


# Impaired fear extinction in serotonin transporter knockout rats is associated with increased 5-hydroxymethylcytosine in the amygdala

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## Summary

**Aims:** One potential risk factor for posttraumatic stress disorder (PTSD) involves the low activity (short; s) allelic variant of the serotonin transporter-linked polymorphic region (5-HTTLPR), possibly due to reduced prefrontal control over the amygdala. Evidence shows that DNA methylation/demethylation is crucial for fear extinction in these brain areas and is associated with neuronal activation marker c-Fos expression. We hypothesized that impaired fear extinction in serotonin transporter knockout (5-HTT<sup>-/-</sup>) rats is related to changes in DNA (de) methylation and c-Fos expression in the prefrontal cortex (PFC) and/or amygdala.

**Methods:** 5-HTT<sup>-/-</sup> and 5-HTT<sup>+/+</sup> rats were subjected to fear extinction. 2 hours after the extinction session, the overall levels of DNA methylation (5-mC), demethylation (5-hmC), and c-Fos in fear extinction and nonfear extinction rats were measured by immunohistochemistry.

**Results:** 5-HTT<sup>-/-</sup> rats displayed decreased fear extinction. This was associated with reduced c-Fos activity in the infralimbic PFC. In the central nucleus of the amygdala, c-Fos immunoreactivity was increased in the fear extinction group compared to the no-fear extinction group, regardless of genotype. 5-hmC levels were unaltered in the PFC, but reduced in the amygdala of nonextinction 5-HTT<sup>-/-</sup> rats compared to nonextinction wild-type rats, which caught up to wild-type levels during fear extinction. 5-mC levels were stable in central amygdala in both wild-type and 5-HTT<sup>-/-</sup> extinction rats. Finally, c-Fos and 5-mC levels were correlated with the prelimbic PFC, but not amygdala.

**Conclusions:** In the amygdala, DNA demethylation, independent from c-Fos activation, may contribute to individual differences in risk for PTSD, as conferred by the 5-HTTLPR s-allele.

## KEYWORDS

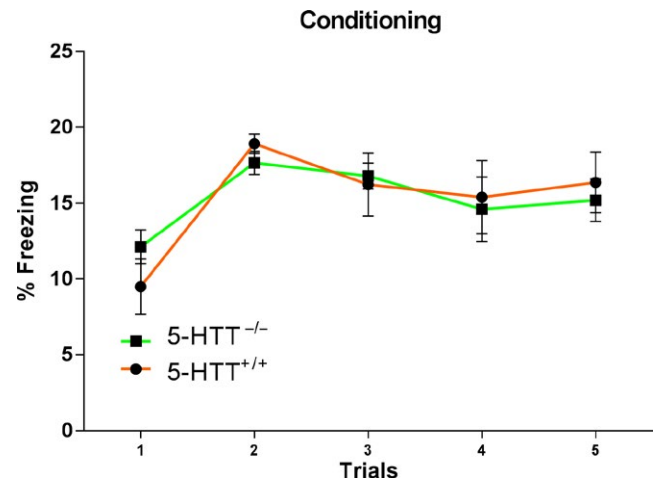
5-hydroxymethylcytosine (5-hmC), 5-methylcytosine (5-mC), DNA (de) methylation, epigenetics, fear extinction, serotonin transporter knockout

## 1 | INTRODUCTION

Posttraumatic stress disorder (PTSD) is a burdensome disease characterized by re-experiences of the traumatic event, avoidance of stimuli related to the trauma, and increased arousal and irritability.<sup>1</sup> One gene that has been associated with increased risk to PTSD under conditions of severe stress is the 5-HTT-linked polymorphic region (5-HTTLPR).<sup>2</sup> The low activity short (s) allelic variant of this polymorphism has been linked to increased fear acquisition<sup>3</sup> and reduced fear extinction.<sup>4</sup> The latter is seen as a hallmark of PTSD.<sup>5</sup> Furthermore, fear extinction has been attributed to reduced prefrontal cognitive control over the amygdala,<sup>6</sup> and this brain phenotype has been found in healthy 5-HTTLPR s-allele carriers.<sup>7</sup> The mechanism(s) in the prefrontal cortex and amygdala underlying the fear extinction deficit in s-allele carriers are to date not fully clear.

There is accumulating evidence showing that epigenetic mechanisms, especially DNA methylation/demethylation, are vital for the extinction of fear memory.<sup>8-12</sup> The mammalian DNA methylations occurred on cytosine (5mC). 5-mC is converted by 10-11 translocation (TET) family proteins into 5-hydroxymethylcytosine (5-hmC), the hydroxylated form of 5-mC. These modified bases may then function as DNA demethylation intermediates subject to deamination, glycosylase-dependent excision, and repair resulting in a reversion back to unmodified cytosine.<sup>13</sup> As DNA methylation/demethylation varies across tissues, rodents—which allow investigation of experimentally obtained brain tissue—are particularly suited to investigate the epigenetic mechanisms associated with fear extinction. In support for a role of DNA methylation in fear extinction, it has been demonstrated that pharmacological inhibition of amygdala DNA methyltransferases (DNMTs) activity resulted in deficits in fear memory.<sup>9</sup> Given that 5-mC is often associated with transcriptional silencing of genes, a decrease in 5-mC as a consequence of DNMT inhibition hypothetically leads to an upregulation of gene expression, which then may interfere with fear memory (re)consolidation.<sup>14</sup> There is also a relationship between demethylation and fear extinction. The overexpression of TET1 or a catalytically inactive mutant (TET1 m) resulted in impaired expression of contextual fear.<sup>11</sup> Furthermore, TET1 knockout mice exhibited impaired fear extinction.<sup>10</sup> The TET1 knockout animals showed significant upregulation of general 5-mC and downregulation of general 5-hmC, and the neuronal activity-regulated gene *c-Fos* was downregulated in cortex and hippocampus.<sup>10</sup> In response to fear extinction learning and fear extinction, a Tet3-mediated accumulation of 5-hmC has been observed, which led to a permissive epigenetic state.<sup>12</sup> These studies demonstrate that fear extinction is, at least in part, dependent on or regulated by changes in methylation and demethylation.

Could changes in methylation and demethylation contribute to impaired fear extinction in 5-HTTLPR s-allele carriers? In rodents, the 5-HTTLPR s-allele is modeled by knockout of the 5-HTT.<sup>15</sup> In line, 5-HTT knockout (5-HTT<sup>-/-</sup>) rats and mice show normal fear acquisition but impaired fear extinction (recall),<sup>16,17</sup> and structural and functional changes in the prefrontal cortex and amygdala.<sup>16-19</sup> Hence, 5-HTT<sup>-/-</sup> rodents can be of help to elucidate whether DNA methylation/



**FIGURE 1** Rats were exposed to 5 tone-shock pairings. Data are expressed as mean  $\pm$  SEM of percentage of freezing during tone (CS) presentation

demethylation contributes to the fear extinction deficits and risk for PTSD associated with the 5-HTTLPR s-allele.

Based on the aforementioned data, we hypothesized that impaired fear extinction in those characterized by inherited 5-HTT downregulation is related to changes in general DNA methylation and/or demethylation and *c-Fos* expression in the prefrontal cortex and/or amygdala. To test this explorative hypothesis, we subjected 5-HTT<sup>-/-</sup> rats and wild-type controls to fear conditioning and subsequent fear extinction testing. Using a complementary immunohistochemistry study, we investigated in the prefrontal cortex and amygdala the expression of the neuronal activity marker *c-Fos* to assess its association with changes in general DNA methylation and demethylation as previous study established.<sup>10</sup>

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. Experimental animals were derived from crossings between 5-HTT<sup>+/-</sup> rats. 5-HTT knockout rats (5-HTT<sup>-/-</sup>, Slc6a41Hubr) were generated on a Wistar background by ENU-induced mutagenesis<sup>20</sup> and have been described previously.<sup>21</sup> All animals (nonlittermates) were 3 months old and weighing 280-320 g. The rats had ad libitum access to food and water. A 12-hour light-dark cycle was maintained, with lights on at 08.00 AM.

### 2.2 | Fear conditioning and extinction

Fear conditioning was performed in Med Associates Inc. (St. Albans, USA) conditioning boxes. 12 5-HTT<sup>+/+</sup> and 12 5-HTT<sup>-/-</sup> adult male rats were placed in the conditioning boxes for 10 minutes, during which they were subjected to a tone (20 seconds, 2k Hz, 85 dB, conditioned stimulus [CS]) 5 times followed by a mild footshock (0.5 mA, 1 seconds; unconditioned stimulus [US]) with a 1 minute interval, preceded

and followed by 2 minutes of habituation and consolidation, respectively. In a separate group of rats ( $n = 8$  each), we found that freezing during conditioning does not differ across genotypes (Figure 1).

To measure cued fear extinction, the rats were tested in a room and in chambers that were different to those used during conditioning. We tested 4 groups of rats: (i) fear extinction-5-HTT<sup>-/-</sup>; (ii) nonfear extinction-5-HTT<sup>-/-</sup>; (iii) fear extinction 5-HTT<sup>+/+</sup>, and (iv) nonfear extinction 5-HTT<sup>+/+</sup> ( $n = 6$  each). After a habituation period of 2 minutes, the animals were exposed to the CS 24 times without shock in a period of 15 minutes. Nonextinction animals were exposed to the extinction context for an equal duration of time as fear extinction rats without exposure to the CS. The details of the freezing measurement have been described in our previous publications.<sup>18,22</sup> In short, conditioned freezing of the rats was manually scored using homemade behavioral observation software. The behavioral software provided event logging functionality, similar to "Noldus Observer." The observers were blind to subject genotype and housing conditions. Freezing behavior was defined as complete lack of movement except for the muscle movements needed for respiration. The freezing was expressed as percentage of time spent on freezing during CS presentation (or as percentage of the pre-CS period). Freezing was only measured for the fear extinction groups; CS-induced freezing was absent in the nonextinction animals.

### 2.3 | Brain tissue processing

Two hours after the start of the extinction session, the animals were deeply anesthetized and transcardially perfused with 20 mL phosphate buffered saline and 20 mL of fixative solution (4% paraformaldehyde, 0.1 mol/L PBS) followed by 30 mL of the second fixative solution. The time point was chosen based on the early-immediate expression of Fos- and Jun-like proteins<sup>23</sup> which was modulated by epigenetic mechanisms.<sup>23,24</sup> The brains were immersed in 30% sucrose solution and thereafter cut by a freezing microtome in 40- $\mu$ m-thick slices. The section was suspended in 0.1 mol/L PBS containing 0.1% azide, until further histological processing.

### 2.4 | 5-mC, 5-hmC and c-Fos immunoreactivity

The experiment was performed in a blinded fashion. The sections were rinsed in PBS (3  $\times$  15 minutes) and preincubated with 1% hydrogen peroxide (30% H<sub>2</sub>O<sub>2</sub> Merck 30 minutes). After washes in PBS, the sections were incubated with PBS-BT (0.1% bovine serum albumin and 0.5% Triton X-100). The sections were incubated with a primary anti-5-methylcytidine mouse monoclonal antibody (dilution 1:1000; GenWay Biotech, Catlog No. GWB-CB561B, San Diego, CA, USA)<sup>25</sup> or a 5-hydroxymethylcytosine rabbit polyclonal antibody (dilution 1:1000; Active motif, Catlog No. 39770, Tokyo, Japan)<sup>26</sup> or a polyclonal anti-c-Fos antiserum raised in rabbit (diluted 1:10 000; Santa Cruz Biotechnology, California, USA), respectively, overnight at room temperature (RT).

The specificity of the 5-hmC antibody, discriminating between 5-hmC and 5-mC, was tested by methyl-DNA immunoprecipitation and dot blot analysis. (<https://www.activemotif.com/catalog/>

details/39769.html). Both are validated for use in immunohistochemistry and immunofluorescence.<sup>26-28</sup>

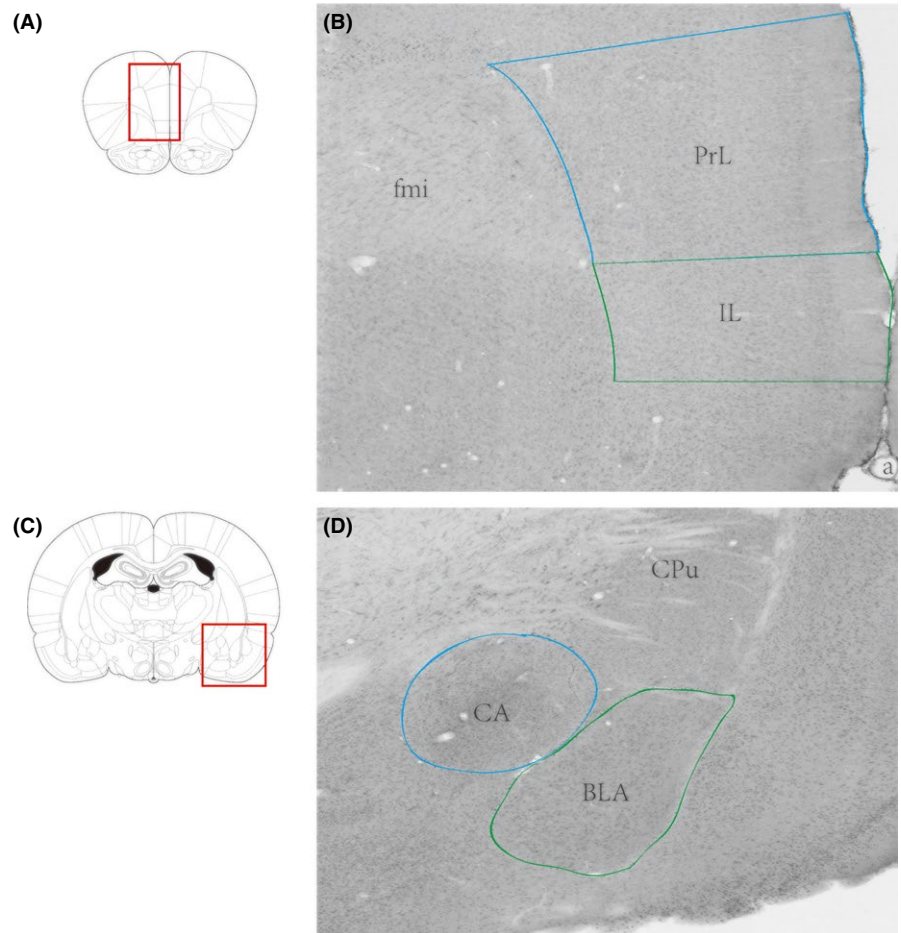
The next day, the sections were immersed into a second antibody donkey anti-mouse antibody solution (dilution 1:200 in PBS, Jackson Westgrove, PA, USA) for 60 minutes. Detection was performed using the avidin-biotin complex (ABC, Vector), and its signal was visualized by incubating the sections in PBS containing 3.3'-diaminobenzidine (DAB) (Sigma, Steinheim, Germany) at 0.5 mg/mL, 0.23% (wt/vol) nickel ammonium sulfate (Merck, Whitehouse Station, NJ, USA) and 0.01% (vol/vol) H<sub>2</sub>O<sub>2</sub> (Merck, Whitehouse Station, NJ, USA) for 5 minutes (RT).

### 2.5 | Image analysis

The microscope (Zeiss Axioskop Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Jena, Germany) had a motorized scanning stage (Märzhäuser, Wetzlar, Germany) and was connected to a camera (SonyXC-77CE) with a  $\times$  20 objective in front. To get the same amount of light, the intensity of light was adjusted for unstained control areas. The collected images were transformed into optical density (OD) images by use of a standard transformation curve.

For each brain area, 3 sections were selected at rostral, medial, and caudal levels, for medial prefrontal cortex (bregma 3.72, 3.00, 2.52 including prelimbic prefrontal cortex [PrL] and infralimbic prefrontal cortex [IL]) and amygdala (bregma -1.72, -1.80, -1.92, including basolateral amygdala [BLA] and central nucleus of the amygdala [CA]), in which individual structures were manually delineated according to Paxinos and Watson's atlas<sup>29</sup> (Figure 1A, C). All brain areas are identified by their shape and size with apparent anatomical structures as references including forceps minor of the corpus callosum, the azygous pericallosal artery for the PrL and IL, and the lateral external capsule and caudate putamen for BLA and CA (Figure 1B, D). Analysis was performed with the help of the software Image Pro Plus 6.2 (Media Cybernetics, Bethesda, USA).

The pattern of the staining revealed densely stained small particles, which is consistent with a previous report<sup>25</sup> where colocalization with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) has been qualitatively verified (Figure 2). The methods of determining the 5-mC- or 5-hmC-ir area and the optical density (OD) of 5-mC or 5-hmC signals have been described in detail in previous work,<sup>30</sup> where this OD measurement is positively correlated with protein levels.<sup>30</sup> In short, the background was defined in the adjacent area with absence of positive staining on the tissue. OD values 2 times above background for 5-mC and 1.5 times above background for 5-hmC were considered as positive signals. These threshold levels were determined by the coefficient of variation ((SD/mean) $\times$ 100%) which was lower than 10% (calculated by measuring 1 complete control 3 times). The average OD was calculated by multiplying the percentage of the positive stained area by the OD of 5-mC- or 5-hmC-ir signal in each section. The total average OD was summed up from 3 sections (rostral, medial, and caudal) of each structure. The OD levels are consistent among rostral, medial, and caudal. Only those sections with 3 different levels or clear completeness of



**FIGURE 2** Representative image of 5-hydroxymethylcytosine (5-hmC) immunoreactivity in rat brains. A, Prefrontal cortex (bregma 3.72 mm), the red box indicates the region for image analysis; B, manual delineation of the prelimbic prefrontal cortex (PrL) and infralimbic prefrontal cortex (IL). C, Amygdala region (bregma -1.92 mm), the red box indicates the region for image analysis; D, basolateral amygdala (BLA) and central nucleus of the amygdala (CA). Both 5-methylcytosine (5-mC) (not shown) and 5-hmC showed a similar pattern of immunoreactivity. Previous colocalization studies have found that the densely stained 5-mC<sup>25</sup> and 5-hmC<sup>26</sup> signals reflect nuclear staining. Abbreviations: a, the azygous pericallosal artery; CPu, caudate and putamen; fmi, forceps minor of the corpus callosum

anatomical structures of interest were included for further analysis. This resulted in group sizes of 4-6 (see Figure 4-6 for vertical scatter plots). Despite the small group sizes, sufficient statistical power was retained (effect size: 2.5-4;  $\alpha$ : 0.05;  $N$  = 4-6;  $1-\beta \geq 0.90$ ).

The number of c-Fos-positive cells was counted in the same brain regions (IL, PrL, BLA and CA) using a drawn overlay that corresponded to the shape and size of each brain area.

## 2.6 | Statistics

Statistical analyses were carried out using SPSS Statistics 21.0 (SPSS Inc, Chicago, IL). The statistical power for independent t-tests was tested by PASS 13.0 (NCSS, LLC, Kaysville, UT). We found that the data were not always normally distributed. Therefore, log<sub>10</sub> transformation was applied to the original data. Baseline freezing behavior in the 2 minutes prior to CS-exposure in the extinction sessions was analyzed using an independent Student's t-test. CS-induced freezing (expressed as percentage of time spent on freezing during CS presentation) was analyzed in trial blocks of 3 CSs and subjected to a genotype x trial-block repeated measures ANOVA. The staining differences were analyzed based on a genotype x fear extinction two-way ANOVA. Significant ANOVA tests were followed up with Bonferroni post hoc comparisons. Pearson correlations were performed to assess the correlations between 5-mC-, 5-hmC-ir, and c-Fos-ir.  $P$  values

lower than 0.05 were considered statistically significant. Values are presented as mean  $\pm$  standard error of the mean (SEM).

## 3 | RESULTS

### 3.1 | Condition freezing

A group of rats ( $n$  = 8 each) was included to measure fear conditioning. We found that the freezing during conditioning did not differ across genotypes (Figure 1). No significant trial-block effect was observed for conditioned freezing ( $F_{(4,56)} = 0.288$ ,  $P = 0.791$ ), nor a significant genotype x trial-block effect ( $F_{(4,56)} = 0.824$ ,  $P = 0.468$ ).

### 3.2 | Fear extinction

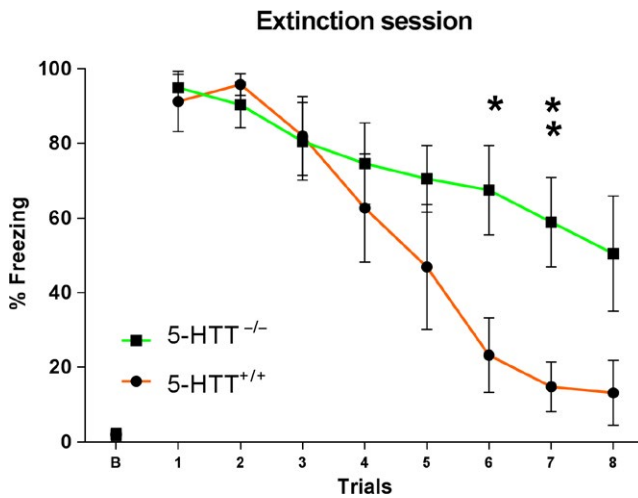
No differences in pre-CS freezing were observed during the extinction session ( $T_{(10)} = 0.000$ ,  $P = 0.996$ ; see Figure 3). In contrast, a significant trial-block effect was observed for CS-induced freezing ( $F_{(7,70)} = 20.304$ ,  $P = 0.000^{**}$ ), as well as a significant genotype x trial-block effect ( $F_{(7,70)} = 3.574$ ,  $P = 0.028^*$ ). Subsequent analysis of the genotype x trial-block interaction, followed up with Bonferroni post hoc comparisons indicated that 5-HTT<sup>-/-</sup> rats showed significantly higher levels of freezing behavior during trial blocks 6 ( $F_{(1,11)} = 8.085$ ,  $P = 0.017^*$ ), 7 ( $F_{(1,11)} = 10.402$ ,  $P = 0.009^{**}$ ), and a trend in trial block

8 ( $F_{(1,12)} = 4.412, P = 0.062$ ), compared to their wild-type counterparts (Figure 3).

### 3.3 | c-Fos-ir

As illustrated in Figure 4, fear extinction had no effect on the number of c-Fos immunopositive neurons in the PrL cortex (genotype  $F_{(1,20)} = 1.472, P = 0.243$ ; treatment  $F_{(1,20)} = 0.120, P = 0.733$ ; genotype x treatment  $F_{(1,20)} = 0.368, P = 0.553$ ). However, for the IL cortex a significant treatment ( $F_{(1,20)} = 7.799, P = 0.013$ ) but not genotype ( $F_{(1,20)} = 0.156, P = 0.698$ ), main effect and a trend genotype

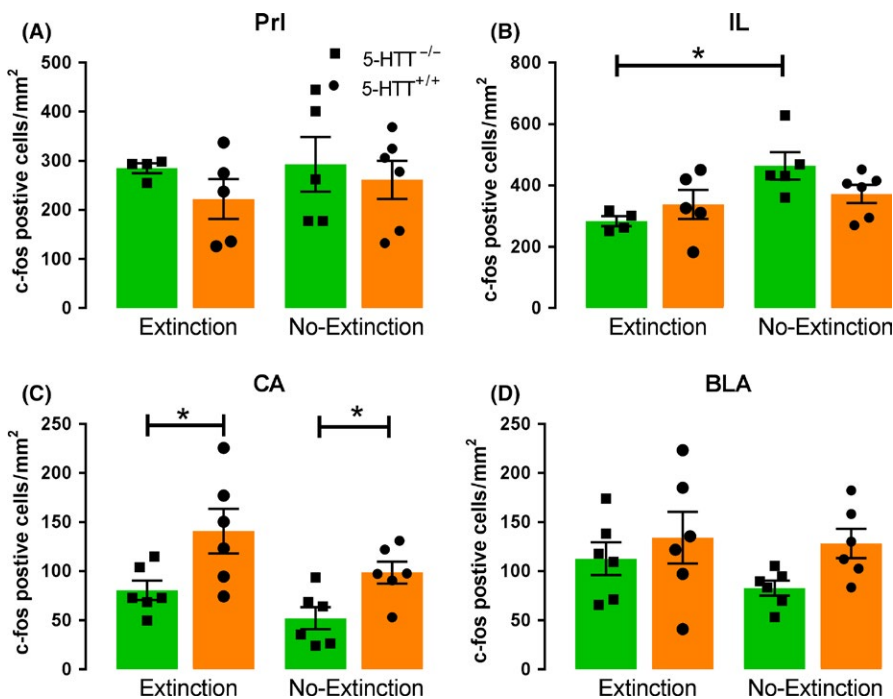
x treatment interaction ( $F_{(1,20)} = 2.682, P = 0.121$ ) was obtained. Subsequent analysis of the interaction indicated that 5-HTT<sup>-/-</sup> rats that received extinction training had reduced c-Fos immunopositive cells in the IL cortex compared to their no-extinction training counterparts ( $2.45 \pm 0.02$  vs  $2.65 \pm 0.04, F_{(1,7)} = 17.852, P = 0.004^*$ ), an effect not observed in 5-HTT<sup>+/+</sup> animals ( $F_{(1,9)} = 0.534, P = 0.483$ ). Fear extinction had no effect in the BLA (genotype  $F_{(1,24)} = 2.644, P = 0.120$ ; treatment  $F_{(1,24)} = 0.486, P = 0.494$ ; genotype x treatment interaction  $F_{(1,24)} = 0.971, P = 0.336$ ), while fear extinction showed a genotype effect ( $F_{(1,24)} = 14.057, P = 0.001$ ) and a treatment effect ( $F_{(1,24)} = 6.454, P = 0.019$ ) on the number of c-Fos immunopositive neurons in the CA, but with no genotype x treatment interaction ( $F_{(1,24)} = 0.342, P = 0.565$ ). Furthermore, c-Fos-ir tended to be higher in the CA of 5-HTT<sup>-/-</sup> rats that underwent fear extinction compared to no-fear extinction 5-HTT<sup>-/-</sup> animals ( $1.89 \pm 0.05$  vs  $1.66 \pm 0.10, F_{(1,10)} = 4.070, P = 0.071$ ). In the 5-HTT<sup>+/+</sup> rats, no significant difference was observed between the fear extinction and no-fear extinction group ( $F_{(1,10)} = 2.390, P = 0.153$ ). The c-Fos-ir was significantly higher in the 5-HTT<sup>+/+</sup> rats that underwent fear extinction than 5-HTT<sup>-/-</sup> rats that underwent fear extinction ( $2.12 \pm 0.07$  vs  $1.89 \pm 0.05, F_{(1,10)} = 6.49, P = 0.029^*$ ). Finally, a significantly higher c-Fos-ir was found in 5-HTT<sup>+/+</sup> rats with no-fear extinction compared to 5-HTT<sup>-/-</sup> rats with no-fear extinction ( $1.98 \pm 0.06$  vs  $1.66 \pm 0.10, F_{(1,10)} = 7.65, P = 0.020^*$ ).



**FIGURE 3** Data represent mean percentage of freezing ( $\pm$  SEM) in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> rats during the 2 min prior to CS-exposure (baseline, B), and during CS-exposure. CSs were presented during 8 (1-8) extinction trial blocks of 3 trials each. \* $P < 0.05$  and \*\* $P < 0.01$

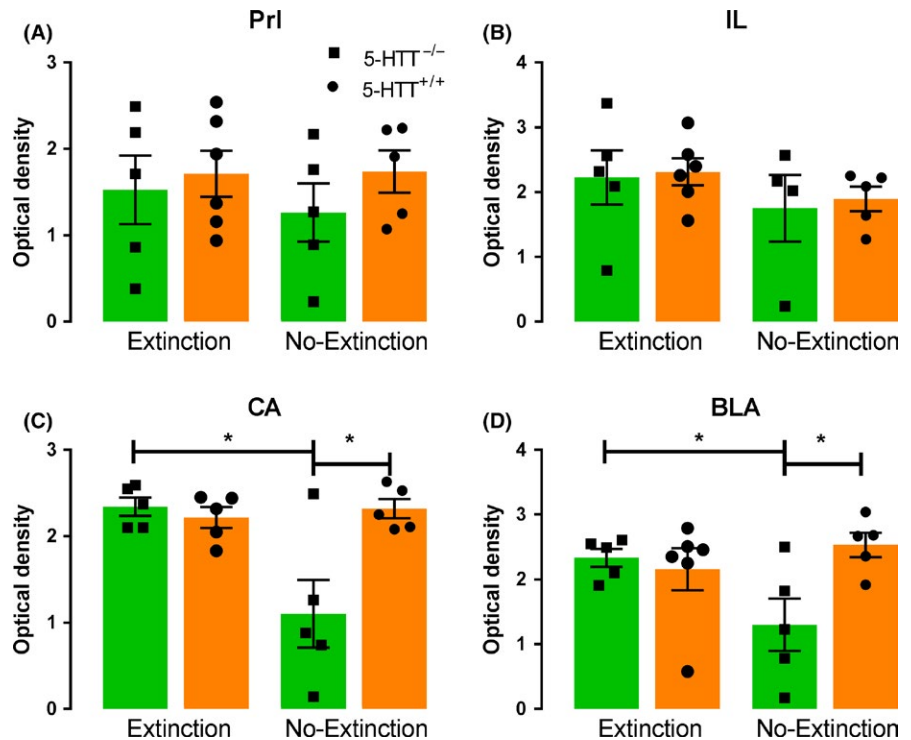
### 3.4 | 5-hmC-ir

Fear extinction had no effect on the total area covered by 5-hmC-ir in the PrL (genotype  $F_{(1,19)} = 1.101, P = 0.309$ ; fear extinction  $F_{(1,19)} = 0.141, P = 0.712$ ; genotype x fear extinction  $F_{(3,17)} = 0.207, P = 0.655$ ) and IL cortex (genotype  $F_{(1,19)} = 0.666, P = 0.426$ ; fear extinction



**FIGURE 4** c-Fos-ir in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> rats exposed to the fear conditioned stimulus in the absence of footshock in the test chamber (fear extinction group) or exposed to the test chamber only (nonfear extinction group). Data represent the mean relative percentage of matched nonextinction groups  $\pm$  SEM at 2 h after the start of extinction session in the PrL (A), IL (B), CA (C) BLA (D). \*Bonferroni-corrected,  $P < 0.05$





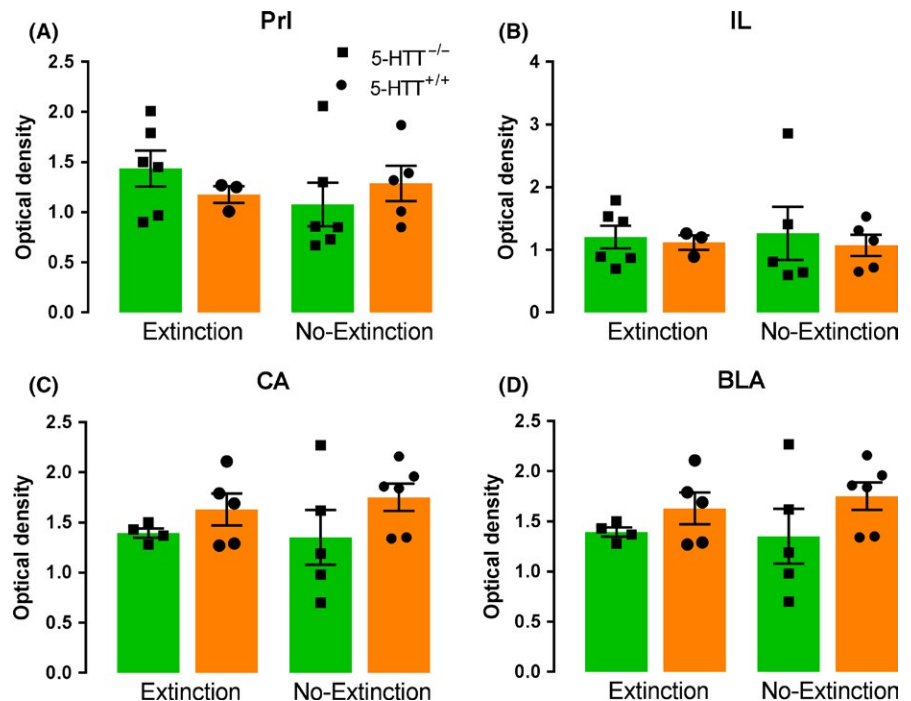
**FIGURE 5** 5-hmC-ir in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> rats exposed to the fear conditioned stimulus in the absence of footshock in the test chamber (fear extinction group) or rats exposed to the test chamber only (nonfear extinction group). Percentage ir (of nonextinction group set at 100%). Data represent the mean of 5-hmC-ir expressed as percentage of matched nonextinction groups  $\pm$  SEM in the PrL (A), IL (B), CA (C) and BLA (D). \*Bonferroni-corrected,  $P < 0.05$

$F_{(1,19)} = 3.084$ ,  $P = 0.097$ ; genotype  $\times$  treatment  $F_{(3,17)} = 0.327$ ,  $P = 0.575$ ) (Figure 5A, B).

However, in the CA, we found a significant genotype  $\times$  fear extinction interaction ( $F_{(3,16)} = 9.443$ ,  $P = 0.007^*$ ) as well as fear extinction ( $F_{(1,18)} = 6.777$ ,  $P = 0.019^*$ ) and genotype ( $F_{(1,18)} = 6.249$ ,  $P = 0.024^*$ ) effects. Subsequent post hoc analysis of the no-extinction group revealed that 5-HTT<sup>+/+</sup> rats exhibited more intense 5-hmC-ir compared to 5-HTT<sup>-/-</sup> rats ( $2.321 \pm 0.112$  vs  $1.102 \pm 0.391$ ,  $F_{(1,8)} = 8.968$ ,

$P = 0.017^*$ ) (Figure 5C). In addition, the 5-HTT<sup>-/-</sup> rats that underwent fear extinction showed higher 5-hmC-ir compared to no-fear extinction 5-HTT<sup>-/-</sup> animals ( $2.344 \pm 0.106$  vs  $1.102 \pm 0.391$ ,  $F_{(1,9)} = 9.374$ ,  $P = 0.016^*$ ) (Figure 5C). In contrast, among 5-HTT<sup>+/+</sup> animals, there were no differences between fear extinction and no-fear extinction groups ( $2.218 \pm 0.120$  vs  $2.321 \pm 0.112$ ,  $F_{(1,9)} = 0.392$ ,  $P = 0.549$ ).

Finally, in the BLA, a significant genotype  $\times$  fear extinction interaction was found ( $F_{(3,17)} = 5.865$ ,  $P = 0.027^*$ ), but without genotype



**FIGURE 6** 5-mC-ir in 5-HTT<sup>+/+</sup> and 5-HTT<sup>-/-</sup> rats exposed to the fear conditioned stimulus in the absence of footshock in the test chamber (fear extinction group) or exposed to the test chamber only (nonfear extinction group). Percentage ir (of nonextinction group set at 100%). Data represent the mean of 5-mC-ir expressed as percentage of matched nonextinction groups  $\pm$  SEM in the PrL (A), IL (B), CA (C) and BLA (D). \*Bonferroni-corrected,  $P < 0.05$

( $F_{(1,21)} = 3.303, P = 0.087$ ) or fear extinction ( $F_{(1,21)} = 1.285, P = 0.273$ ) main effects. A subsequent post hoc test revealed significantly higher 5-hmC-ir in the BLA of 5-HTT<sup>+/+</sup> rats compared to 5-HTT<sup>-/-</sup> rats within the nonfear extinction group ( $2.533 \pm 0.189$  vs  $1.301 \pm 0.404$ ,  $F_{(1,9)} = 7.653, P = 0.024^*$ ). The 5-HTT<sup>-/-</sup> rats that underwent fear extinction exhibited higher 5-hmC-ir compared to the no-fear extinction animals ( $2.335 \pm 0.310$  vs  $1.301 \pm 0.903$ ,  $F_{(1,9)} = 5.869, P = 0.042^*$ ) (Figure 5D).

### 3.5 | 5-mC-ir

Regarding 5-mC-ir, we did not observe any genotype or fear extinction effects in the PrL cortex (genotype  $F_{(1,18)} = 0.014, P = 0.906$ ; fear extinction  $F_{(1,18)} = 0.358, P = 0.558$ ; genotype x fear extinction interaction  $F_{(3,16)} = 1.342, P = 0.264$ ) (Figure 6A). Likewise, in the IL cortex, no significant changes in 5-mC-ir were found (genotype  $F_{(1,17)} = 0.250, P = 0.624$ ; fear extinction  $F_{(1,17)} = 0.001, P = 0.978$ ; genotype x fear extinction  $F_{(3,15)} = 0.035, P = 0.854$ ) (Figure 6B). Finally, neither in the CA nor in the BLA changes in 5-mC-ir were found (CA: genotype  $F_{(1,18)} = 0.001, P = 0.974$ , fear extinction  $F_{(1,18)} = 1.029, P = 0.326$ ; genotype x fear extinction  $F_{(3,16)} = 0.017, P = 0.897$ ; BLA: genotype  $F_{(1,18)} = 3.088, P = 0.098$ ; fear extinction  $F_{(1,18)} = 0.046, P = 0.832$ ; and genotype x fear extinction  $F_{(3,16)} = 0.203, P = 0.658$ ) (Figure 6C, D).

### 3.6 | Correlations

A positive correlation was observed between c-Fos-ir and 5-mC in the PrL of 5-HTT<sup>-/-</sup> rats (Table 1). No significant correlation was found between 5-mC, 5-hmC, c-Fos within the PFC and amygdala of the same rats.

## 4 | DISCUSSION

In line with previous findings from our group<sup>18,22,31</sup> and others,<sup>16,17</sup> 5-HTT<sup>-/-</sup> animals showed reduced fear extinction compared to their wild-type counterparts. This behavioral impairment was associated with reduced neuronal activity in the IL, but not PrL cortex, as measured by c-Fos immunohistochemistry. No genotype differences in c-Fos expression were found in the CA and BLA. Nonextinction 5-HTT<sup>-/-</sup> rats exhibited significantly lower 5-hmC levels compared to extinction 5-HTT<sup>-/-</sup> rats in the BLA and CA. The 5-hmC level increased significantly in 5-HTT<sup>-/-</sup> rats exposed to the fear predicting conditioned stimulus during extinction. No differences in either 5-mC or 5-hmC were observed in the IL and PrL. Finally, we observed that c-Fos expression correlates with DNA methylation in the PrL cortex. These findings indicate that our hypothesis was partially confirmed: impaired fear extinction in those characterized by inherited 5-HTT downregulation is related to changes in general DNA demethylation in the amygdala.

The reduced c-Fos levels in the IL of 5-HTT<sup>-/-</sup> rats in association with fear extinction are in line with earlier observations indicating that resistance to extinction is associated with reduced c-Fos

**TABLE 1** Correlations between DNA (de) methylation OD and c-Fos immunoreactivity in the prefrontal cortex and amygdala of 5-HTT<sup>-/-</sup> and 5-HTT<sup>+/+</sup> rats

Brain region	PrL-5-HTT <sup>+/+</sup>	IL-5-HTT <sup>+/+</sup>	BLA-5-HTT <sup>+/+</sup>	CA-5-HTT <sup>+/+</sup>	PrL-5-HTT <sup>-/-</sup>	IL-5-HTT <sup>-/-</sup>	BLA-5-HTT <sup>-/-</sup>	CA-5-HTT <sup>-/-</sup>
c-fos	R = 0.415, P = 0.306	R = 0.249, P = 0.552	R = 0.364, P = 0.272	R = -0.070, P = 0.838	<b>R = 0.671, P = 0.048*</b>	R = 0.147, P = 0.728	R = -0.264, P = 0.492	R = 0.324, P = 0.434
5-hmC	R = 0.196, P = 0.587	R = -0.328, P = 0.355	R = 0.016, P = 0.965	R = -0.191, P = 0.622	R = 0.562, P = 0.115	R = -0.097, P = 0.804	R = -0.062, P = 0.884	R = -0.048, P = 0.903

Bold indicate statistical significance (\* $P < 0.05$ ).

expression in the IL cortex.<sup>32-34</sup> This region plays a critical role in both extinction learning and its extinction recall.<sup>35,36</sup> Thus, reversible IL cortex inactivation in rats shortly prior to extinction training resulted in impaired fear extinction and consolidation of the extinction memory.<sup>35</sup> We found no genotype and extinction differences in c-Fos immunoreactivity in the PrL cortex, a region involved in the expression of fear.<sup>35,37</sup> This is somewhat unexpected, as the PrL is involved in the expression of fear.<sup>38</sup> Possibly, other immediate early gene markers are needed to uncover a difference between extinction and no-extinction groups. In the CA, which is critically involved in fear expression,<sup>39</sup> c-Fos immunoreactivity was increased in the fear extinction group compared to the no-fear extinction group, regardless of genotype.<sup>16</sup> Increased neural activity within the CA corresponds to the freezing response of the animals.<sup>6</sup> As the animals were sacrificed 2 hours after the start of the extinction session, the lack of genotype difference may relate to the lack of freezing differences at the start of the session. Together, these observations suggest that abnormal IL cortex function may contribute to impaired fear extinction in 5-HTT<sup>-/-</sup> animals.

The normalization of reduced 5-hmC in the amygdala after fear extinction in 5-HTT<sup>-/-</sup> rats fits the report that extinction training induced the expression of synaptic signaling genes along with 5-hmC peaks.<sup>12</sup> Possibly, increased or altered gene expression in the amygdala during fear extinction due to higher levels of 5-hmC is related to an active process attempting to silence the emotional value of the CS or to form an extinction memory in 5-HTT<sup>-/-</sup> animals. It is not likely that genotype differences in 5-hmC in the amygdala are related to differences in the strength of the fear memory or baseline freezing, given that we (see materials and methods section) and others did not observe 5-HTT genotype differences in these measures.<sup>16,17</sup>

In the PrL and IL, no genotype and fear extinction effects for 5-mC and 5-hmC were observed. It has been demonstrated that fear extinction leads to a dramatic genome-wide redistribution of 5-hmC and that this is associated with promotion of gene expression as well as formation of fear extinction memory in the prefrontal cortex.<sup>12</sup> In the same study, it was reported that 7-10, but not 1-5 hours postextinction upregulation of Tet3 mRNA emerged.<sup>12</sup> This result is consistent with our finding that the overall 5-hmC levels were stable in the PrL and IL, 2 hours postextinction. In light of the idea that the IL is implicated in fear extinction by inhibiting the BLA, it may be surprising that we did not observe any significant difference between 5-HTT<sup>-/-</sup> and 5-HTT<sup>+/+</sup> rats in 5-hmC and 5-mC in this prefrontal area. Possibly, other IL mechanisms may be involved in the fear extinction deficit in 5-HTT<sup>-/-</sup> rats.

A previous *in vitro* study showed that c-Fos was highly associated with DNA methylation modifications.<sup>24</sup> TET1 knockout mice exhibited impaired fear extinction which was associated with a significant upregulation of general 5-mC and downregulation of general 5-hmC and c-Fos in the cortex and hippocampus.<sup>10</sup> A key upstream neuronal activity-regulated gene, Npas4, was found to exhibit hypermethylation of its promoter region in TET1 knockout animals compared to controls, both in naive mice and after extinction training. This hypermethylation could lead to the reduced expression of Npas4 and its

downstream product c-Fos.<sup>10</sup> Interestingly, we observed a positive correlation between 5-mC-ir and c-Fos activation in the PrL of 5-HTT<sup>-/-</sup> rats (Table 1). This implies that c-Fos is either an upstream or a downstream factor in the regulation of DNA methylation, although neither 5mC nor c-Fos was different between genotypes in the PrL. Future research is required to further investigate these potential relationships.

This study has some limitations. Firstly, we did not include female rats, while PTSD is more prevalent in females compared to males.<sup>40</sup> Secondly, although our study had sufficient statistical power, a larger sample size would have been more ideal. Furthermore, the global DNA methylation and hydroxymethylation levels need to be confirmed by other techniques like mass spectroscopy or specific 5-hmC and 5-mC antibody/chemical-based captures.<sup>12,41</sup> Our preliminary data do show that 5-hmC and 5-mC levels are not different between naive 5-HTT<sup>-/-</sup> and 5-HTT<sup>+/+</sup> rats in the PFC as measured by mass spectroscopy (Y. Kroeze, L. Kroeze, J. Homberg, & J. H. Jansen, unpublished findings), which is in line with current findings. Future studies should also elucidate the specific gene promoters at which the amygdala changes in 5-hmC levels in response to fear extinction take place. Candidate genes are those encoding for BDNF<sup>42,43</sup> and gamma-aminobutyric acid (GABA) signaling components<sup>44</sup> and the glucocorticoid receptor.<sup>45,46</sup> Finally, given that PTSD is associated with fear extinction deficits due to impaired recall of the fear extinction memory,<sup>47</sup> a similar study should be followed up to investigate the association between epigenetic mechanisms and the recall of the fear extinction memory in 5-HTT<sup>-/-</sup> rats.<sup>18</sup>

In sum, we show that early-immediate 5-hmC modification in the amygdala is involved in impaired fear extinction in 5-HTT<sup>-/-</sup> rats, which is independent of c-Fos activation. As 5-HTT<sup>-/-</sup> rats model the 5-HTTLPR s-allele,<sup>15</sup> this finding may add a new dimension to the understanding of individual differences in risk for PTSD, as conferred by the 5-HTTLPR s-allele.<sup>2</sup>

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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