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The winner and loser effect, serotonin transporter genotype, and the display of offensive aggression

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ABSTRACT

Aggressive behaviour results from a complex interplay between genetic and environmental factors. Key modulators of aggression include the serotonergic system on the molecular level and experience in prior aggressive contests as an environmental factor. The aim of this study was to elucidate the effects of fighting experience on the display of offensive aggressive behaviour in adult male mice varying in serotonin transporter (5-HTT) genotype. 5-HTT +/+, 5-HTT +/− and 5-HTT −/− mice were given either a winning or a losing experience on each of three consecutive days and were subsequently observed for their offensive aggressive behaviour as residents against a docile intruder from the C3H strain in a resident–intruder paradigm. The main findings were: There was no significant difference between the amount of offensive aggressive behaviour displayed by the genotypes. Winners showed more engagement with the intruder, attacked him faster and exhibited overall higher aggression scores than losers. There was no significant genotype × social experience interaction: winning and losing had a similar effect on offensive aggressive behaviour in all three 5-HTT genotypes. We conclude that social experience in terms of having been a winner or having been a loser rather than the 5-HTT genotype determines the behaviour towards a docile intruder.

1. Introduction

Aggression in its maladaptive and escalated form is a major burden on the health and well-being of populations and exacts an economic toll from nations [1]. Aggressive traits and behaviours are the result of a complex interplay between genetic and environmental factors [2]. On the molecular level, serotonin (5-HT) has been implicated in the neural control of the expression of aggressive behaviour in a wide variety of animal species, more than any other neurochemical system [3–6]. However, it is not clear if the 5-HT system generally dampens aggression or if the neurotransmitter plays opposite roles in adaptive and escalated forms of aggressive behaviour [3,7–9].

Among several other functional components of the 5-HT system, the 5-HT transporter (5-HTT) has been linked to aggressive behaviour [10–12]. The 5-HTT is a key regulator for central serotonergic activity. That is, it functionally inactivates 5-HT molecules by active transport from the extracellular space to the 5-HT terminals and thus determines the magnitude and duration of postsynaptic receptor-mediated signalling [13,14]. The transcriptional activity of the human 5-HTT gene is modulated by a length polymorphism in the transcriptional control region (5-HTTLPR) with the short variant (*s*) being associated with a lower transcriptional activity and therefore a reduced amount of 5-HTT protein compared to the long (*l*) gene variant [10]. Low 5-HTT function induced by the *s* allele of the 5-HTTLPR has been associated with anxiety- and depression-related personality traits as well as with neuropsychiatric diseases [15–17]. Individuals carrying one or two copies of the *s* allele are more likely to develop major depression following stressful life events [18–21] but see also [22]. Thus, the 5-HTTLPR seems to moderate the response to environmental influences, potentially facilitating the development of mental illness [11].

In modelling the neurobiological implications of the 5-HTTLPR, mice with a partial or complete inactivation of 5-HTT function are an indispensable tool for measuring effects of 5-HTT depletion [23,24]. The loss of functional 5-HTT results in more than 50 different phenotypic changes, such as increased anxiety [25–29] and reduced aggressive behaviour [30,31]. Interestingly, some of these alterations

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can be shaped by environmental influences such as an adverse early environment or negative life experiences during adulthood, probably modelling the 5-HTTLPR-by-stress risk factor for behavioural pathology reported in humans [28,29,32–34].

So far, gene × environment interaction studies in the 5-HTT knockout mouse model have mainly focused on anxiety and depression-related behaviours, while the aggressive behaviour of the mouse model has been largely neglected in this context. Nevertheless, aggressive behaviour should be of special interest, since several disorders, including depression, personality disorders and drug abuse, which are associated with 5-HTT gene variants in humans, can also manifest in inappropriate aggression [35]. Moreover, there seems to be a systematic relationship between offensive aggression in laboratory rodents and angry aggression in humans. Although cognitive representations of emotions and motives associated with angry aggression in humans are more elaborate and differentiated, there are a number of detailed correspondences between human anger/aggression and conditions that produce offensive aggression in laboratory rodents, especially regarding its antecedents, its response characteristics, and its outcomes [36].

The majority of stressful stimuli involved in human psychopathologies are of a social nature. Indeed, social stressors probably constitute the most frequent and persisting sources of stress [37]. Furthermore, social status appears strongly associated with the number of stressful events experienced [38]. Animal models of social stress were found to have both face and predictive validity in modelling the implications of stressful social stimuli in psychopathologies in humans, with most involving the establishment of clear relationships of dominance/subordination in agonistic encounters [37,39]. While social defeat is one of the most stressful social stimuli in most species, dominant animals also can experience considerable amounts of stress [37,40]. Winning as well as losing agonistic encounters can strongly, and differentially, affect both the physiology and subsequent behaviour of the participants, with winners being generally more active and aggressive than losers in future fights [35,41]. Therefore, experience in prior aggressive contests has also emerged as an important environmental modulator of aggressive behaviour in animals [35,41–43].

Against the background that the 5-HTT genotype can be significantly involved in the processing of stressful life experiences, the aim of the present study was to investigate whether the 5-HTT genotype is also involved in the modulation of aggressive behaviour by previous fighting experience. For this purpose, wildtype mice (5-HTT +/+) as well as heterozygous (5-HTT +/−) and homozygous (5-HTT −/−) 5-HTT knockout mice were given the social experience of either being a winner, or being a loser, and were afterwards analysed for their offensive aggressive behaviour.

In line with the literature [41,43], we hypothesised that animals with repeated experiences as winner would show increased aggression scores compared with animals with repeated experiences as loser (hypothesis 1). Based on the findings of reduced aggression in 5-HTT −/− mice [30,31], we further expected a main effect of genotype with lowest levels of offensive aggressive behaviour in 5-HTT −/− mice (hypothesis 2). Moreover, we hypothesised that the 5-HTT genotype would interact with social experience in modulating aggressive behaviour (hypothesis 3), because 5-HTT depletion in humans as well as laboratory mice has been shown to interact significantly with environmental factors in shaping the behavioural profile [18,28,32]. In mice, this includes also fighting experience, which can result in genotype-dependent differences in the anxiety- and explorative behaviour of the 5-HTT knockout mouse model, suggesting a similar relationship for aggressive behaviour [29]. Since changes in aggressive behaviour by previous fighting experience are often related to increases or decreases in circulating steroid hormone levels [40,41,44–46], we also monitored adrenocortical activity as well as testosterone titres. Firstly, we expected adrenocortical activity to be differentially influenced by winning and losing and these influences also to have an impact on the stress response in future fights (hypothesis 4). Since a previous study indicated a 5-HTT genotype-dependent modulation of the adrenocortical stress response to agonistic experiences [29], we also expected the changes in corticosterone levels to be modulated by genotype with the effects of winning and losing being most pronounced in mice with impaired 5-HTT function (hypothesis 5). Finally, we expected testosterone titres to be influenced by 5-HTT genotype after different social experiences (hypothesis 6).

2. Methods

2.1. Animals and housing conditions

5-HTT +/+, 5-HTT +/− and 5-HTT −/− mice [47], backcrossed into a C57BL/6J genetic background for >10 generations, originated from the internal stock of the Department of Behavioural Biology at the University of Münster, Germany. The original breeding stock was obtained from the Department of Psychiatry at the University of Würzburg, Germany. Breeding pairs each consisted of a male and a female 5-HTT +/− mouse and resulting offspring were thus 5-HTT +/+, 5-HTT +/−, and 5-HTT −/− mice. Genotyping was accomplished using ear tissue to extract genomic DNA, amplified by PCR. Genotypes were identified by agarose gel electrophoresis of DNA-fragments of either 225 bp (5-HTT +/+), 272 bp (5-HTT −/−) or both (5-HTT +/−).

In total, 111 male mice (37 5-HTT +/+, 38 5-HTT +/−, and 36 5-HTT −/−) were used for the behavioural investigations (deviations from these sample sizes were for technical reasons). Pups were weaned on postnatal day (PND) 21 ± 1 and maintained in sibling groups of two to five animals of the same sex. Only in rare cases were age-matched males from different litters housed together. From PND 61 ± 3 of age all mice were housed individually to provoke isolation-induced aggressiveness necessary for the following resident intruder paradigm (RIP) and to exclude a possible influence of social interactions with conspecifics on the offensive aggressive behaviour.

To generate winning experiences and to assess aggressive behaviour (see Section 2.3), 20 males of the C3H strain (obtained from Harlan Winkelmann GmbH, Borchen, Germany) served as subordinate opponents, since C3H mice are characterised by a low level of intermale aggression [48]. To further minimise the probability of increased aggression resulting from prolonged isolation, C3H males were housed in groups of three.

To generate losing experiences 12 males of the NMRI strain served as opponents (obtained from Harlan Winkelmann GmbH, Borchen, Germany), since this strain is characterised by a high level of intermale aggression [49]. To further stimulate aggressiveness and therefore allow for a high success rate when generating experiences as a loser, NMRI males were housed individually during the whole experimental phase. At the time of the experiments C3H males, as well as NMRI males, were at least 60 days of age.

All experimental mice, as well as the C3H and NMRI opponents, were housed in standard Macrolon cages type III (38 cm × 22 cm × 15 cm) with a paper towel and sawdust as bedding material (Allspan, Höveler GmbH & Co. KG, Langenfeld, Germany). To guarantee that experimental males and NMRI males defended their cages as their territory against an intruding opponent, cages were not cleaned at least for four days prior to testing. The housing room was maintained at a 12 h light/dark cycle (lights on at 08:00 a.m.) and a temperature of 22 ± 3 °C. Commercial mouse diet (Altromin 1324, Altromin GmbH, Lage, Germany) and water were available *ad libitum*. Tests were conducted between 08:00 a.m. and 10:00 a.m..

The present 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 Münster (reference number: 8.87–50.10.46.08.151).

2.2. Experimental design

The experiment consisted of two parts in which the agonistic behaviour of the mice was assessed after different social experiences (SE) that is having been a winner or a loser in previous fights (Fig. 1). As a first step, mice of all three genotypes were given a total of three defined experiences as either a winner or a loser using the RIP on PND 76 ± 3 (SE1), 77 ± 3 (SE2), and 78 ± 3 (SE3). Afterwards, winners and losers experienced three additional RIPs on PND 82 ± 3 (RIP1), 84 ± 3 (RIP2), and 86 ± 3 (RIP3) against a C3H male in their home cages and were observed for offensive aggressive, social interest and defensive behaviour.

To investigate stress reactivity non-invasively (see Section 2.4.1), faecal samples were collected on the day before SE1, after SE1 and after RIP1.

For the determination of plasma corticosterone and testosterone levels, mice were decapitated immediately after RIP3 and trunk blood was collected (see Section 2.4.2).

2.3. Behavioural investigations

The RIP is based upon isolation-induced aggressiveness in male mice and elicits agonistic behaviour between a singly housed resident male and an intruding subordinate opponent male [50,51]. It was used for two different purposes in this study: At first the RIP was applied to generate repeated experiences as a winner or loser for the mice, as established by Jansen et al. [29]. Afterwards, it was carried out to assess the influence of these experiences on future agonistic behaviour of the mice.

To create experience as a winner, mice were confronted three times as residents in their home cage with an intruding docile C3H male. To create experience as a loser, mice were placed three times as intruders into the home cage of an aggressive NMRI male. Animals of an additional control group stayed naive at the same time. To ensure an equal handling like winner and loser animals, control animals were also transferred to the testing room, but instead of confronting them with a C3H or NMRI male, they were only taken out of their cage for approximately three seconds held by their tail and afterwards returned to their home cage.

In order to assess the agonistic behaviour afterwards, winners, losers, and control animals were confronted three times in their home cage with an intruding C3H male. For behavioural investigations, only the first of these three RIPs was analysed, since it reflects best the direct influence of the three preceding winning/losing experiences on the agonistic behaviour. Moreover, recent results of our lab point to stable effects of 5-HTT genotype and environmental influences over three consecutive confrontations [52].

To avoid a confounding effect of familiarity, each C3H/NMRI male and each experimental animal were matched only once during SE1–3 and RIP1–3. Thus, winners and losers were confronted with six different opponents and control animals with three different opponents during the whole experimental period. C3H and NMRI males were used pseudo-randomly.

The test lasted 10 min and was observed via video camera and an attached monitor. To prevent mice from injury, confrontation was stopped before expiration of the testing time when fighting became too escalated, defined as one of the opponents showing persistent defensive postures, while the other showed continuous offensive aggression. During RIP3, a partition grid was attached in the resident's cage after 5 min to separate the opponents for the remaining 5 min. Thus, a comparable testing time of 10 min was possible for all animals before blood samples were taken, without endangering any animal's health.

The classification of winners and losers was performed according to Jansen et al. [29]. An animal was categorised as a winner, if it showed at least five behavioural patterns of winners (see Table 1) each time in SE1, SE2, and SE3. In addition, these patterns had to occur twice as often as 'loser' behavioural patterns. Accordingly, an animal was categorised as a loser, if it showed at least five behaviour patterns of a loser each time in SE1, SE2, and SE3 and these patterns had to occur twice as often as 'winner' behavioural patterns. Animals which did not meet the criteria for winning and losing were excluded from further experiments and final analysis. When mice of the three 5-HTT genotypes were confronted with an intruding C3H male, they were winners in 38 out of 46 cases (see Table 2). When mice were placed as intruders into the home cage of an NMRI male, they emerged as losers in 37 out of 45 cases. It cannot be fully excluded that by sorting out animals that did not met the criteria, successfully trained winners were probably intrinsically more aggressive than successfully trained losers. However, 82.6% of the winner trainings and 82.2% of the loser trainings were successful and numbers of animals that had to be excluded did not differ between the genotypes (Fisher's Exact Test for Count Data, winners: $p = 0.394$, losers: $p = 0.074$), thus we do not consider this as a significant factor. Please note: When winner and loser experiences were pooled for analysis, there was a significant association between genotype and training outcome ($p = 0.048$).

In order to assess social interest, offensive aggressive and defensive behaviour, a total of seven behaviour patterns were recorded during RIP1 for mice of all three genotypes, using the software Observer XT 8.0 (Noldus Information Technology BV, Wageningen, NL). A definition of each behavioural parameter is given in Table 1. To characterise the aggressiveness of the C3H opponents, two behaviour patterns were recorded (see Table 1).

All behavioural investigations were carried out by an experienced observer (V.K.), who remained blind to genotype. Residents and intruders could be easily distinguished by coat colour. Data were collected using *focal animal sampling* and *continuous recording*.

2.4. Endocrinological investigations

2.4.1. Corticosterone metabolites (CM)

On the day before SE1, after SE1 and after RIP1, faecal samples voided between 04:00 p.m. and 08:00 p.m. (that is 8–12 h after the

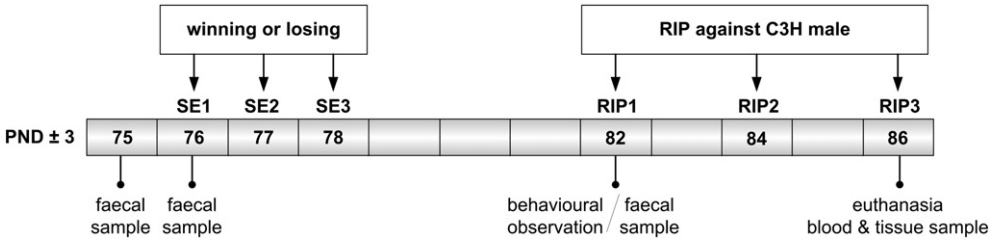

Fig. 1. Experimental design. 5-HTT +/+, 5-HTT +/−, and 5-HTT −/− mice experienced either winning or losing an aggressive encounter on three consecutive days (SE1, SE2, SE3). Afterwards three resident intruder paradigms with C3H males were performed (RIP1, RIP2, RIP3), with the first one being analysed for agonistic behaviour. Faecal samples for the determination of corticosterone metabolites were collected as well as blood and tissue samples were taken; PND = postnatal day.

Table 1
Description of behaviour patterns.

Behaviour	Definition
Social interest behaviour	
Approaching	Direct movement towards another mouse at a walking or running pace until the distance between both mice is at most one body length.
Offensive aggressive behaviour/winner behaviour patterns	
Following	A mouse runs after another mouse, while the head of the following 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 for at least three seconds the behaviour starts again.
Chasing	*Following* subsequent to an agonistic interaction (*attack*, *bite attack* or *escalated fighting*).
Attack[1]	A mouse contacts the body of another mouse with its mouth, making that mouse react with winced movements of either single extremities, the tail or the whole body. Attacks are single countable events of low intensity. (*Latency to attack*: Time that elapses until an attack is performed for the first time by the focal animal. If no attack occurred, the latency was set to the maximal testing time of 10 min.)
Bite Attack[1]	A series of attacks with rushing and leaping at another mouse. As the behaviour is of higher intensity than an attack itself, single attacks are not countable anymore.
Escalated fighting	Physical struggle between two mice which is initiated by an *attack* and usually involves further attacks, 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 broke apart.)
Defensive behaviour/Loser behaviour patterns	
Avoiding*	Directed movement away from another mouse at a walking or running pace.
Flee*	*Avoiding* subsequent to an agonistic interaction (*attack*, *bite attack* or *escalated fighting*).
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.

Note: For description of behaviour patterns see also [29,53,54]. The *latency to attack* is given as duration in seconds. For all other behaviour patterns frequencies were recorded. For data analysis frequencies of *following*, *chasing*, *attack*, *bite attack* and *escalated fighting* were added to form the *sum of offensive aggression*. *: These behaviour patterns were only assessed for the evaluation of winning and losing during the generation of social experiences. [1]: These behaviour patterns were also assessed for the C3H opponents.

RIP) were collected and frozen at −20 °C until assayed for CM (corticosterone is the major glucocorticoid in mice [55]). The time frame was chosen according to Touma et al. [56] who found a peak of CM in the faeces 8 to 12 h after the exposure to a stressor. During sample collection, mice were placed in a standard Macrolon cage type II (27 cm × 22 cm × 15 cm) provided with three fresh paper towels. This procedure resembled the routinely performed transfer to clean cages for animal maintenance and was therefore not considered to be a stressor that could possibly corrupt subsequent samples.

The collected faecal samples were analysed for immunoreactive 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 essay are described in Touma et al. [56,57]. Before EIA analysis, faecal 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 5.7% and 11.1%, respectively. Samples of less than 0.02 g of faeces were excluded from data analysis.

2.4.2. Corticosterone/testosterone

Immediately after RIP3, mice were anaesthetized with Isofluran as inhalation anaesthetic (Forene, Abbott GmbH, Wiesbaden, Germany) and decapitated. Trunk blood was collected in heparinised capillaries within a maximum of 3 min after moving the cage to avoid an impact of handling and blood sampling on the parameters investigated [55,58,59]. Blood plasma was separated from cellular components by centrifugation (5 min at 13,000 rpm) and frozen at −20 °C until assayed.

Table 2
Evaluation of winning and losing.

Social experience				
Genotype	Winning		Losing	
	Criterion met	Criterion not met	Criterion met	Criterion not met
5-HTT +/+	13	5	12	6
5-HTT +/−	13	1	13	2
5-HTT −/−	12	2	12	0

Blood samples were analysed for plasma corticosterone and testosterone concentrations by use of an established DEMEDITC Enzyme Immunoassay Kit (EIA, corticosterone: DE4164, testosterone: DE1559, Demeditec Diagnostics GmbH, Kiel, Germany). All standards, samples and controls were run in duplicate concurrently. For measurement of plasma corticosterone, samples were diluted 1:5, while controls were diluted 1:50. The intra- and inter-assay coefficients of variation were 3.3% and 6.0%, respectively. Results were only accepted if within the range of 1.44–69.17 ng/ml, which applied to all samples. For measurement of plasma testosterone samples and controls were used undiluted. The intra- and inter-assay coefficients of variation were 5.7% and 7.2%, respectively. Results were only accepted if within the range of 0.2–16 ng/ml, which applied to all but two samples (1 5-HTT +/− loser, 1 5-HTT +/+ winner).

2.5. Statistical analysis

All data sets were checked for normal distribution by descriptive analysis of the histogram as well as by applying the Kolmogorov–Smirnov test.

To evaluate differences between more than two independent samples normally distributed data were analysed using two-way analysis of variance (ANOVA). Post hoc multiple comparisons were conducted using the Scheffé test for equal variances (*approaching*, *latency to bite*) and the Tamhane's T2 test where the homogeneity of variances assumption was violated (*sum of offensive aggression*). Statistical significance was set at $p<0.05$.

For CM concentrations more than two dependent samples were compared using a Repeated Measures ANOVA. Since the sphericity assumption was not met, Huynh–Feldt correction was applied. Pairwise comparisons between independent samples were performed using independent samples *t*-tests (two-tailed). Dependent samples were compared by means of paired samples *t*-tests (two-tailed). Results for CM concentrations were considered significant at an alpha level of 0.05 with Bonferroni correction [60] for 18 tests, which corresponds to a p-value of $0.05/18 = 0.003$.

All tests were calculated using the software package PASW Statistics 18 (Release 18.0.0, SPSS Inc., 2009).

3. Results

3.1. Behaviour

The repeated RIPs to assess the agonistic behaviour typically began with the opponents *approaching* each other and demonstrating their social interest, prior to the onset of the various patterns of offensive aggression. Therefore, frequencies of *approaching* are presented at first, followed by the offensive aggressive behaviour (*latency to attack* and the *sum of offensive aggression)*. Thereafter, the defensive behaviour of the mice is given. Finally, the docile C3H opponents are characterised.

3.1.1. Social interest

Concerning the frequency of *approaching* (Fig. 2), there was a significant main effect of social experience ($F(2, 102) = 39.288$, $p<0.001$), that is having been a winner or a loser in previous fights. While winners showed more *approaching* than naive control animals ($p<0.001$), losers showed less *approaching* than controls ($p = 0.002$). Moreover, winners and losers differed significantly from each other, with winners *approaching* their opponent more frequently than losers ($p<0.001$).

There was also a significant main effect of the 5-HTT genotype ($F(2, 102) = 3.245$, $p = 0.043$) on the frequency of *approaching* with 5-HTT −/− mice showing less *approaching* than 5-HTT +/− mice ($p = 0.048$).

There was no significant interaction between social experience and genotype ($F(4, 102) = 0.857$, $p = 0.492$).

3.1.2. Offensive aggressive behaviour

Concerning the *latency to attack* (Fig. 3), the ANOVA revealed a main effect of social experience ($F(2,102) = 9.273$, $p<0.001$). Post hoc analysis showed that winners attacked their opponent significantly sooner than losers ($p<0.001$) as well as naive control animals ($p = 0.022$).

There was no significant main effect of genotype ($F(2, 102) = 0.363$, $p = 0.697$), as well as no significant social experience × genotype interaction ($F(4, 102) = 1.166$, $p = 0.330$).

For a comprehensive analysis, the frequencies of the behaviour patterns *following*, *chasing*, *attack*, *bite attack* and *escalated fighting* were added to form the *sum of offensive aggression* (Fig. 4). The ANOVA

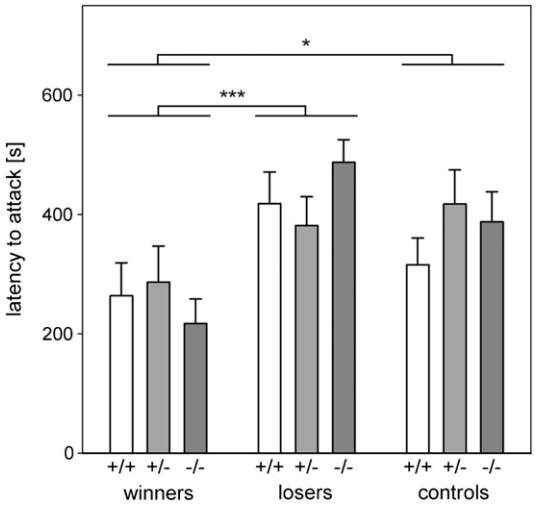

Fig. 3. *Latency to attack* for 5-HTT +/+, 5-HTT +/−, and 5-HTT −/− mice. Data are shown as mean + SEM. Statistics: ANOVA; post hoc testing: Scheffé test: ***: $p \le 0.001$, *: $p \le 0.05$. Sample sizes: 38 winners (13 +/+, 13 +/−, 12 −/− mice), 37 losers (12 +/+, 13 +/−, 12 −/− mice), and 36 controls (12 +/+, 12 +/−, 12 −/− mice). There was a significant main effect of social experience ($p<0.001$).

detected a significant main effect of social experience on the *sum of offensive aggression* ($F(2,102) = 9.464$, $p<0.001$) with winners showing significantly more offensive aggressive behaviour than losers ($p<0.001$). Higher frequencies of offensive aggression in winners than in controls were indicated by an insignificant statistical trend ($p = 0.062$).

The ANOVA revealed neither an effect of genotype ($F(2, 102) = 1.024$, $p = 0.363$), nor a significant interaction between social experience and genotype ($F(4, 102) = 0.448$, $p = 0.774$).

3.1.3. Defensive behaviour

As intended by the experimental design, mice of all three 5-HTT genotypes showed only very low rates of defensive behaviour. Therefore, the median frequency of the behaviour pattern *defensive upright posture* was 0.00/min in all nine experimental groups. Thus, statistical analysis was not appropriate.

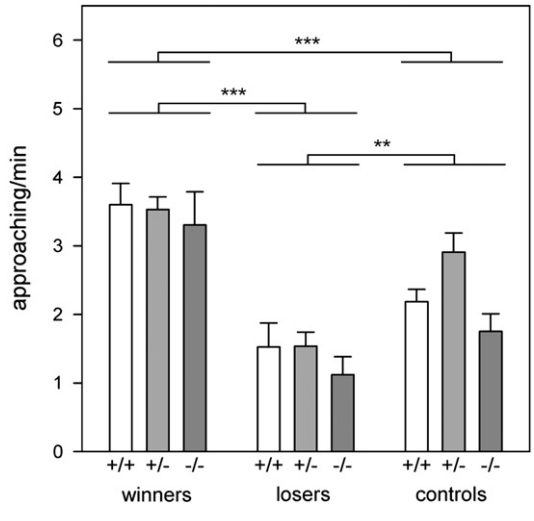

Fig. 2. *Social interest* (*approaching*) for 5-HTT +/+, 5-HTT +/−, and 5-HTT −/− mice. Data are shown as mean + SEM. Statistics: ANOVA; post hoc testing: Scheffé test: ***: $p \le 0.001$, **: $p \le 0.01$. Sample sizes: 38 winners (13 +/+, 13 +/−, 12 −/− mice), 37 losers (12 +/+, 13 +/−, 12 −/− mice), and 36 controls (12 +/+, 12 +/−, 12 −/− mice). There was a significant main effect of social experience ($p<0.001$). Not shown: main effect of genotype ($p = 0.043$) with 5-HTT −/− mice showing less *approaching* than 5-HTT +/− mice ($p = 0.048$).

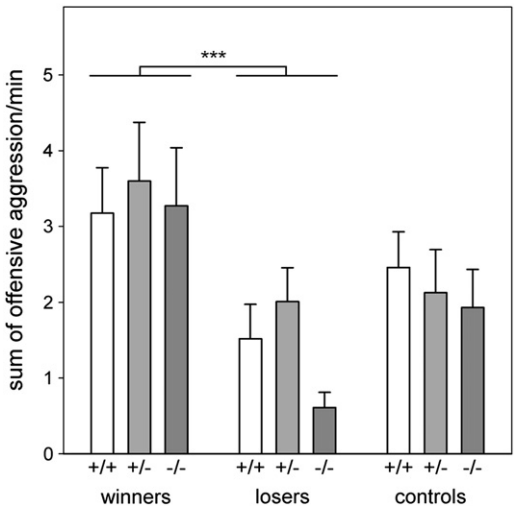

Fig. 4. *Sum of offensive aggression (*added frequencies of *following*, *chasing*, *attack*, *bite attack* and *escalated fighting)* for 5-HTT +/+, 5-HTT +/−, and 5-HTT −/− mice. Data are shown as mean + SEM. Statistics: ANOVA; post hoc testing: Tamhane's T2 test: ***: $p \le 0.001$. Sample sizes: 38 winners (13 +/+, 13 +/−, 12 −/− mice), 37 losers (12 +/+, 13 +/−, 12 −/− mice), 36 controls (12 +/+, 12 +/−, 12 −/− mice). There was a significant main effect of social experience ($p<0.001$).

3.1.4. C3H males

C3H males were chosen as intruders because of their low rate of intermale aggression. They were expected to function as subordinate opponents, provoking offensive aggression in the focal animals instead of expressing aggressive behaviour themselves. Accordingly, *attacks* and *bite attacks* initiated by C3H males occurred only sporadically confirming the aim of the experimental design. For both behaviour patterns the median frequency in all groups was 0.00/min, so that a statistical analysis was not appropriate.

3.2. Endocrinological investigation

3.2.1. Corticosterone metabolites (CM)

The Repeated Measures ANOVA of faecal CM concentrations (Fig. 5) of samples collected on the day before SE1 (A), after SE1 (B), and after RIP1 (C) resulted in a significant within-subject main effect of the sampling point on the CM concentrations (Huynh–Feldt adjusted $F(1.953, 123.054) = 7.620$, $p = 0.001$). That is, mean CM concentrations changed over the course of the experiment. Moreover, there was an interaction effect of sampling point and social experience (Huynh–Feldt adjusted $F(3.906, 123.054) = 3.347$, $p = 0.013$), which indicated that the pattern of changes in CM concentrations differed between winners, losers and controls.

Pairwise comparisons revealed a significant increase in CM concentrations at sampling point B compared to baseline values at sampling point A for losers ($t = -3.691$, $p = 0.001$). Further post hoc comparisons for each social experience across time or within each time point between the social experiences did not bring about statistically significant differences apart from significance levels and trend levels that again did not withstand Bonferroni correction (winners: A vs C: $t = -2.911$, $p = 0.008$; losers: B vs C: $t = 1.788$, $p = 0.088$; controls: A vs C: $t = -2.552$, $p = 0.017$, B vs C: $t = -1.921$, $p = 0.066$; B: winners vs. losers: $t = -2.239$, $p = 0.031$).

3.2.2. Plasma corticosterone (CORT)

The analysis of the CORT concentrations (Fig. 6 A) immediately after the last confrontation revealed neither an effect of social experience ($F(2, 100) = 0.544$, $p = 0.582$), nor of the 5-HTT genotype ($F(2,100) = 0.866$, $p = 0.424$). There also was no interaction effect of social experience and genotype ($F(4, 100) = 0.544$, $p = 0.704$).

3.2.3. Plasma testosterone (TEST)

The ANOVA revealed neither a significant main effect of social experience ($F(2, 100) = 1.361$, $p = 0.261$), nor of genotype ($F(2,100) = 0.132$, $p = 0.877$) on the TEST concentrations (Fig. 6 B) immediately after the last confrontation. Also no interaction effect of these variables was detectable ($F(4,100) = 0.559$, $p = 0.693$).

4. Discussion

The overall aim of the present study was to investigate how 5-HTT genotype and the previous social experience of winning and losing agonistic encounters shape the display of offensive aggressive behaviour. For this purpose, mice varying in 5-HTT genotype were given three repeated experiences as either winner or loser, and were later evaluated for their behaviour during an RIP. To elicit offensive aggressive behaviour in the focal animals, and to prevent their behaviour being dominated by the intruder, a docile opponent was chosen. Indeed, the C3H mice displayed aggressive behaviour only in isolated cases, regardless of the 5-HTT genotype of their opponent. Accordingly, 5-HTT +/+, 5-HTT +/−, and 5-HTT −/− mice displayed defensive behaviour very rarely. Using this experimental design, we found that winning and losing shape the offensive aggressive behaviour in the same direction in all animals without genotype-dependent differences.

4.1. Effects of social experience

4.1.1. Winners showed more engagement with the intruder, attacked him faster and exhibited overall higher aggression scores than losers

The repeated experience of being a winner or being a loser exerted a significant influence on the social interest, as well as on the offensive aggressive behaviour of the mice. Three repeated social victories against a subordinate C3H opponent tended to increase aggressive behaviour, whereas three repeated social defeats against a superior NMRI male tended to decrease the aggressive behaviour of the mice. This results in significant differences between winner and loser animals in the observed behaviours, which confirms our first hypothesis and is in good agreement with observations about the modulation of aggressive behaviour by fighting experience in a wide range of animals (as a review see: [61]).

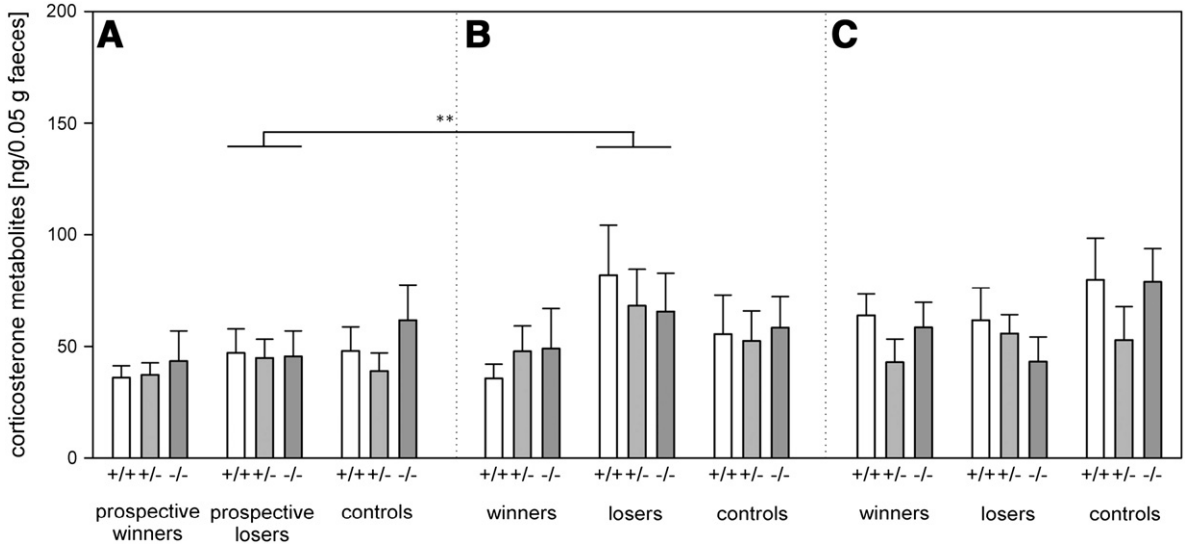

Fig. 5. Concentration of corticosterone metabolites [ng/0.05 g faeces] in the faeces of 5-HTT +/+, 5-HTT +/−, and 5-HTT −/− mice before SE1 (A), after SE1 (B), and after RIP1 (C). Data are shown as mean + SEM. Statistics: Repeated Measures ANOVA; post hoc testing: paired-samples *t*-test and independent-samples *t*-test with sequential Bonferroni correction for multiple comparisons ($\alpha = 0.05/18 = 0.003$): **: $p \leq 0.01$. Sample sizes: 23 winners (8 +/+, 9 +/−, 6 −/− mice), 23 losers (7 +/+, 10 +/−, 6 −/− mice), 26 controls (9 +/+, 9 +/−, 8 −/−mice). There was a significant main effect of sampling point ($p = 0.001$) and a significant sampling point × social experience interaction ($p = 0.013$).

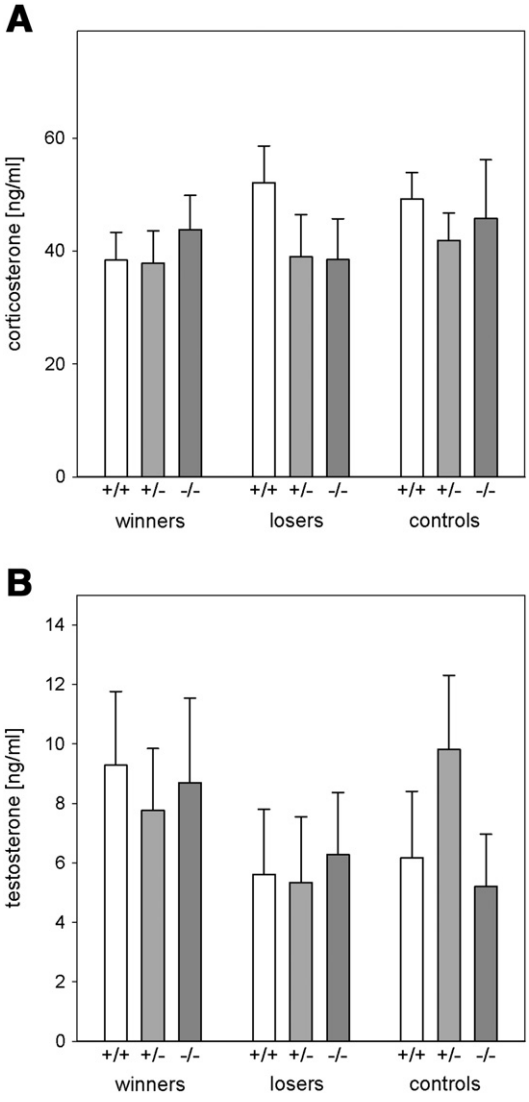

Fig. 6. Concentration of (A) plasma corticosterone and (B) plasma testosterone [ng/ml] of 5-HTT +/+, 5-HTT +/−, and 5-HTT −/− mice. Data are shown as mean + SEM. Sample sizes for corticosterone: 37 winners (12 +/+, 13 +/−, 12 −/− mice), 37 losers (12 +/+, 13 +/−, 12 −/− mice), and 35 controls (12 +/+, 12 +/−, 11 −/− mice). Sample sizes for testosterone: 37 winners (12 +/+, 13 +/−, 12 −/− mice), 36 losers (12 +/+, 12 +/−, 12 −/− mice) and 36 controls (12 +/+, 12 +/−, 12 −/− mice).

In general, contest behaviour depends upon the level of information a contestant has about the costs and benefits of possible behavioural options in the contest. Prior fighting experience is hypothesised to influence an animal's assessment of its own fighting ability and estimated costs in later contests [41]. Animals with winning experience may assess their own fighting ability as good and expect low costs in future fights. Due to the perceived reduction in the ratio of costs to benefits, they are generally more willing to engage in a contest, exhibit an increased activity level and a readiness to adopt more costly behaviour. By contrast, animals with losing experience may assess their own fighting ability as poor and expect high costs. Due to an increased costs/benefits ratio they act more passively and cautiously and often exhibit a decreased willingness to engage in a contest [41,62]. Since the winners in the present study showed more engagement with the intruder, attacked him faster and exhibited overall higher aggression scores than losers, mice acted in agreement with this schema.

Interestingly, winner and loser effects were most distinct during the early phase of the confrontation, in which social investigation by *approaching* the opponent took place. In this phase winners and losers differed not only significant from each other but also from control animals. By contrast, for the *sum of offensive aggressive behaviour*, representing a later stage of the contest, only the direct comparison of winners and losers resulted in significant differences, while neither winners nor losers individually differed from the controls. This might result from prior experience influencing how an animal assesses its perceived fighting ability, instead of altering its actual fighting ability [63]. As soon as animals had gathered information about the intruding C3H male by *approaching* him and were able to directly compare their actual fighting ability during the physical interaction, they probably adjusted their behaviour to the more recent and reliable information, while information from past experiences diminished to some extent [41,63].

4.2. Effects of genotype and genotype × social experience interaction

4.2.1. 5-HTT genotype did not influence the aggressive behaviour towards a docile C3H opponent

Against all expectations and in contrast to our second hypothesis, there were no genotype-dependent differences for the *latency to attack*, or the *sum of offensive aggression* within the groups of different social experiences. This result contradicts findings of Holmes et al. [30] who found longer attack latencies and reduced aggression in 5-HTT −/− mice. However, Holmes et al. used DBA/2J male mice as intruders, a strain which is characterised by higher aggressiveness than C3H mice [48]. Since genetic effects on aggressive behaviour critically depend upon the type of intruder used in the RIP [64–67], the opposing results might be traced back to the differences between C3H and DBA/2J mice. Also in a previous study by our lab, aggressive behaviour, and particularly the ability to attain dominance, strongly depended on the opponents and the context in which aggression was expressed: In a direct confrontation with 5-HTT +/+ mice, 5-HTT −/− mice were clearly inferior to 5-HTT +/+ mice. But when housed in established social groups of males of the same genotype, 5-HTT −/− mice were just as able to establish and maintain dominance relationships, and to show significant amounts of aggressive behaviour, even if overall aggression was lower than in groups of 5-HTT +/+ mice [31]. Thus, the loss of 5-HTT function does not necessarily bring about a peaceful behavioural profile or impairs the ability to display normal patterns of offensive aggression [31]. In the context of the present study, the 5-HTT genotype did not impair the aggressive behaviour towards a docile opponent in the home cage and all mice showed comparable amounts of offensive aggressive behaviour when defending their territory against a C3H male.

Nevertheless, genotype influenced the social interest behaviour of the animals, since 5-HTT −/− mice approached their opponents less frequently than 5-HTT +/− mice. Although this difference was surprisingly not found in comparison with 5-HTT +/+ mice, it generally stands in line with studies reporting of reduced social interactions in 5-HTT −/− mice [27,68] but see [30]. However, reduced social interactions in 5-HTT −/− mice are less likely to result from an intrinsic deficit in social interest, but rather they might be associated with the increased anxiety-like behaviour and the hypoactive phenotype of the mouse model [27]. While reduced social interaction is mainly observed under conditions where mice are confronted with unfamiliar conspecifics in an artificial test situation, 5-HTT −/− mice show no deficit in social behaviour when living in stable social groups with well-established dominance hierarchies. Under these conditions they show equal amounts of social exploration and even more socio-positive behaviours than 5-HTT +/+ mice [31].

4.2.2. 5-HTT genotype did not interact with social experience in shaping aggressive behaviour

There is increasing evidence that variations in behavioural profiles can result from specific gene × environment interactions, that is, some genotypes are more responsive to their environment than others [69]. In terms of the 5-HTT, findings in both humans and laboratory mice

underscore this view, with effects of 5-HTT depletion on anxiety and depression-related behaviours being significantly modulated by, and depending, on environmental influences. For example in humans, there is a number of studies reporting of an increased risk of depressive symptoms and diagnosable depression in carriers of the 5-HTTLPR short allele, but only in interaction with stressful life events [18,21,70,71] but see also [22]. With regard to mice, 5-HTT +/− mice show a more pronounced increase of anxiety-related behaviour than 5-HTT +/+ mice after experiencing low maternal care [32] and the simulation of a 'dangerous environment' during pregnancy and lactation increases anxiety-related behaviour and reduces exploratory locomotion most markedly in 5-HTT −/− offspring [28]. Relating to social experiences during adulthood, both winning and losing have been shown to elevate anxiety-like behaviour and decrease locomotion in mice of all three 5-HTT genotypes, with the effects of losing being most pronounced in 5-HTT −/− mice [29].

Accordingly, we also expected the expression of aggressive behaviour to be influenced by gene × environment interactions resulting in differential effects of repeated winning and losing in the three 5-HTT genotypes. However, the ANOVA did not detect any significant genotype × social experience interaction and therefore we have to reject our third hypothesis.

It seems probable that the lack of an interaction effect is related to some characteristics of the context in which the aggressive behaviour was assessed. For 5-HTT +/− mice in particular it is known that behavioural changes due to genotype often manifest only under challenging environmental conditions [26]. Since we did not find any genotype-dependent changes in aggressive behaviour – although those have been described in different experimental contexts – our experimental design might not have been challenging enough to induce these differences, and the same might also apply to possible genotype × social experience interactions. It might be speculated that testing the mice in the home cage of the opponent would have revealed a more varied picture. Indeed, in a recent study [52] assessing the offensive aggressive behaviour in different environmental situations (own territory, opponent's territory, neutral area) resulted in a significant interaction of the 5-HTT genotype with the environmental situation in which the contest took place. Furthermore, a more challenging opponent (e.g. NMRI male) might help to further investigate the interaction of the 5-HTT genotype with fighting experience in shaping future aggressive behaviour.

However, under the present experimental conditions it must be concluded that mice with reduced or abolished 5-HTT function were equally able to integrate the information from previous fights and obviously assessed their own fighting ability and estimated costs of later contests in the same way like wildtype animals.

4.3. Endocrinological parameters

Adrenocortical activity was monitored non-invasively by measuring faecal corticosterone metabolite (CM) concentrations with a recently developed enzyme immunoassay [56,57]. By applying this technique we showed that mice of the three genotypes did not differ in baseline levels of this measure of stress hormone activity before undergoing the social experiences, which confirms previous results of our lab [29]. Together with previous results of unaltered baseline levels of plasma corticosterone [72], this indicates that the 5-HTT genotype does not affect adrenocortical activity under baseline resting conditions (but see also [73,74]).

CM concentrations changed significantly across the three times of faecal sampling in dependency of the social experience, what confirms our fourth hypothesis in which we expected winning and losing to differentially affect adrenocortical activity. Losers showed an increase in CM concentrations after their first losing experience, pointing to a stress response in those animals, while winners exhibited no significant increase in CM concentrations in response to their first winning experience. This stands in line with numerous findings in a variety of species indicating higher levels of stress in losers than in winners [29,40,45,75] and underlines that social defeat is one of the most stressful social stimuli in animals [37].

As for the results of the behavioural investigations, there was again no significant genotype × social experience interaction for CM concentrations. We therefore have to reject our fifth hypothesis of a genotype-dependent hormonal response to the different social experiences. This contrasts with previous results of Jansen et al. [29] who found indications of an increased stress-responsive phenotype in 5-HTT +/− mice in response to winning and losing. However, it should be noted that Jansen et al. tested the mice in several paradigms for anxiety-like and explorative behaviours before faecal samples were collected. Thus, differences in the two studies' results might arise from these additional experimental procedures.

At the end of the experimental course, levels of plasma CORT also were determined to monitor the activity of the HPA axis, and no differences between mice with different genotypes or social experiences emerged. It must be considered that all animals had experienced at least three social victories at that time, since all focal animals dominated the C3H opponents in the RIP. Results of plasma CORT therefore suggest that the 5-HTT genotype is obviously not involved in the modulation of the adrenocortical stress response to repeated fighting against a subordinate opponent, which is in agreement with recent data regarding CORT concentrations in 5-HTT +/− and 5-HTT −/− mice after confrontations in varying environmental situations [29].

Besides the HPA axis, TEST concentrations were monitored, since the gonadal hormone is an important mediator of aggressive behaviour, and changes in TEST concentrations can often be observed following an aggressive encounter [46,75–78]. In the present study, neither an effect of the 5-HTT genotype nor the social experience was revealed and we therefore have to reject hypothesis six of a genotype-dependent modulation of testosterone titres. Due to the timing of blood sampling, this suggests that the 5-HTT genotype is obviously not involved in the modulation of the gonadal hormone response to positive fighting experiences, which is again consistent with the data on TEST concentrations in 5-HTT +/− and 5-HTT −/− mice after confrontations in varying environmental situations [52].

4.4. Conclusions

The results demonstrate that the 5-HTT genotype does not interact with winning or losing experiences in shaping the offensive aggressive behaviour that a male shows against a docile intruder in its own territory. The effects of previous social experiences on future offensive aggressive behaviour were the same in all three genotypes: Three social victories tended to increase, and three social defeats tended to decrease, offensive aggressive behaviour, most probably through the effect of past contest experience on the perceived cost/benefit ratio of future contests. We conclude that social experience, rather than 5-HTT genotype, is crucial in determining the behaviour towards a docile intruder.

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