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Research report

Modulation of behavioural profile and stress response by 5-HTT genotype and social experience in adulthood

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ABSTRACT

Behavioural profiles can be shaped by genotype and environmental factors during early phases of life. The aim of this study was to investigate whether anxiety-like behaviour, exploration and adrenocortical stress responses can be modulated by genotype and social experiences in adulthood. Male mice lacking the serotonin transporter gene which is under scrutiny for anxiety disorders were compared with heterozygous and wildtype controls. Concerning social experiences, the males of all three genotypes were provided with a winner or a loser experience in a resident-intruder paradigm on three consecutive days. Anxiety-like behaviour and exploration were recorded in the dark-light, elevated plus-maze and open-field test. To non-invasively assess adrenocortical activity, corticosterone metabolites were determined from feces. The main findings were: Repeated social experience, irrespective of winning or losing, elevated levels of anxiety-like behaviour and decreased exploration. In losers a distinct effect of genotype occurred, with homozygous knockout males showing more anxiety-like behaviour and less exploration than the other genotypes. In winners no genotype-dependent variation was found. Genotypes did not differ in basal stress hormone secretion. There was, however, a main effect of social experience with higher activation of the stress hormone system in losers than in winners. This effect was strongest in the heterozygous genotype. In conclusion, our data show that anxiety circuits retain their plasticity throughout adulthood and can be shaped by genotype and social experiences during this phase of life. Moreover, responsiveness towards negative life experiences is influenced significantly by the 5-HTT genotype.

1. Introduction

Individual differences in traits of anxiety and even in the etiology of anxiety disorders can be modulated by both, environmental and genetic factors [24]. Regarding the molecular genetic basis, candidate genes have been identified which are associated with anxiety disorders (e.g., [15]). Of particular interest for this study was the serotonin transporter (5-HTT) which plays a key-role in serotonergic neurotransmission by removing the serotonin that is released into the synaptic cleft [6]. In humans, a repeat length polymorphism in the transcriptional control region of the 5-HTT gene (SLC6A4) was found, resulting in allelic variation of 5-HTT expression and function, and associated traits of negative emotionality including anxiety, depression and aggressiveness [3,33,34]. The generation of mice with a targeted disruption of the 5-HTT allows to investigate the consequences of its diminished or absent function. In fact, 5-HTT knockout mice were shown to display increased anxiety-like behaviour and 'behavioural despair' (e.g., depression-related behaviour) [26,47,68].

Interestingly, phenotypic consequences of the 5-HTT polymorphism in humans and monkeys and 5-HTT knockout mice seem to critically depend on adverse and stressful environmental influences during early development [24]. Humans with one or two copies of the short allele of the 5-HTT promoter polymorphism have been reported to exhibit more depressive symptoms, and suicidality behaviour than individuals homozygous for the long allele but only in relation with stressful life events ([8]; but see: [53]). Heterozygous 5-HTT knockout mice display increased anxiety- and depression-related behaviours compared to wildtypes but only when they had received low maternal care [7].

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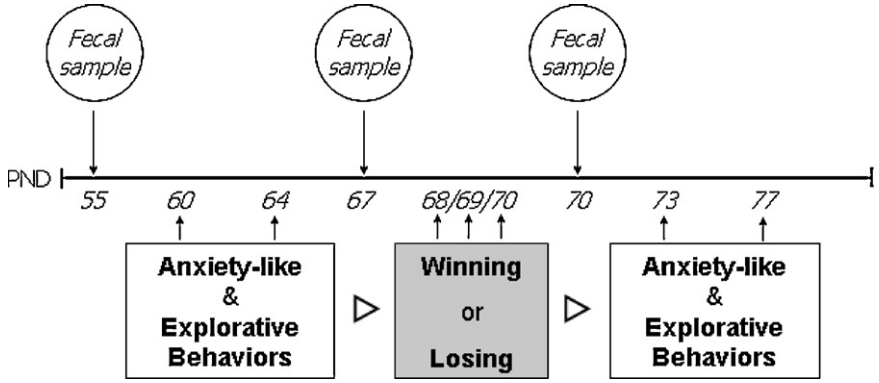

Fig. 1. Experimental design. Males of all three genotypes were provided with winner and loser experiences on postnatal days (PND) 68, 69, and 70. Before and after these experiences anxiety-like and explorative behaviours were assessed in a battery of tests. On PND 55, 67, and 70 fecal samples for investigation of corticosterone metabolites were collected.

Anxiety and fear circuits seem to be particularly vulnerable to environmental influences in times when synaptic connections are developing and refining, i.e., when brain circuits are highly plastic [9]. Accordingly, research on environmental influences on anxiety-related traits has mainly focused on early phases so far (e.g., [40,45,63]). However, these circuits seem to retain their plasticity in adulthood, as is indicated by the efficacy of psychotherapy and pharmacotherapy in later life [24]. Moreover, in rodents exposure to an enriched environment in adulthood decreases anxiety and fear [52]. On the other hand, a single social defeat in adulthood is related to increases in anxiety-like behaviour; at the same time the functionality of hippocampal serotonergic 5-HT_{1A} receptors is decreased [5].

Based on the knowledge that anxiety circuits seem to remain plastic during adulthood and that social defeat can enhance anxiety-like behaviour, the aim of the present study was to investigate possible involvement of the 5-HTT in maintaining this plasticity during adulthood in a genotype- and social experience-dependent way. Therefore, we focused on the modulation of anxiety-like behaviour by different social experiences in adulthood in wildtype as well as in heterozygous and homozygous 5-HTT knockout mice. We hypothesised that repeated losing (social defeat) as well as repeated winning (social victory) would influence anxiety-like behaviour, but in a differential way (hypothesis 1) and that these effects might be modulated by 5-HTT genotype (hypothesis 2). Since the endocrine stress response of winners and losers can differ significantly [57,66], we also expected winning and losing to differentially influence adrenocortical activity (hypothesis 3) and this hormonal response to be modulated by genotype (hypothesis 4).

Table 1
Description of behavioural patterns.

Behaviour	Definition
Winner behavioural patterns	
Attack	Rushing and leaping at another mouse with biting.
Escalated fight	Physical struggle between two mice which is initiated by a bite and usually involves further biting, kicking, wrestling, and rolling over and over. In-between, mice locked jaws. (A score was given for each escalated fight from the onset until the mice separated.)
Chasing	A mouse follows another mouse, while the head of the chasing mouse is directed to the backside of the other individual. The maximum distance between the animals is one body length. After stopping in forward motion the behaviour starts again.
Rushing	*Chasing* subsequent to an agonistic interaction (biting, attack or escalated fight).
Biting	A mouse contacts the body of another mouse with its mouth, making that mouse react with winced movement of either single extremities or the tail or the whole body.
Loser behavioural patterns	
Avoiding	Directed movement away from another mouse at a walking or running pace.
Flee	*Avoiding* subsequent to an agonistic interaction (biting, attack or escalated fight).
Defensive upright posture	Rearing up on the hind paws and keeping still, with the head up in the air, and the forepaws rigidly stretched out toward another mouse.
Defensive sidewise posture	Rearing up on the hind paws and keeping still, with shoulder and flank presented to another mouse.

Note: For description of behavioural patterns see also Marashi et al. [41,42].

2. Materials and methods

2.1. Animals and housing conditions

5-HTT knockout mice (5-HTT KO) [4] backcrossed into a C57BL/6J genetic background for >10 generations derived from our local stock. Parents were bred in pairs of heterozygous knockout males and females, and resulting offspring were of three different genotypes. To distinguish between wildtype (5-HTT+/+), heterozygous knockout (5-HTT+/−) and homozygous knockout (5-HTT−/−) mice, genomic DNA was extracted from tail tissue (sampling was performed on day 21 ± 1 of life). PCR amplicons of 225 bp (5-HTT+/+), 272 bp (5-HTT−/−) or both (5-HTT+/−) were identified by agarose gel electrophoresis.

Table 2
Validation of winner or loser experiences.

Genotype	Social experience		
	Winning	Losing	Unclear
(a) Confrontation of 5-HTT males with males from the strain C3H			
+/+[a]	10	0	1
+/−[b]	8	0	1
−/−[c]	6	0	5
(b) Confrontation of 5-HTT males with males from the strain NMRI			
+/+[a]	0	9	0
+/−[b]	0	10	3
−/−[c]	0	8	0

[a] Wildtype mice.
[b] Heterozygous knockout mice.
[c] Homozygous knockout mice.

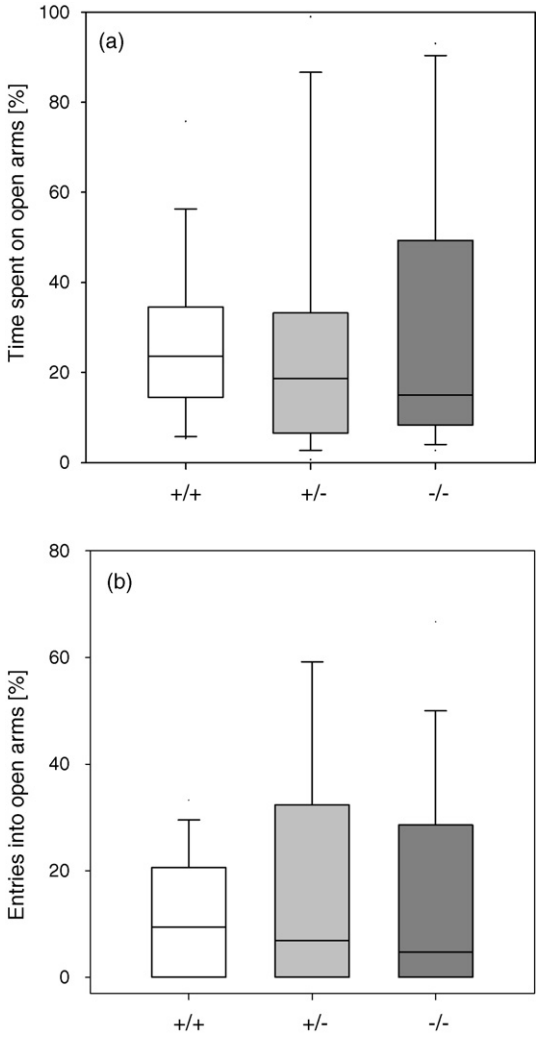

Fig. 2. Behavioural performance of mice of the three 5-HTT genotypes (5-HTT+/+, +/−, −/−) in the elevated plus-maze test. Data are shown as medians with the 25th and 75th percentile. Whiskers show the 5th and 95th percentile. $N_{(+/+)} = 20$, $N_{(+/-)} = 22$, $N_{(-/-)} = 19$.

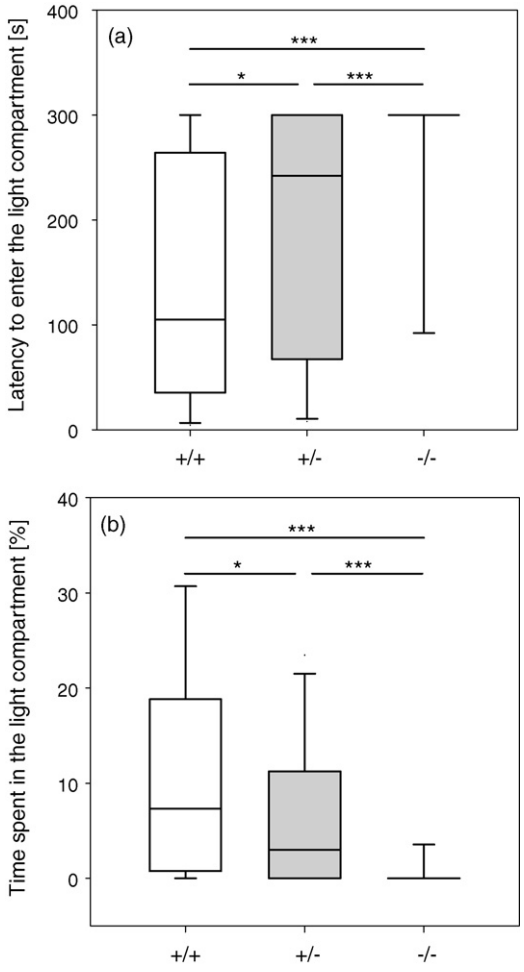

Fig. 3. Behavioural performance of mice of the three 5-HTT genotypes (5-HTT+/+, +/−, −/−) in the dark-light test. Data are shown as medians with the 25th and 75th percentile. Whiskers show the 5th and 95th percentile. $N_{(+/+)} = 22$, $N_{(+/-)} = 22$, $N_{(-/-)} = 18$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

In total, 65 male 5-HTT mice (19 5-HTT−/−, 24 5-HTT+/−, and 22 5-HTT+/+ mice) were used for the behavioural investigations (deviations from these sample sizes were due to technical reasons). After weaning at day 21 ± 1 of age, the animals were housed in groups of two to five littermates of the same sex in standard cages (Macrolon cages type III, 38 cm × 22 cm × 15 cm). From day 50 of age, i.e. before beginning of behavioural testing and during the whole experimental phase, all 5-HTT animals were housed singly. This measure was used to exclude possible influence of social interaction with conspecifics on the anxiety-like behaviour.

Males of the strains C3H and NMRI (obtained from Harlan Winkelmann GmbH (Borchen, Germany) at an age of 21 days of life) served as opponents for the generation of winner and loser experiences (see below). At the time of experiments, these mice were 60 days of age or older. Since C3H males present a very low rate of intermale aggression [29], it was possible to house them in groups of three individuals. By contrast, NMRI males had to be housed singly from day 40 because males of this strain show a high level of aggressiveness [48]. All experimental animals as well as the opponents were housed in standard polycarbonate cages with sawdust as bedding material (Allspan Höveler GmbH & Co. KG, Langenfeld, Germany). The housing room was temperature and humidity controlled and had a 12-h light/dark cycle with lights on at 7.00 a.m. Commercial mouse diet (Altromin 1324, Altromin GmbH, Lage, Germany) and water were available *ad libitum*. Tests were conducted between 8.00 a.m. and 10.00 a.m.

The presented work complies with current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86/609/EEC). All experiments were announced to the local authority and were approved by the 'Animal Welfare Officer' of the University of Muenster (reference number: 9.93.2.10.55.07.005).

2.2. Health check

At day 40, a general health check using a standard protocol (see [22,36,54]) was performed to guarantee that only healthy animals were included into the study and that data were not the result of deteriorated physical conditions of the animals. All animals passed the health check, indicating that no differences concerning general health state, sensory functions, reflexes and motor abilities existed between 5-HTT+/+, 5-HTT+/−, and 5-HTT−/− mice.

2.3. Experimental design

In order to gain converging evidence from more than one single test for differentially modulated anxiety we used a test battery of three different tests [43].

The experiment consisted of three parts (see Fig. 1). In a first step, 5-HTT males of the three genotypes were tested for anxiety-like and explorative behaviours in the elevated plus-maze (days 60 ± 2) and dark-light test (days 64 ± 2). Afterwards, three defined winner or three defined loser experiences were generated using the resident-intruder paradigm (days 68, 69, and 70; see Section 2.5). In a third step, anxiety-like and explorative behaviours were assessed again in the elevated plus-maze (days 73 ± 2) and in the open-field test (days 77 ± 2). The experiments were conducted by F.J., who was blind to the genotype of the tested animals.

To determine non-invasively levels of stress, corticosterone metabolites (CM) were investigated in fecal samples on different days. For analysing basal values, feces produced over 24 h were collected on day 55. To investigate stress reactivity, fecal samples voided during a 4 h time interval were collected before the first (day 67) and after the last (day 70) winner/loser experience (see Section 2.6.1).

2.4. Anxiety-like and explorative behaviours

Since the applied behavioural tests are all based on the exploratory locomotion of the mice, some of the measured parameters cannot unequivocally be inter-

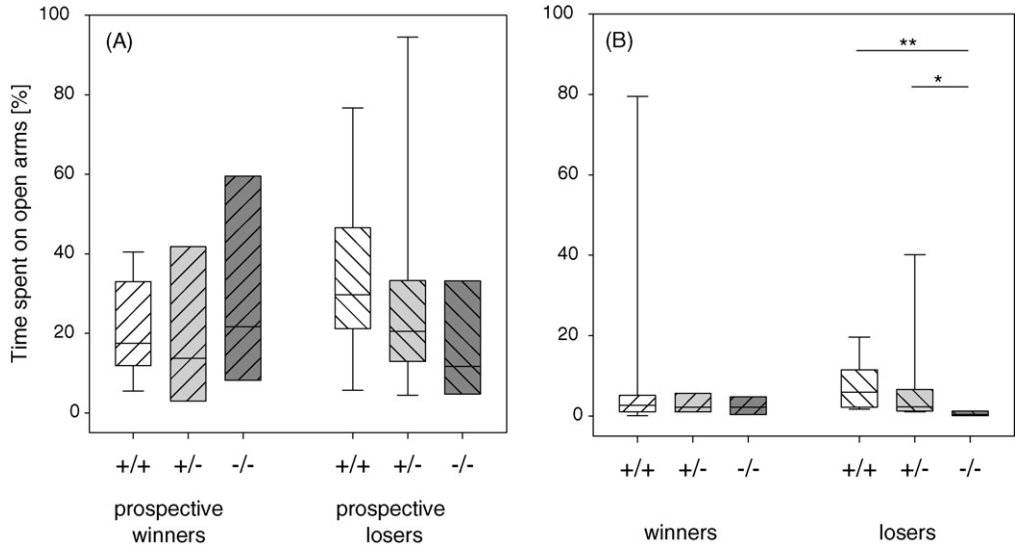

Fig. 4. Anxiety-like behaviour of mice of the three 5-HTT genotypes (5-HTT+/+, +/−, −/−) in the elevated plus-maze test before (A) and after (B) repeated winner or loser experiences. Data are shown as medians with the 25th and 75th percentile. Whiskers show the 5th and 95th percentile. $N_{(+/+\text{prospective winners})} = 10$, $N_{(+/-\text{prospective winners})} = 8$, $N_{(-/-\text{prospective winners})} = 6$, $N_{(+/+\text{prospective losers})} = 9$, $N_{(+/-\text{prospective losers})} = 10$, $N_{(-/-\text{prospective losers})} = 8$; $N_{(+/+\text{winners})} = 10$, $N_{(+/-\text{winners})} = 8$, $N_{(-/-\text{winners})} = 6$, $N_{(+/+\text{losers})} = 9$, $N_{(+/-\text{losers})} = 10$, $N_{(-/-\text{losers})} = 7$; $^{*}p < 0.05$; $^{**}p < 0.01$.

preted as indicating the anxiety-related approach avoidance conflict, but are also influenced by the basal locomotor activity and exploratory drive of the animals [25,39].

2.4.1. Elevated plus-maze test

Anxiety-like and explorative behaviours were measured at days 60 ± 2 (before winning/losing) and 73 ± 2 (after winning/losing) by means of the elevated plus-maze in which mice have the choice to choose freely between opposing open and closed arms [39,51]. The maze was elevated 50 cm above the floor and its arms were 30 cm long and 5 cm wide. The test apparatus was lit by a bulb above its centre (23 lux). Mice were placed into the centre of the maze randomly facing one of the arms. Each entry into an open or closed arm was counted and the time the animals spent in either type of arm was measured over 5 min by the use of an automatic tracking system (see http://www.phenotyping.com/digital.html). The percentage of time spent on open arms and the percentage of entries into open arms were analysed as parameters measuring anxiety-like behaviour.

2.4.2. Dark-light test

The test is based on the inborn tendency of mice to avoid brightly lit places [14]. Dark-light tests lasted 5 min and were performed with mice on day 64 ± 2. A longer latency to enter the light compartment and more time spent in the dark section of the test box indicated a higher level of anxiety-like behaviour. The test apparatus consisted of a standard Macrolon cage type III and was divided into two different sections by a small sliding door (11 cm × 5.5 cm). One section was transparent (26 cm × 22 cm × 15 cm) and the other (12 cm × 22 cm × 15 cm) was coated with black paint (impermeable for light) and closed by a black cap. The light compartment of the box was lit by a bulb suspended above its centre (13 lux). To habituate mice to the testing apparatus before starting the data collection, they were placed for 1 min into the dark compartment of the test apparatus. After habituation, the sliding door was opened and mice had the chance to choose freely between the dark and light part of the box. The latency to enter the light compartment as well as the time spent in the dark part of the box were analysed.

2.4.3. Open-field test

In the open-field test, mice had the opportunity to explore a square-shaped arena for a fixed amount of time [1,67]. The open-field was a moderately lit (13 lux) square arena of 80 cm × 80 cm with walls of 40 cm in height. On day 77 ± 2 each mouse was given one test session of 5 min during which the animals' locomotor behaviour was measured using an automatic tracking system (see above). At the beginning of the test, mice were placed into the centre of the arena. The distance travelled in the test arena as well as the percentage of time spent in the centre of the open-field (defined as the area of the open-field being located at least 20 cm distant from the walls) were analysed.

2.5. Providing winner or loser experiences

We used the resident-intruder paradigm to generate repeated winner or loser experiences for the 5-HTT males. It is based on isolation-induced aggressiveness of male mice that enhances defensive aggression against intruding conspecifics [25,46]. In order to generate losers, 5-HTT mice were placed (days 68, 69, and 70) as intruders into the home cage of male mice of the (aggressive) strain NMRI on three consecutive days. In contrast, to create winners, 5-HTT mice stayed in their own home cage as residents and a male of the (docile) strain C3H was introduced into the cage as an intruder on three consecutive days (days 68, 69, and 70). The test lasted at most 10 min. Via video camera and an attached monitor, F.J. observed the confrontation and stopped it, when fighting was too escalated. By this, the mice could be prevented from severe injury. Intruders were placed individually into the resident's home cage. The sums of the total number of winner behavioural patterns as well as loser behavioural patterns (for definition of behavioural patterns see Table 1) were recorded by an experienced observer who remained blind to genotype, using the software Observer XT 7.0 (Noldus Information Technology BV, Wageningen, NL). Residents and intruders could be easily distinguished by the light coat color of the C3H and NMRI males. Data was collected using *focal animal sampling* and *continuous recording*. An animal was categorised as 'winner', if it showed at least five winner behavioural patterns in each of three consecutive confrontations. In addition, these patterns had to occur at least twice as often as loser behavioural patterns. Accordingly, an animal was categorised as 'loser', if it showed at least five loser behavioural patterns in each of three consecutive confrontations. In addition, these patterns had to occur at least twice as frequently as winner behavioural patterns. Tests in which mice could not be clearly categorised as winners or losers were designated as 'unclear'.

When males of the three 5-HTT genotypes were confronted with an intruder C3H male in their home cage, they proved as winners in 24 out of 31 cases (see Table 2). In no case a loser experience was made. In seven cases the experience was defined as 'unclear'. Mice labeled as 'unclear' did not differ significantly between genotypes (Fisher-test: $p = 0.123$). When 5-HTT males of the three genotypes were placed as intruders into the home cage of resident NMRI males, they emerged as losers in 27 of 30 cases. In three cases the experience was categorised as 'unclear'. Mice labeled as 'unclear' did not differ between genotypes ($p = 0.167$). Subsequent data analysis was only performed for winners and losers.

2.6. Endocrinological investigations: non-invasive monitoring of glucocorticoid metabolites

2.6.1. Sample collection

On day 55 ± 2 the individually housed animals were placed in a cage identical to their home cage containing fresh bedding material for 24 h. All feces defecated during this 24 h period (starting at 6 p.m.) were collected and frozen at −20 °C until assayed for corticosterone metabolites (corticosterone is the major glucocorticoid in mice [58]). This procedure resembled the routinely performed transfer to clean cages for animal maintenance and thus was not considered to be associated with severe stress that might have corrupted subsequent samples. On days 67 ± 2 and 70 ± 2 (i.e., before beginning of the first confrontation and after the last one) fecal samples voided between 4 p.m. and 8 p.m (that is 8–12 h after the winner/loser experience) were collected by placing mice in a standard Macrolon cage with fresh bedding. The time frame was chosen according to Touma et al. [60], showing that a peak of corticosterone metabolites can be found in the feces 8–12 h after the exposure to a stressor.

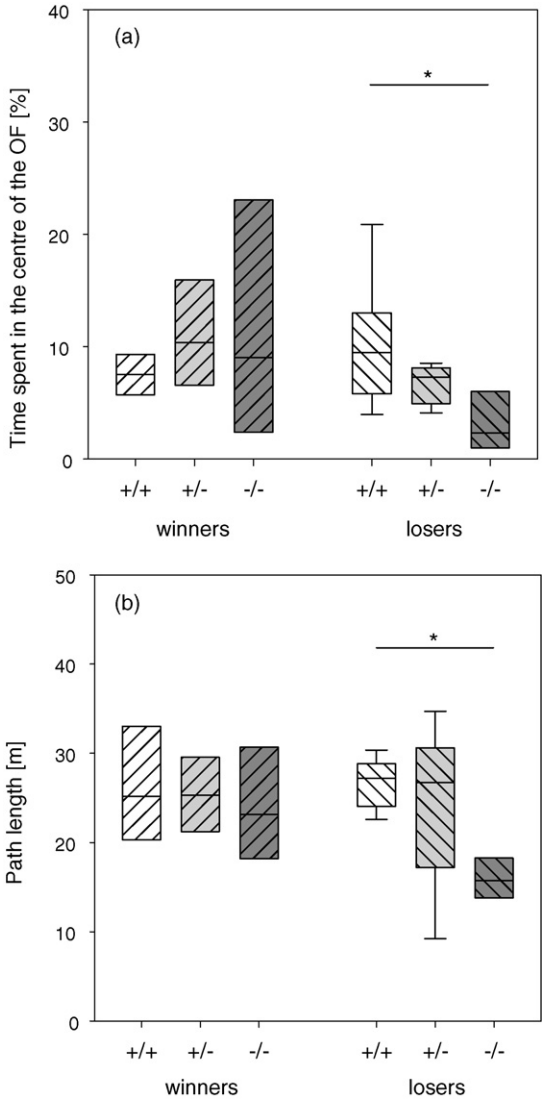

Fig. 5. Behavioural performance of mice of the three 5-HTT genotypes (5-HTT+/+, +/−, −/−) in the open-field test. Data are shown as medians with the 25th and 75th percentile. Whiskers show the 5th and 95th percentile. $N_{(+/+\text{winners})} = 8$, $N_{(+/-\text{winners})} = 8$, $N_{(-/-\text{winners})} = 6$, $N_{(+/+\text{losers})} = 10$, $N_{(+/-\text{losers})} = 10$, $N_{(-/-\text{losers})} = 7$; $^*p < 0.05$.

2.6.2. Hormone analysis

To monitor the activity of the hypothalamic-pituitary-adrenocortical (HPA) axis, the collected fecal samples were analysed for immunoreactive corticosterone metabolites (CM) using an established 5α-pregnane-3β,11β,21-triol-20-one enzyme-immunoassay (EIA). Details regarding development, biochemical characteristics, and physiological validation of this assay are described in [60,61]. Before EIA analysis, the fecal samples were homogenised and aliquots of 0.05 g were extracted with 1 ml of 80% methanol. The intra- and inter-assay coefficients of variation were 8.8% and 13.4%, respectively. Samples of less than 0.05 g of feces were excluded from data analysis.

2.7. Statistical analysis

Since behavioural data were not normally distributed and could not be transformed to a normal distribution, these data were analysed using non-parametric statistical tests. For a comparison of anxiety-like and explorative behaviours between the three 5-HTT genotypes in the elevated plus-maze, dark-light and open-field test, Kruskal–Wallis *H*-test was performed and, in cases of significance ($p < 0.05$), the Schaich–Hamerle test was used for subsequent pairwise comparisons. Paired data from behavioural parameters before and after the repeated winner or loser experiences were tested using the Wilcoxon Signed Ranks statistics.

As a considerable number of animals refused to leave the dark compartment of the apparatus, a chi-square test was calculated to reveal whether performance was differentially expressed between genotypes.

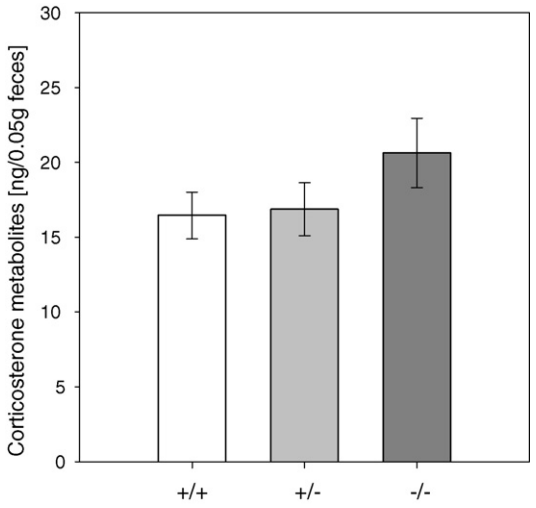

Fig. 6. Concentration of corticosterone metabolites in the feces (24 h fecal sample) of mice of all three 5-HTT genotypes (5-HTT+/+, +/−, −/−) on day 55 of life. Data are shown as means ± SEM. $N_{(+/+)} = 22$, $N_{(+/-)} = 24$, $N_{(-/-)} = 18$.

For analyses of corticosterone metabolites parametric statistical tests were used. 24 h samples were compared by an ANOVA with the factor *genotype*. The 4 h samples were analysed by a Repeated Measures ANOVA, with *genotype* and *social experience* (winning/losing) as independent factors. Subsequently, Bonferroni corrected independent *t*-tests were performed.

3. Results

3.1. Anxiety-like and explorative behaviours

3.1.1. Baseline genotype effects on anxiety

Before the animals were subjected to repeated winner or loser experiences, a basic comparison between all three genotypes indicated no significant difference in the elevated plus-maze test. The percentage of time spent on open arms as well as the percentage of entries into open arms did not differ significantly between 5-HTT wildtype, heterozygous, and homozygous mice (Kruskal–Wallis *H*-test: $\chi^2 = 1.343$, df = 2, $p = 0.511$; $\chi^2 = 0.375$, df = 2, $p = 0.829$; see Fig. 2). In contrast, the dark-light test showed significant effects of genotype, measured by the latency to enter the light compartment ($\chi^2 = 14.551$, df = 2, $p = 0.001$; see Fig. 3) and the percentage of time spent therein ($\chi^2 = 16.793$, df = 2, $p = 0.001$). Post hoc comparisons indicated that 5-HTT−/− mice took the longest time to enter the light part of the box (Schaich–Hamerle test, Δ_{Rcrit}(5-HTT+/+ vs. 5-HTT−/−) = 9.34, $p < 0.001$, Δ_{Rcrit}(5-HTT+/− vs. 5-HTT−/−) = 9.34, $p < 0.001$) and spent the longest time in the dark compartment (Δ_{Rcrit}(5-HTT+/+ vs. 5-HTT−/−) = 9.34, $p < 0.001$, Δ_{Rcrit}(5-HTT+/− vs. 5-HTT−/−) = 9.34, $p < 0.001$). The lowest level of anxiety-like behaviour was found in 5-HTT wildtype mice. Heterozygous 5-HTT knockout mice showed anxiety-like behaviour on an intermediate level (Δ_{Rcrit}(5-HTT+/+ vs. 5-HTT+/−) = 5.83, $p < 0.05$). Chi-square analysis of the percentage of animals that entered the light compartment as a function of genotype revealed a significantly worse performance of 5-HTT−/− mice compared to both other genotypes (5-HTT+/+ vs. 5-HTT−/−: $\chi^2 = 14.83$, $p < 0.001$; 5-HTT+/− vs. 5-HTT−/−: $\chi^2 = 7.79$, $p < 0.01$; 5-HTT+/+ vs. 5-HTT+/−: $\chi^2 = 0.9429$, $p = 0.332$).

3.1.2. Winner and loser effects on anxiety

If mice were assigned to groups of prospective winners or prospective losers (see Fig. 4) again no effect of genotype occurred in the elevated plus-maze test before these experiences were actually made (time spent on open arms: $\chi^2 = 1.016$, df = 2, $p = 0.602$; entries into open arms: $\chi^2 = 4.269$, df = 2, $p = 0.118$). After the repeated experience of winning or losing, the level of anxiety-like

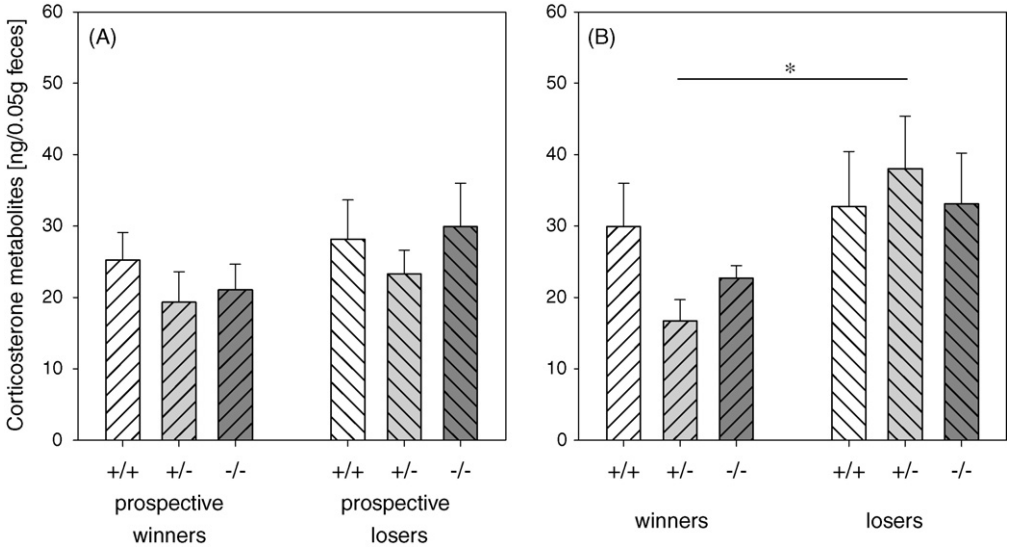

Fig. 7. Concentration of corticosterone metabolites in the feces (4 h fecal samples) of mice of all three 5-HTT genotypes (5-HTT+/+, +/−, −/−) before (A) and after (B) repeated winner or loser experiences. Data are shown as means + SEM. $N_{(+/+\text{prospective winners})} = 9$, $N_{(+/-\text{prospective winners})} = 7$, $N_{(-/-\text{prospective winners})} = 6$, $N_{(+/+\text{prospective losers})} = 8$, $N_{(+/-\text{prospective losers})} = 10$, $N_{(-/-\text{prospective losers})} = 8$; $N_{(+/+\text{winners})} = 4$, $N_{(+/-\text{winners})} = 7$, $N_{(-/-\text{winners})} = 3$, $N_{(+/+\text{losers})} = 5$, $N_{(+/-\text{losers})} = 5$, $N_{(-/-\text{losers})} = 5$; $^*p < 0.05$.

behaviour, measured by the 'percentage of time spent on open arms', was elevated in all experimental groups irrespective of the 'quality' of life experience, i.e., winning or losing (Wilcoxon Signed Ranks test: $Z_{+/+\text{winners}} = -1.784$, $p = 0.074$; $Z_{+/+\text{losers}} = -2.666$, $p = 0.008$; $Z_{+/-\text{winners}} = -2.336$, $p = 0.018$; $Z_{+/-\text{losers}} = -2.803$, $p = 0.005$; $Z_{-/-\text{winners}} = -2.201$, $p = 0.028$; $Z_{-/-\text{losers}} = -2.366$, $p = 0.018$; see Fig. 4). The percentage of entries into open arms differed significantly between the first and the second trial of the elevated plus-maze test in wildtype individuals who were confronted with a loser experience ($Z_{+/+\text{winners}} = -1.342$, $p = 0.180$; $Z_{+/+\text{losers}} = -2.201$, $p = 0.028$; $Z_{+/-\text{winners}} = -1.214$, $p = 0.225$; $Z_{+/-\text{losers}} = -1.752$, $p = 0.080$; $Z_{-/-\text{winners}} = -1.826$, $p = 0.068$; $Z_{-/-\text{losers}} = -1.604$, $p = 0.109$).

Strikingly, a significant effect of genotype concerning the time spent on open arms was only detected within the group of losers ($\chi^2 = 14.688$, df = 2, $p = 0.001$; see Fig. 4) with 5-HTT−/− males showing significantly more anxiety-like behaviour than heterozygotes (Δ_{Rcrit}(5-HTT+/− vs. 5-HTT−/−) = 9.22, $p < 0.05$) and wildtypes (Δ_{Rcrit}(5-HTT+/+ vs. 5-HTT−/−) = 11.70, $p < 0.01$). In contrast, no significant differences were found between the winners of all three genotypes ($\chi^2 = 0.551$, df = 2, $p = 0.759$). Consistent results could be detected in the open-field test: Concerning the parameter 'percentage of time spent in the centre of the open-field', a significant effect of genotype was only detected within the losers ($\chi^2 = 5.992$, df = 2, $p = 0.050$; see Fig. 5) with 5-HTT−/− mice spending less time in the centre of the test arena than wildtypes (Δ_{Rcrit}(5-HTT+/+ vs. 5-HTT−/−) = 9.57, $p < 0.05$). Consistent with previous results, winners of the three genotypes did not differ ($\chi^2 = 1.921$, df = 2, $p = 0.383$). A significant effect of genotype was again found within the losers ($\chi^2 = 9.984$, df = 2, $p = 0.007$) concerning the level of explorative behaviour measured by the travelled distance of mice in the open-field with 5-HTT−/− males travelling lower distances than heterozygous (Δ_{Rcrit}(5-HTT+/− vs. 5-HTT−/−) = 9.57, $p < 0.05$) and wildtype mice (Δ_{Rcrit}(5-HTT+/+ vs. 5-HTT−/−) = 11.87, $p < 0.01$). In contrast, the distance winners travelled in the test apparatus did not differ between the genotypes ($\chi^2 = 0.219$, df = 2, $p = 0.896$).

3.2. Endocrinological investigations

Before winning or losing no significant difference between mice of the three genotypes were found regarding corticosterone metabolite (CM) concentrations (day 55: ANOVA: $F_{(2,61)} = 1.384$, $p = 0.258$; see Fig. 6). The analysis of 4 h fecal samples collected before (day 67) and after (day 70) repeated confrontations with a conspecific, revealed a main effect of social experience, that is being a winner or loser (Repeated Measures ANOVA: $F_{(1,22)} = 6.453$, $p = 0.019$; see Fig. 7). Post hoc comparisons showed that 5-HTT+/− loser mice had significantly higher CM concentrations than 5-HTT+/− winners ($t = -3.013$, df = 10, $p = 0.013$). In contrast, wildtype and homozygous 5-HTT winners did not differ from wildtype and homozygous losers (5-HTT+/+ winners vs. 5-HTT+/+ losers: $t = -0.081$, df = 7, $p = 0.938$; 5-HTT−/− winners vs. 5-HTT−/− losers: $t = -1.093$, df = 6, $p = 0.316$).

4. Discussion

The main finding of this study is: Anxiety-like behaviour as well as exploration and stress hormone secretion can be modulated significantly by 5-HTT genotype as well as by social experiences in adulthood.

In a first step, we confirmed genotype-dependent levels of anxiety-like behaviour in the 5-HTT knockout mouse model by the use of the dark-light test [26]. In contrast to Holmes et al. [26], we did not find as high a variation in the elevated plus-maze test. Basically, this is not surprising because different laboratories can detect different results in the same test even under highly standardised conditions [13,37]. In addition, subtle differences in the test procedure can influence findings significantly. Holmes et al. [26] worked with an illumination of 200 lux, whereas our laboratory used a more dimmed light (23 lux). It was shown earlier that behaviour in the elevated plus-maze can depend upon lighting conditions [12].

In a second step, we modified the resident-intruder paradigm so that winners and losers could be generated predictably. Contrary to hypothesis 1, anxiety-like behaviour was elevated in all individuals regardless of winning or losing on three consecutive days. Notwithstanding the fact that an enhancement of anxiety-like behaviour can be due to repeated testing [19,20], it is well-known that social defeat increases anxiety (for a review see [5]). Considering this, the increase in anxiety-like behaviour in winners was rather unexpected. A possible explanation might be that the involvement in agonistic interactions usually causes an immediate activation of the hypothalamic-pituitary-adrenocortical (HPA) axis resulting in a distinct increase in circulating glucocorticoid

concentrations [28,35,55]. This acute stress response adaptively provides the organism with energy and puts it in a state of increased arousal, alertness and vigilance [11,66]. Accordingly, both opponents of a dyadic encounter are affected and only when individuals stratify into winners and losers a differential pattern develops. The activation of the HPA axis involves the secretion of corticotropin releasing factor (CRF) from the paraventricular nucleus of the hypothalamus. This neuropeptide exerts a strong anxiogenic-like effect [2,17,18]. In our experiment, 5-HTT mice lived a non-arousing life until, in adulthood, they were confronted with same-sex conspecifics. Possibly, these dyadic interactions caused the hitherto strongest activation of the HPA axis in their lives, including a distinct secretion of CRF. Exogenous CRF administered centrally can enhance 5-HT turnover in different brain regions [10] and it is likely that serotonergic neurotransmission is both influenced by and can influence hypothalamic CRF function [30]. Thus, the involvement in agonistic interactions may shape anxiety circuits in the adult brain through the action of CRF or through its interaction with the serotonergic system. As a consequence, individuals might behave in a more anxious and less explorative way in the future which was the case in the second testing phase (elevated plus-maze and open-field test). From an ecological perspective, such a behavioural response to the experience of repeated agonistic interactions is highly adaptive because the individual prospectively lives in a 'socially dangerous' and competitive world. In such a situation, more anxious individuals will avoid interactions with rivals, thereby minimising their costs of fighting [49].

A distinct effect of genotype within the group of losers confirmed hypothesis 2: 5-HTT−/− mice showed more anxiety-like behaviour and reduced exploration compared to 5-HTT+/+ and 5-HTT+/− mice as indicated by measures of the elevated plus-maze as well as those of the open-field test. In contrast to our hypothesis 2, however, no genotype-dependent variation occurred in winners. How to explain these results? Pavlovian fear conditioning and fear extinction do not vary between 5-HTT−/− and HTT+/+ mice [68]. However, when re-exposed to the conditioned stimulus (CS; tone) in the absence of the unconditioned stimulus (US; footshock) in the extinction context 24 h later, 5-HTT−/− mice exhibit a significant deficit in extinction recall, i.e. 5-HTT−/− mice have a deficit in the ability to retain extinction memory. In our resident-intruder paradigm, losing possibly represents a negative experience comparable with electric foot-shock in the conditioned fear paradigm. If so, individuals lacking the 5-HTT might not extinguish the negative experience of losing as good as wildtypes and might therefore show higher degrees of anxiety-like behaviour when tested for this trait in an unfamiliar environment. Moreover, it seems that losing *per se* is not fatal for an individual as long as this experience does not interact with a genetic predisposition for enhanced anxiety-like behaviour. Last, the finding that no differences existed between the winners of all three genotypes suggests: While the 5-HTT gene is significantly involved in the processing of negative experiences, it does not seem to play an essential role concerning positive experiences, i.e., winning in this study.

The serotonergic system is significantly involved in the modulation of anxiety and fear and 5-HTT is a key regulator of central serotonergic activity [24]. Noteworthy, the serotonergic system can be affected considerably by agonistic experiences. Social defeat in particular, can have pronounced and long-lasting effects [5,16,21,44,64,69]. In defeated rats, for example, a desensitisation of postsynaptic 5-HT_{1A} receptors has been described, modelling findings in clinically depressed patients [5]. In addition, winners and losers exhibit different time courses concerning 5-HT metabolism after fighting with loser individuals being affected distinctly longer [50,70]. In our study we compared 5-HTT+/+, 5-HTT+/− and 5-HTT−/− genotypes. Overall, the inactivation of the 5-HTT gene leads to markedly altered 5-HT homeostasis in 5-HTT−/− mice and to a lesser extent in 5-HTT+/− mice with depleted tissue stores that are inadequately compensated for by increased 5-HT synthesis [4,31]. This considerable variation in 5-HT homeostasis is likely to release differential responses of the three genotypes to the pronounced and long-lasting changes of the serotonergic system induced by the repeated experience of losing.

Concerning adrenocortical activity, two earlier studies showed lower basal plasma corticosterone values in 5-HTT−/− mice than in wildtypes [32,59]. We monitored adrenocortical activity non-invasively by measuring fecal corticosterone metabolites with a recently developed enzyme-immunoassay [60], avoiding confounding effects of the blood collection itself. By applying this technique and choosing a sampling interval of 24 h our data robustly show that no genotype-dependent variation in basal degrees of stress existed under our housing conditions. However, it has to be considered that slight differences in the circadian hormone rhythm are not displayed by this sampling method [61,62,65]. Regarding the effects of winning and losing on glucocorticoid secretion, a main effect of social experience was found with higher levels of stress in losers than in winners, as suggested in hypothesis 3. These data agree with numerous findings in a variety of species [56,57,66]. Interestingly, the post hoc analysis points to a modulation of this stress response by genotype and thereby confirms hypothesis 4: While in heterozygous animals winners had significantly lower concentrations of corticosterone metabolites than losers, this effect could not be shown for wildtype and 5-HTT−/− mice. These two genotypes displayed comparable corticosterone response patterns, as has also been shown before [38,59]. Thus, a 5-HTT genotype-dependent modulation of adrenocortical stress response to agonistic experience seems likely. 5-HTT+/− mice exhibited a 50% reduction in 5-HTT expression and therefore closely model the profile of the short allele combination of the 5-HTT polymorphism in humans (see Introduction; [27]). Human individuals carrying the short allele combination show a marked increase in cortisol production during and following exposure to a stressor [23]. The animal data match these findings in so far as 5-HTT+/− mice respond to the acute stressor of saline injection with a stronger increase of ACTH than 5-HTT+/+ mice [38]. The present study supports this view of an increased stress-responsive phenotype in 5-HTT+/− mice. In addition, this suggests negative social experiences as a key factor in shaping the adrenocortical stress response of this genotype.

In conclusion, our data firstly underline the view that anxiety circuits retain their plasticity during adulthood and can be shaped by social experiences during this phase of life. Secondly, we can point out two important modulating factors for anxiety: the 5-HTT genotype and the social experience of being a loser. Thus, negative life experiences have the strongest effect in mice lacking the 5-HTT, increasing anxiety-like and decreasing explorative behaviour. Further studies have to reveal if these results apply to both sexes or whether they are valid exclusively for male 5-HTT knockout mice.

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