HTT^{-/-}$ fathers, compared to $5\text{-}HTT^{+/-}$ offspring from $5\text{-}HTT^{+/+}$ fathers, showed lower

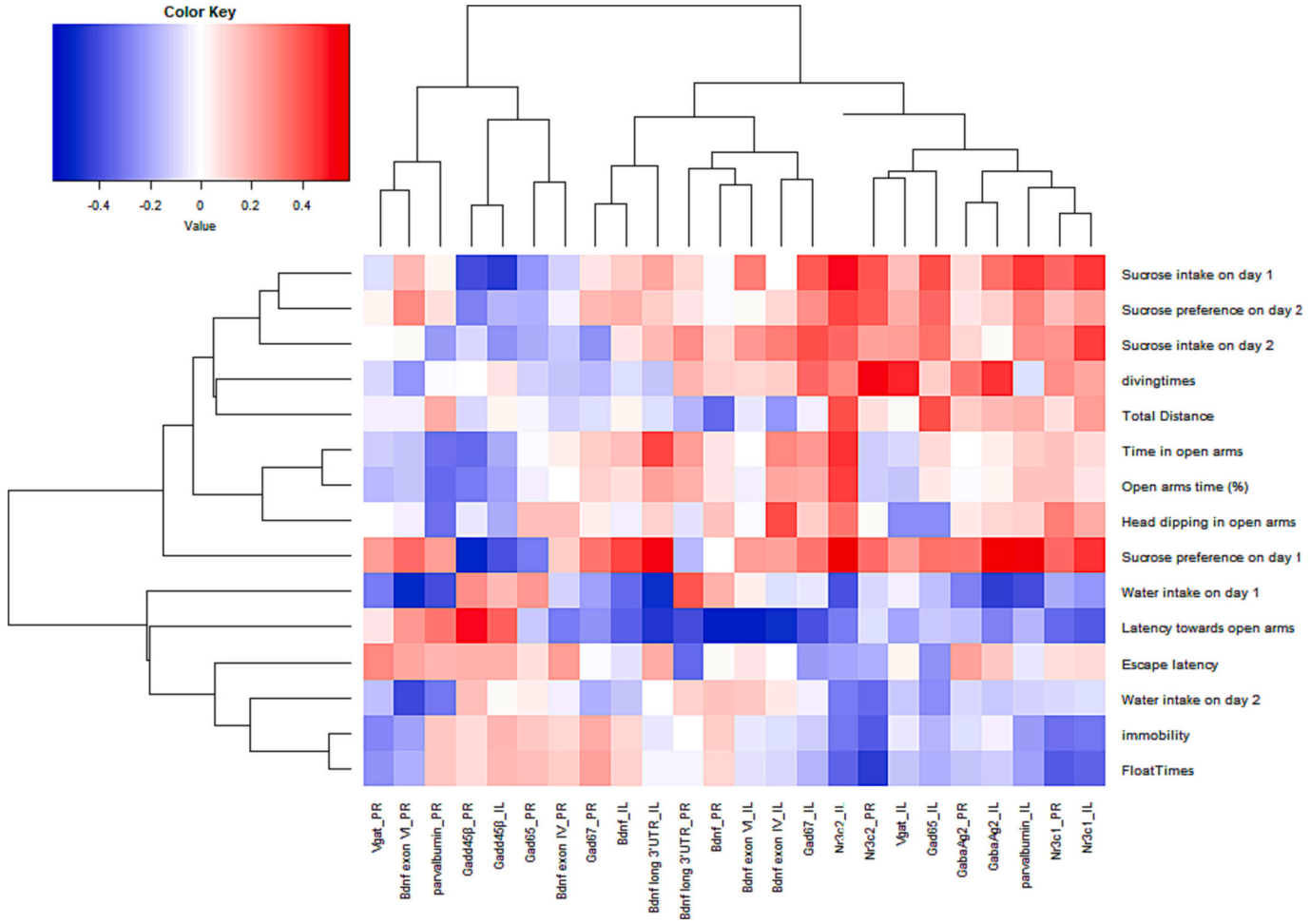

Fig. 7. Correlations between molecular and neurochemical changes and behavior. The gene expression in different subregions is in the columns and the behaviors are in the rows; PR means prelimbic cortex and IL means infralimbic cortex. Columns are mean-centered, with correlation represented by color (blue, lower correlation; red, higher correlation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expression levels of *Vgat* ($F_{(1,11)} = 7.758$, $p = 0.018$, $p_{corr} = 0.142$) (Supplementary Fig. 6A). A similar effect was found for *parvalbumin* with 5-HTT$^{+/-}$ rats from 5-HTT$^{-/-}$ fathers having lower *parvalbumin* mRNA levels ($F_{(1,11)} = 14.534$, $p = 0.003$, $p_{corr} = 0.069$) (Supplementary Fig. 6B). For other genes, no significant paternal genotype effects were found.

Regarding neuroplasticity-related genes, we found a significant effect of paternal 5-HTT genotype on two genes in the prelimbic cortex: *Bdnf isoform VI* ($F_{(1,10)} = 9.158$, $p = 0.013$, $p_{corr} = 0.153$) (Supplementary Fig. 6C) and *Bdnf long 3'UTR* ($F_{(1,\ 10)} = 7.435$, $p = 0.020$, $p_{corr} = 0.118$) (Supplementary Fig. 6D) levels. 5-HTT$^{+/-}$ adult male offspring with 5-HTT$^{-/-}$ fathers, compared to those with 5-HTT$^{+/+}$ fathers, showed a lower expression of *Bdnf isoform VI*, while for *Bdnf long 3'UTR*, an opposite effect was found.

Finally, we found that *Nr3c1* but not *Nr3c2* mRNA expression levels were significantly affected by paternal 5-HTT genotype. Specifically, 5-HTT$^{+/-}$ adult male offspring from 5-HTT$^{-/-}$ fathers showed a relative reduction in the expression levels *Nr3c1* ($F_{(1,11)} = 5.358$, $p = 0.041$, $p_{corr} = 0.197$) (Supplementary Fig. 6E). Regarding the other measured genes, no significant parental genotype effect was found (Supplementary Fig. 7).

In sum, a significant down-regulation was found in prelimbic cortex for the vesicular GABA transporter *Vgat,* the GABAergic marker *parvalbumin, Bdnf exon isoform VI* and *Nr3c1,* and an upregulation of *Bdnf long 3'UTR* was also found in prelimbic cortex, in 5-HTT$^{+/-}$ offspring with 5-HTT$^{-/-}$ fathers. None of the effects held after FDR correction.

3.7. The influence of 5-HTT paternal genotype on HPA-axis function in 5-HTT$^{+/-}$ offspring

Regarding the HPA-axis function, there was no effect of paternal 5-HTT genotype in 5-HTT$^{+/-}$ rats on CORT levels at baseline ($F_{(1,22)} = 0.840$, $p = 0.369$), at 20 min ($F_{(1,\ 15)} = 0.574$, $p = 0.460$) or at 60 min ($F_{(1,\ 19)} = 2.054$, $p = 0.168$) after stress exposure (Supplementary Table 10) (Supplementary fig. 8).

4. Discussion

The current rat study demonstrates that the offspring's own 5-HTT$^{-/-}$ genotype increases anxiety- and depression-like behaviors and affects gene expression in mPFC subregions. Specifically, genes encoding for GABAergic markers, GABA synthesis, BDNF promoter demethylation and glucocorticoid signaling were altered in 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ offspring. No changes in plasma corticosterone levels were observed. Since the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats were all born from 5-HTT$^{+/-}$ mothers, we excluded possible confounding effects from placental-derived 5-HT levels and maternal care. Moreover, we hypothesized that 5-HTT$^{+/-}$ offspring's behavior and gene expression could have been influenced by their fathers' genotype, either 5-HTT$^{-/-}$ or 5-HTT$^{+/+}$. Indeed, analyzing by grouping the data according to the paternal 5-HTT genotype revealed that the paternal 5-HTT$^{-/-}$ genotype affects anxiety- and depression-like behavior, and GABAergic, BDNF and HPA-axis-related gene expression in the prelimbic cortex, in 5-HTT$^{+/-}$

offspring.

We subjected 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats to a series of behavioral tests measuring anxiety- and depression-like behavior to assess the effect of their own 5-HTT genotype. In the elevated plus maze test, 5-HTT$^{-/-}$ rats traveled the longest distance. Regarding other parameters, 5-HTT$^{-/-}$ rats showed the highest latency to reach the open arms and the lowest percentage of time spent there. Moreover, they also exhibited the lowest head dipping time in the open arms. These parameters indicate that 5-HTT$^{-/-}$ rats avoided the open arms, which is indicative for increased anxiety. This is in line with previous studies of 5-HTT$^{-/-}$ rats (Olivier et al., 2008; Sbrini et al., 2020b; Verheij et al., 2018) and 5-HTT$^{-/-}$ mice (Holmes et al., 2003). In the sucrose preference test, 5-HTT$^{-/-}$ rats displayed lower sucrose preference and intake, which is indicative for anhedonia. We hereby again confirm previous reports on increased anhedonia in 5-HTT$^{-/-}$ rats (Olivier et al., 2008; Sbrini et al., 2020a) and 5-HTT$^{-/-}$ mice (Kloke et al., 2013; Popa et al., 2008; Popp et al., 2021; Rogers et al., 2017; Wang et al., 2022), although not all 5-HTT$^{-/-}$ mouse studies found a decrease in sucrose preference (Kalueff et al., 2006). The genetic background potentially plays a role, since the differential findings in the 5-HTT$^{-/-}$ mice were obtained with different mouse strains. In the learned helplessness test, no genotype difference was found. This finding was inconsistent with previous studies in 5-HTT$^{-/-}$ rats, which demonstrated that 5-HTT$^{-/-}$ rats had a lower escape latency (Schipper et al., 2015; van der Doelen et al., 2013). The maternal genotype (5-HTT$^{+/-}$) was the same in all studies and can therefore not explain the finding inconsistencies. Alternatively, procedural changes, such as the opening of the door in between the stress trials in this study but not our previous study, might explain these differences (Schipper et al., 2015; van der Doelen et al., 2013). On the other hand, findings in 5-HTT$^{-/-}$ mice substantially differ from our 5-HTT$^{-/-}$ rat findings, as the mice showed a longer escape latency and higher rate of escape failures (Lira et al., 2003). Finally, in the forced swim test no effects of the offspring's own 5-HTT genotype were found, comparable to a previous study from our laboratory (Diniz et al., 2021), but inconsistent with a 5-HTT$^{-/-}$ rat study (Olivier et al., 2008) and two 5-HTT$^{-/-}$ mouse studies (Lira et al., 2003; Rogers et al., 2017). Because the rat study also used 5-HTT$^{+/-}$ mothers, the reason for this non-replication is yet unclear. Taken together, the increased anxiety-like behavior and anhedonia in 5-HTT$^{-/-}$ rodents appear to be stable phenotypes across studies, including 5-HTT$^{-/-}$ mouse studies, and to be primarily related to their own 5-HTT genotype, while learned helplessness and behavioral despair as measured in the forced swim test are less consistent between studies.

At the molecular level we found that the own 5-HTT genotype had a significant effect on BDNF-related gene expression in the mPFC, specifically *BDNF exon VI.* The downregulation of *BDNF exon VI* in 5-HTT$^{-/-}$ rats is in line with previous findings (Molteni et al., 2010). 5-HTT$^{-/-}$ rats also showed major changes in the expression of GABAergic system markers. For instance, *Gad67* expression was reduced in 5-HTT$^{-/-}$ rats, in line with our previous study (Guidotti et al., 2012).

We also evaluated the expression of mineralocorticoid (*Nr3c2*) receptor, which is implicated in HPA-axis functioning. We demonstrated a lower expression level of *Nr3c2* in the mPFC of 5-HTT$^{-/-}$ rats. A previous study found higher mineralocorticoid receptor mRNA expression levels in 5-HTT$^{-/-}$ rats in comparison to 5-HTT$^{+/+}$ rats (Van der Doelen et al., 2014b).

We presume that this difference is due to maternal care or placental effects. In fact, in this study, the glucocorticoid receptor (*Nr3c1*) gene expression level was not influenced by the offspring's own 5-HTT genotype. However, in unpublished work from our group, 5-HTT$^{-/-}$ offspring from 5-HTT$^{-/-}$ mothers displayed reduced glucocorticoid gene expression, suggesting that the maternal 5-HTT genotype influences glucocorticoid genes expression.

We also analyzed the correlations between behavioral and brain measures. The correlational analyses suggest associations between the molecular and neurochemical changes and behavioral changes in the offspring. Specifically, there was a positive correlation between anxiety-like behavior and *Gadd45β* mRNA expression levels, and a negative correlation between anxiety-like behavior and *Bdnf exon IV* expression levels in the prelimbic cortex. These correlations suggest that anxiety-like behavior in 5-HTT$^{-/-}$ rats can be explained by increased *Gadd45β* mRNA expression levels and decreased *Bdnf exon IV* levels.

5-HTT$^{+/-}$ rats had 5-HTT$^{-/-}$ or 5-HTT$^{+/+}$ fathers, and therefore received their 5-HTT^{-} allele from, respectively, their father or their mother. The 5-HTT$^{+/-}$ offspring that received the 5-HTT^{-} allele from their father showed more anxiety-like behavior than 5-HTT$^{+/-}$ offspring receiving the 5-HTT^{-} allele from their mother. Regarding *parvalbumin* and *Bdnf exon VI*, we found significant expression differences in prelimbic cortex which were related to both the offspring own genotype and the paternal genotype. More specifically, we found the lowest expression of *parvalbumin, Vgat* and *Bdnf exon VI* in 5-HTT$^{+/-}$ offspring from 5-HTT$^{-/-}$ fathers. Furthermore, 5-HTT$^{+/-}$ offspring from 5-HTT$^{-/-}$ fathers, i.e. that received the 5-HTT^{-} allele from their fathers, showed lower *Nr3c1* expression compared to 5-HTT$^{+/-}$ offspring from 5-HTT$^{+/+}$ fathers, i.e. that received the 5-HTT^{-} allele from their mothers. These observed differences in 5-HTT$^{+/-}$ offspring can be due to one of two causes: the paternal 5-HTT genotype, affecting the offspring through, for example, differential paternal behavior influencing the mother, or the parental origin of the 5-HTT^{-} allele. Paternal behavior could lead to epigenetic modifications in the females (Mendonça et al., 2019). By applying in vitro fertilization techniques in animal experiments, the influence of males on females can be avoided (Takeo et al., 2022). Notably, it was previously reported that methylation of certain CpG sites were only correlated between fathers and children, but not between mothers and children (Cimino et al., 2017). For future research, more attention can be directed towards the factors influencing male epigenetics, such as social behavior.

While we attempted to design our study as complete as possible, some limitations need to be considered. First, we only have data from male offspring. Therefore, the experimental findings do not generalize to females. We also did not examine whether the sex of the offspring influences the potential effects of the offspring's 5-HTT genotype. Another point worth considering is that even though we attempted to cancel out confounding effects of maternal care by only using 5-HTT$^{+/-}$ mothers, we cannot completely exclude differences in maternal care quality, because offspring, which differ by their 5-HTT genotype, could influence maternal behavior. To investigate whether dams provide different levels of maternal care depending on the offspring's 5-HTT genotype, future studies could incorporate specific measures of maternal-pup interaction, such as the ink mark-fading test (Cavigelli et al., 2010). Finally, because the rats underwent a test battery, we cannot exclude the possibility that the behavioral testing itself had an effect on neurotransmitter and gene expression levels.

Taken together, our findings indicate that the own 5-HTT genotype of the offspring, independent of the maternal 5-HTT genotype, increases anxiety and anhedonia, and potentially does so via regulating neuroplasticity, the GABA system as well as the HPA-axis functionality. Interestingly, we also found evidence that the paternal 5-HTT genotype also contributed to anxiety and depression behaviors. These behavioral effects were accompanied by changes in gene expression levels. Altogether, these results further deepen our understanding of how 5-HTT genotype shapes individual differences in affective behavior.

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CRediT authorship contribution statement

Menghan Sun: Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Paola Brivio:** Data curation. **Ling Shan:** Conceptualization, Writing – review & editing. **Sylvia Docq:** Data curation. **Lisa C.M.W. Heltzel:** Data curation. **Celine A.J. Smits:** Data curation. **Anthonieke Middelman:** Resources. **Roel Vrooman:** Software. **Marcia Spoelder:** Writing – review & editing. **Michel M.M. Verheij:** Methodology, Supervision, Writing – review & editing. **Jan K. Buitelaar:** Conceptualization, Funding acquisition, Writing – review & editing. **Morgane Boillot:** Supervision, Writing – review & editing. **Francesca Calabrese:** Supervision, Writing – review & editing. **Judith R. Homberg:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Sabrina I. Hanswijk:** Conceptualization, Data curation, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data are put on accessible servers and made available upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jad.2024.01.114.

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