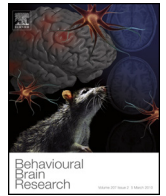




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Research report

The attribution of incentive salience to an appetitive conditioned cue is not affected by knockout of the serotonin transporter in rats



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HIGHLIGHTS

- Attribution of incentive salience to conditioned stimuli & motivation for reward were studied.
- Serotonin transporter knockout rats were compared with wild-type counterparts.
- Knockout did not affect conditioned stimulus salience attribution.
- Knockout animals showed an increased motivation for reward.

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ABSTRACT

Understanding the neurobiological basis underlying individual differences in conditioned stimulus (CS) sensitivity is pertinent, given that excessive conditioned responses to CSs is a key feature of anxiety-related disorders and drug addiction. We have previously shown that behaviour of serotonin transporter knockout (5-HTT^{-/-}) rats-mimicking the common 5-HTT promoter polymorphism in humans-is strongly driven by Pavlovian CSs. To investigate whether the knockout rats attribute greater incentive salience to CSs, we tested the 5-HTT^{-/-} rats and their wild-type counterparts in the sucrose-reinforced sign-versus goal-tracking task. We also assessed whether motivational properties of the unconditioned stimulus (sucrose pellet) are involved in the individual differences under investigation, by testing the animals in a sucrose-reinforced progressive ratio schedule of reinforcement. We found no genotype differences in sign-versus goal-tracking behavior, despite that progressive ratio responding was increased in 5-HTT^{-/-} rats. In conclusion, the high CS sensitivity in 5-HTT^{-/-} rats cannot be explained by enhanced incentive salience attribution to the CS as measured by the sign- versus goal-tracking paradigm. Rather, 5-HTT^{-/-} rats may be more sensitive to the motivational properties of the unconditioned stimulus.

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1. Introduction

Behaviour is strongly driven by Pavlovian conditioned stimuli (CSs). These are stimuli that predict unconditioned stimuli (USs) that have emotionally and/or motivationally relevant aversive

or rewarding properties. CSs may elicit 'automatic' conditioned responses (CRs), which help organisms to respond quickly and properly to environmental stimuli. Whereas CRs are highly adaptive, sometimes they go awry and can trigger pathological conditions like anxiety-related disorders [1] and drug addiction [2,3]. Understanding the neurobiological mechanisms contributing to excessive CRs is essential to further our insight into these neuropsychiatric disorders.

Pavlovian CSs are associated with complex psychological properties. First, they attract attention and thereby trigger approach (in case of a rewarding CS) or avoidance (in case of an aversive CS) behaviour. Secondly, CSs can become 'wanted' in the sense that individuals will work to get them, and they can even reinforce learning a new instrumental response to get them (i.e., they act as conditioned or secondary reinforcers) [4]. This feature can motivate organisms in such a way that they engage into reward-seeking or punishment-avoidance behaviour for a long period of time in the absence of the rewarding or aversive US itself.

Abbreviations: 5-HTT, serotonin transporter; BP, breaking point; CR, conditioned response; CS, conditioned stimulus; US, unconditioned stimulus; FR, fixed ratio; ITI, intertrial interval; PR, progressive ratio.

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Of interest, there are large individual differences in sensitivity to CSs. These individual differences have been extensively studied in the so-called sign-versus goal-tracking task. In this task, some animals approach and interact with the CS before collecting the reward (sign-trackers), whereas others directly approach the reward location without approaching or paying attention to the CS (goal-trackers). Sign-trackers attribute more incentive salience to CSs, making the CSs more effective reinforcers in sign-than in goal-trackers [5].

The neurobiological basis of individual differences in CS sensitivity may be, at least in part, related to serotonin, given that serotonin is implicated in individual differences in CRs [6–14]. For instance, the low activity short (s) allelic variant of the common serotonin transporter promoter polymorphism (5-HTTLPR) in humans, which hypothetically is associated with increased extracellular serotonin levels due to reduce serotonin reuptake, is associated with attentional vigilance and gaze bias toward negatively [15] and positively valenced stimuli [16,17]. In line, we have shown that behaviour of serotonin transporter knockout (5-HTT^{-/-}) rats is strongly driven by Pavlovian CSs [9], and that these animals show impaired extinction of conditioned fear and reward-seeking behaviour [7,18]. These findings prompted us to hypothesize that besides dopamine, serotonin mediates individual differences in sensitivity to CSs as measured in the sign-versus goal-tracking task.

To test this hypothesis we subjected 5-HTT^{-/-} rats and their wild-type controls to the sign-tracking versus goal-tracking task and studied their behaviour during acquisition (revealing individual differences in CRs) and extinction (indicative for new learning). Furthermore, to assess whether motivational properties of the US are involved in the individual differences under investigation, the animals were tested in a sucrose-reinforced progressive ratio schedule of reinforcement. We used 5-HTT^{-/-} rats as animal model, because they are characterized by a constitutive increase in extracellular serotonin levels (Homberg et al., 2007), model the 5-HTTLPR s-allele in humans [19], and because the sign-versus goal-tracking task has been developed for rats [20].

2. Methods

2.1. Animals

All experiments were in compliance with national regulatory principles and approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. All efforts were made to reduce animal suffering and the number of experimental animals. Experimental animals (Slc6a4^{1-Hubr}) [21] were derived from crossing heterozygous 5-HTT knockout (5-HTT^{+/-}) rats that were outcrossed for at least 10 generations with wild-type Wistar rats (Harlan Laboratories, The Netherlands) at the central animal facility of the Radboud University. Male animal facility reared 5-HTT^{-/-} and 5-HTT^{+/+} offspring was used for the experiments described below. The animals were 10 weeks of age at the start of the experiment.

Animal housing took place in a temperature (21 ± 1 °C) and humidity-controlled room (60% relative humidity) with background music and a ventilation system based upon over-pressurization (15-fold). The room was on a 12 hr reversed light–dark cycle, with lights on at 20:00 p.m. (maximum light intensity: 60 lx; minimal light intensity: 0 lx; transition period: 30 min.). All rats were socially housed (2 animals per cage) under conventional housing conditions in Macrolon type III open cages with sawdust bedding and a shelter. Cages were changed every week, always after experimental sessions. Animals had *ad libitum* access to acidified tap water (pH value 2.6–2.9; weekly change of water

bottles) except during the experimental sessions, and were food deprived for 21 h prior to the experimental sessions. After the daily experimental sessions the animals received 2 h of *ad libitum* access to food (V1534, ssniff Spezialdiäten, Soest, Germany). This food restriction schedule resulted in a nominal loss of body weight as well as well-motivated animals in the experimental paradigms. All rats were extensively handled for 5 days before the start of the experiments. Experimental sessions (1 session/day) were performed from Monday to Friday between 9 a.m. and 17 p.m. The experimenter was blind to the genotype of the rats.

2.2. Apparatus

All behavioural tests were conducted in four identical operant conditioning chambers (24.1 × 20.5 × 29.2 cm (*l* × *w* × *h*); MED Associates, St. Albans, VT, USA) equipped with a red house-light located on the upper right corner of the left wall, and a food cup for 45 mg sucrose pellet delivery and two retractable levers on either side of a food cup incorporated in the right wall of the chamber.

2.3. Experimental paradigms

2.3.1. Sign-versus goal-tracking experiment

Eight 5-HTT^{+/+} and eight 5-HTT^{-/-} animals were tested in an adapted variant of the sign-versus goal-tracking paradigm described in detail by Flagel et al., [20]. In brief, during two pre-acquisition sessions animals received 50 sucrose pellets on a random interval schedule (30s mean inter-trial interval; ITI) to familiarize them with pellet retrieval from the food cup. Subsequently, rats received 20 acquisition sessions during which sign-versus goal-tracking behaviour of the animals was examined. During these experimental sessions animals continued to receive sucrose pellets on the random interval schedule as described above, but prior to each pellet presentation one lever was extended for 8s (CS+; left or right, counter balanced within groups). Thus, the pellet was delivered directly after the retraction of the CS+ lever. In addition, the second lever (CS-) was presented for 8s on a random interval 30s schedule, but explicitly unpaired with the sucrose pellet presentation. This second lever served as a control to examine the animal's tendency to approach and contact a lever in general. Importantly, interaction (~depression of the lever) with the CS+ (or CS-) lever was recorded but didn't have any programmed consequences. As such, animals received a sucrose pellet irrespective of whether they interacted with the CS+ or not.

Rats were given a total of 29 CS+ trials and 29 CS- trials in a randomized order. Following the above described 20 acquisition sessions animals received 8 extinction sessions in which they again received a total of 58 trials (29 CS+ trials, 29 CS- trials) but now no pellet was presented after CS+ presentation.

The total number and latency of lever (CS+ and CS-) and food cup contacts—as detected by interruption of an infrared sensor beam in the food cup—during CS presentation and inter-trial interval (ITI) were recorded using Med Associates (St. Albans, VT, USA) software and analyzed using MATLAB 8.2 (MathWorks, Natick, Massachusetts, USA) by means of a custom written script.

2.3.2. Progressive ratio schedule of reinforcement experiment

Nine 5-HTT^{+/+} and nine 5-HTT^{-/-} rats were tested in a variant of the progressive ratio schedule of reinforcement paradigm as described in full length by Richardson and Roberts [22]. In short, during two sessions animals were trained on a fixed ratio (FR) 1 schedule of reinforcement. During these sessions animals had to choose during distinct trials between a rewarding lever (RL; left or right, counterbalanced within groups) and an unrewarding lever (UL). Successful session completion required fifty correct trials, i.e., animals had to make 50 RL responses. Trials commenced with

house light illumination and insertion of both levers. When one of the levers was pressed both were subsequently retracted. A RL, but not UL, response resulted in the delivery of a sucrose pellet reward to the food cup (45 mg, Bio Serv, Frenchtown USA); The ITI was ten seconds. After FR1 training, animals were tested on a progressive ratio (PR) schedule of reinforcement, in which rats had to press an increasing number of times on the RL to obtain a sucrose reward. The PR series was derived from the equation: Response ratio (rounded to nearest integer) = $[5e^{(\text{reward no.} \times 0.2)}] - 5$ [22]. This resulted in the following PR schedule series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, ... The PR session was terminated and breaking point (BP; point in series at which the animal ceases to respond) value determined when the animal stopped pressing any lever for one hour. A higher BP is considered to be indicative for a higher experienced reinforcing efficacy of the US by the animal, as it presumably reflects the maximum effort that it will expend in order to receive the US [22].

The total number of lever presses on RL and UL was recorded during each trial using Med Associates software.

2.4. Statistics

All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). We considered results to be significant at $p < 0.05$. NS = not significant.

2.4.1. Sign-versus goal-tracking experiment

For all the different measures of the training and extinction phase of the sign-versus goal-tracking paradigm (no. of lever- or foodcup contacts during CS- trials and ITI, difference in probability) a repeated measures analysis of variance (ANOVA) was performed using the 10 (training phase) or 4 (extinction phase) session block (2 sessions/block) data and trial type (CS+, CS-) as within-subject factors, and genotype (5-HTT^{+/+}, 5-HTT^{-/-}) as between-subject factors. The difference in probability measure was calculated as the difference in the probability of lever approach versus the probability of the approach to the food cup [20]. Notably, these values are not mutually exclusive. Thus in one trial both a lever- and food cup contact can be made. A positive value indicates a bias towards lever interaction, whereas a negative value is indicative for a relatively increased food cup interaction.

2.4.2. Progressive ratio schedule of reinforcement experiment

BP values were analyzed using a non-parametric Mann-Whitney test as such values are derived from an escalating function, which will result in a larger variance on the high end of the scale and lower variance at the low end (thereby prohibiting the use of an ANOVA as it violates one of its assumption, namely of homogeneity of variance). The difference between

incorrect responses was analyzed using an independent Student's *t*-test.

3. Results

3.1. Sign-versus goal-tracking experiment

Statistical analysis of the number of made lever contacts revealed a main stimulus-type effect ($F_{(1,14)} = 38.762$, $p < 0.0005$) and stimulus-type x session block interaction ($F_{(9,126)} = 4.633$, $p < 0.0005$), indicative for an increased number of CS+ lever contacts by the animals, compared to the number of CS- lever contacts, across sessions (see Fig. 1a). No other main effects ($F_{(1,14)} \leq 0.009$, NS) or interactions ($F_{(9,126)} \leq 0.934$, NS) were observed for this measure. Furthermore, a main stimulus-type effect was observed for the number of food cup contacts made during CS- presentation ($F_{(1,14)} = 35.363$, $p < 0.0005$) and ITI ($F_{(1,14)} = 72.527$, $p < 0.0005$), next to an additional stimulus-type x session block interaction for the number of food cup contacts made during CS- presentation ($F_{(9,126)} = 3.488$, $p < 0.01$) (see Fig. 1b). Together these data reflect an overall decrease in the number of feeder cup contacts during CS+ presentation relative to CS- presentation for both genotypes across sessions. In contrast, the number of feeder cup contacts was increased during the ITI after CS+ trials compared to CS- trials (see Fig. 1c). Next to these effects, a significant genotype x session block interaction was observed for both measures (CS- related food cup contacts: $F_{(9,126)} = 2.254$, $p < 0.05$; ITI-related food cup contacts: $F_{(9,126)} = 1.971$, $p < 0.05$), in addition to a main genotype effect for CS- related food cup contacts ($F_{(1,14)} = 4.792$, $p < 0.05$). As these significant genotype-related differences could be observed for both conditions (CS- and ITI-related) they reflect an increase in overall food cup visits for 5-HTT^{+/+} animals, relative to 5-HTT^{-/-} animals. No other significant effects were observed for both food cup contact-related measures (main effects: $F_{(1,14)} \leq 1.102$, NS; interactions with session block: $F_{(9,126)} = 1.139$, NS). Analysis of the latencies to contact the lever or food cup after lever presentation yielded no significant differences (data not shown).

The above described genotype-effect on the number of food cup contacts did not affect the sign-tracking versus goal-tracking measure, namely the difference between the probability to interact with the CS+ lever and the probability to visit the feeder cup (see Fig. 2). Thus, only a significant stimulus type effect ($F_{(1,14)} = 92.307$, $p < 0.0005$) and a stimulus type x session block interaction ($F_{(9,126)} = 12.443$, $p < 0.0005$) were observed for this measure (other main effects: $F_{(1,14)} \leq 0.288$, NS; other interactions with session block: $F_{(9,126)} \leq 1.142$, NS), indicating only a difference between behaviour in CS+ and CS- trials: CS+ trials were associated with an increased tendency to approach the lever during later trials, compared to CS- trials.

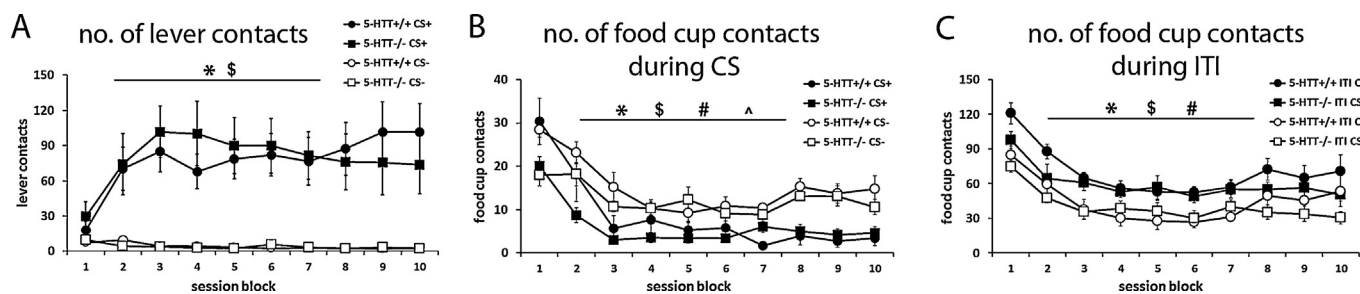


Fig. 1. Number of lever and food cup contacts during the acquisition phase of the sign-versus goal-tracking paradigm. Data represent mean number of (A) lever and (B) food cup contacts (\pm SEM) for 5-HTT^{+/+} and 5-HTT^{-/-} rats, and shown separately for CS+ and CS- trials. (C) number of food cup contacts made during the inter-trial interval period after CS+ and CS- trials for 5-HTT^{+/+} and 5-HTT^{-/-} rats. Data shown as 10 session blocks of 2 sessions each. * $p < 0.0005$, CS+ different from CS-; \$ $p < 0.01$, stimulus-type x session block interaction; # $p < 0.05$, genotype x session block interaction; ^ $p < 0.05$, 5-HTT^{+/+} rats different from 5-HTT^{-/-} rats.

lever vs. food cup approach

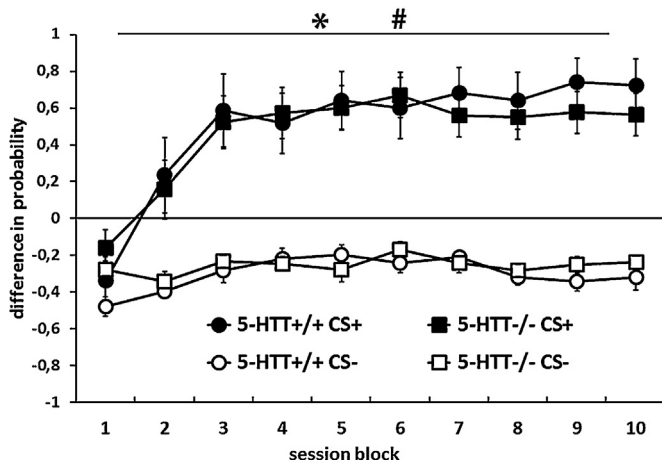


Fig. 2. Difference in probability to approach the lever versus food cup during the acquisition phase of the sign-versus goal-tracking paradigm. Data shown as 10 session blocks of 2 sessions each. Data represent mean difference in probability (\pm SEM) for 5-HTT^{+/+} and 5-HTT^{-/-} rats, and shown separately for CS+ and CS- trials. * $p < 0.0005$, CS+ different from CS-; # $p < 0.0005$, stimulus type \times session block interaction.

Subsequent to the acquisition phase, animals were tested during 8 extinction sessions. Only CS+ measures were included in this analysis. As (1) animals were not trained to an explicitly defined criterion and (2) genotype differences were observed for the food cup contact measures we decided to use relative measures for the analysis of the extinction sessions. Thus, for each animal the total number of CS+ lever contacts made during an extinction session was normalized against the total number of CS+ lever contacts the animal had made during the last acquisition phase session block

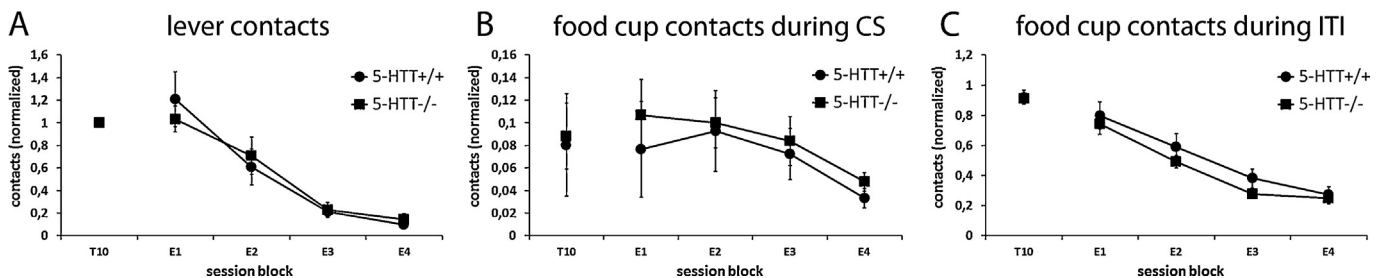


Fig. 3. Lever and food cup contacts during the extinction phase of the sign-versus goal-tracking paradigm. Data represent the normalized number of (A) lever and (B) food cup contacts during CS+ trials (\pm SEM) for 5-HTT^{+/+} and 5-HTT^{-/-} animals. (C) normalized number of food cup contacts (\pm SEM) during the inter-trial interval (ITI) after CS+ trials for 5-HTT^{+/+} and 5-HTT^{-/-} rats. Data are shown as session blocks of 2 sessions each, and normalized against (A) the total number of lever contacts, or (B and C) total number of food cup contacts made by the animal during the last session block of the acquisition phase. T10 = acquisition phase session block 10, E1–4 = extinction phase session block 1–4.

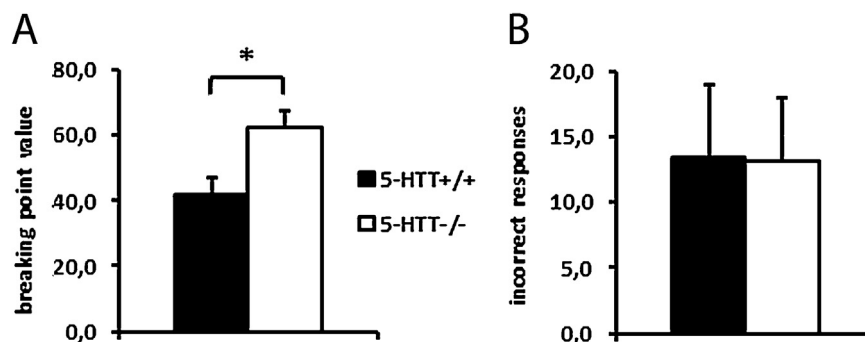


Fig. 4. Progressive ratio schedule of reinforcement. Data represent (A) the mean breaking point value and (B) the mean number of incorrect responses (\pm SEM) during the PR test session for 5-HTT^{+/+} and 5-HTT^{-/-} animals. * $p < 0.05$, 5-HTT^{+/+} different from 5-HTT^{-/-} animals.

(i.e., the average of the last two acquisition sessions). The two food cup contact measures were normalized against the total number of food cup contacts made during the CS+ presentation and CS+ related ITI (i.e., total number of CS+ related food cup contacts). Statistical testing yielded no significant genotype differences for these different measures (main effects: $F_{(1,14)} \leq 1.120$, NS; interactions with session block: $F_{(3,42)} \leq 0.769$, NS) (See Fig. 3).

3.2. Progressive ratio schedule of reinforcement experiment

No significant differences were observed during the FR1 training phase (data not shown). With regard to the results of the PR test session, analysis revealed that the BP was significantly higher in 5-HTT^{-/-} animals relative to 5-HTT^{+/+} animals ($U = 14$, $p < 0.05$) (see Fig. 4a), suggestive for a higher motivation to obtain the US. No genotype differences were observed for unrewarded lever presses ($t_{(16)} = 0.061$, NS; see Fig. 4b).

4. Discussion

Here we show that, unlike our hypothesis, 5-HTT^{-/-} knockout rats do not show increased sign-tracking, neither during acquisition, nor during extinction. Because 5-HTT^{-/-} rats did show an increased motivation to work for a sucrose reward during the progressive ratio schedule of reinforcement, the same reward as used during the sign-versus goal-tracking task, we suggest that the 5-HTT^{-/-} rats are more sensitive to the motivational properties of the US, rather than the incentive salience properties of the CS.

The reversal learning [10], attentional set shifting [8], signal attenuation [9], and fear extinction [7] tasks altogether show that behaviour of the 5-HTT^{-/-} rats is driven by positively and negatively valenced CSs that are emotionally and/or motivationally relevant for the animals. A major difference between these tasks and the sign-versus goal-tracking task is that the reward (sucrose

pellet) is not exclusively dependent on the CS in the sign-versus goal-tracking task. That is, in the sign-versus goal-tracking task the animals receive a pellet regardless whether the animal uses a sign-tracking or a goal-tracking strategy. This mitigates the necessity to track the CS for cost efficient behavioural responding. In line, we have not observed genotype differences during the acquisition of Pavlovian CRs [10,23]. Yet, whereas we previously observed perseverative appetitive responding in the 5-HTT^{-/-} rats during extinction [9], no genotype differences were found during extinction in the present sign-versus goal-tracking task. This implies that the environment sensitive 5-HTT^{-/-} rats [24,25] only acquire tight CS–US associations when the CS is the only cue available signaling the availability of the US. The increase in overall food cup visits (during ITI and CS– presentation) in 5-HTT^{+/+} animals compared to 5-HTT^{-/-} rats may reflect an increase in general exploratory behaviour. Because there are no genotype differences in the probability to approach the food cup or CS+ lever, it is not likely that this increase in general exploratory behaviour interferes with sign-versus goal-tracking. Furthermore, there are no genotype differences in exploratory behaviour between 5-HTT^{-/-} and 5-HTT^{+/+} rats [26].

It has previously been demonstrated that sign-tracking is mediated by dopamine. More specifically, accumbal dopamine was found to attribute incentive salience to reward cues [27,28]. Our data suggest that serotonin's function in incentive salience attribution is different from that of dopamine. Pharmacological studies hint that serotonin is implicated in (i) associative learning processes [29], and tryptophan (serotonin precursor) depletion studies suggest that serotonin mediates (ii) Pavlovian aversive predictions [30]. Furthermore, studies based on 5-HTT genetic variance in humans, non-primates, and rodents suggest that serotonin mediates (iii) environmental sensitivity [16,24,25]. Regarding option (i), the absence of genotype differences during extinction implies that serotonin does not mediate new learning. Also option (ii), is not likely to play a role, since there were no punishments in the current experimental set-up. Yet, regarding option (iii), we have observed that 5-HTT^{-/-} rats show an increased locomotor response to an acute cocaine challenge compared to wild-type rats [18], and increased innate anxiety responses [31]. It is therefore possible that 5-HTT^{-/-} rats are more sensitive than wild-type rats to USs, a factor that is not measured in the sign-versus goal-tracking task. In support, the increased progressive ratio responding in 5-HTT^{-/-} rats suggests that they are more sensitive to the motivational properties of the US.

This study may have some limitations. First, we applied food deprivation in the sign-versus goal-tracking task, which may cause stress. There is extensive evidence that 5-HTT^{-/-} rats are more sensitive to stress than wild-type rats [25]. If food-deprivation stress would have affected task performance, it is most likely that genotype effects would have been found. This was not the case. Therefore, we deem it unlikely that food-deprivation affected our data. Secondly, it can be argued whether the 5-HTT^{-/-} rats model the 5-HTTLPR s-allele in humans, because these human subjects do not completely lack the 5-HTT. However, comparisons from the perspective of 5-HTT expression levels are difficult, because there is no evidence that the 5-HTTLPR s-allele is actually leading to a reduction in 5-HTT expression. Nonetheless, from a behavioural point of view the 5-HTT^{-/-} rats show many traits that correspond to those displayed by 5-HTTLPR s-allele carriers, as described elsewhere [24,32].

In sum, our data do not confirm our hypothesis that serotonin mediates individual differences in sensitivity to CSs as measured in the sign-versus goal-tracking task. This implies that the heightened sensitivity of 5-HTT^{-/-} rats to Pavlovian CSs [9] is not due to increased attribution of incentive salience to the CS, but to another process, possibly increased sensitivity to the US.

Disclosure

All authors report no potential conflicts of interest.

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