Addiction Biology

SSA SOCIETY FOR THE STUDY OF ADDICTION

ORIGINAL ARTICLE

doi:10.1111/adb.12596

# Appetitive to aversive counter-conditioning as intervention to reduce reinstatement of reward-seeking behavior: the role of the serotonin transporter

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## ABSTRACT

Counter-conditioning can be a valid strategy to reduce reinstatement of reward-seeking behavior. However, this has not been tested in laboratory animals with extended cocaine-taking backgrounds nor is it well understood, which individual differences may contribute to its effects. Here, we set out to investigate the influence of serotonin transporter (5-HTT) genotype on the effectiveness of counter-conditioning after extended access to cocaine self-administration. To this end, 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats underwent a touch screen-based approach to test if reward-induced reinstatement of responding to a previously counter-conditioned cue is reduced, compared with a non-counter-conditioned cue, in a within-subject manner. We observed an overall extinction deficit of cocaine-seeking behavior in 5-HTT$^{-/-}$ rats and a resistance to punishment during the counter-conditioning session. Furthermore, we observed a significant decrease in reinstatement to cocaine and sucrose associated cues after counter-conditioning but only in 5-HTT$^{+/+}$ rats. In short, we conclude that the paradigm we used was able to produce effects of counter-conditioning of sucrose seeking behavior in line with what is described in literature, and we demonstrate that it can be effective even after long-term exposure to cocaine, in a genotype-dependent manner.

**Keywords** cocaine, counter-conditioning, reinstatement, serotonin transporter, touch-screen.

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## INTRODUCTION

In humans dealing with drug addiction problems, relapse rates are high (Hubbard, Craddock, & Anderson 2003). In laboratory animals, reinstatement of drug self-administration, a model for human drug relapse, can occur spontaneously, after drug associated cue exposure or after re-exposure to the drug itself (Shaham *et al.* 2003). The fact that reinstatement occurs after prolonged periods of extinction underlines the importance of research into new treatment options for drug addiction.

Generally, during appetitive conditioning, a conditioned stimulus (CS+) is paired with a rewarding unconditioned stimulus (US) and subsequently becomes rewarding itself, driving reward-seeking behavior. The purpose of extinction is to reduce reward-seeking behavior by exposing individuals to the CS+ without reward, thereby forming a new, dominant, memory trace (for review see: Quirk & Mueller 2008). However, multiple studies have shown that this strategy is not sufficient to reduce drug seeking. In counter-conditioning, the previously rewarding CS+ is coupled to an aversive US (e.g. footshock) instead of a CS+ without US. While research using this method is sparse, it has been shown to enhance conventional extinction as well as to reduce reinstatement in laboratory animals with limited cocaine-taking history (Tunstall, Verendeev, & Kearns 2012) and healthy humans receiving natural rewards (Kerkhof *et al.* 2011; Kaag *et al.* 2016). These findings suggest that counter-conditioning may be a valid strategy

to reduce reinstatement. However, it remains unclear if this strategy will prove sufficient in animals with extended cocaine-taking histories. Therefore, it is our first hypothesis that counter-conditioning is an effective tool to reduce reinstatement of reward-seeking in rats with a history of at least 20 cocaine self-administration sessions of 6 hours per session.

One genetic factor influencing the acquisition and extinction of conditioned behaviors involves the low activity short (s)-allelic variant of the serotonin transporter linked polymorphic region (5-HTTLPR). S-allele carriers show increased acquisition of fear (Garpenstrand *et al.* 2001) or increased activation of several brain regions such as the amygdala and anterior cingulate cortex, during fear conditioning (Klucken *et al.* 2015). Increased activation of these regions has been linked to increased attention to salient stimuli (Homberg & Lesch 2011; Klucken *et al.* 2013). Furthermore, s-allele carriers may be at increased risk to develop substance use disorders (Feinn, Nellissery, & Kranzler 2005; Gokturk *et al.* 2008; Cao, Hudziak, & Li 2013). Increased cue reactivity is an important driving factor for fear conditioning and in cocaine seeking and relapse (Volkow *et al.* 2006). Previous research has shown that s-allele carriers have both increased negative emotional cue reactivity and an attentional bias towards positive stimuli compared with l-allele controls (Beevers *et al.* 2009) and show increased reversal learning (Finger *et al.* 2007). In concordance with the 'for-better-or-worse' concept for gene x environment interactions (Belsky *et al.* 2009), this may—on the one hand—provide a potential cause of increased addiction sensitivity and—on the other hand—an opportunity for treatment using counter-conditioning in this population.

To test this idea in a controlled manner, the serotonin transporter knockout (5-HTT$^{-/-}$) rat, modeling the 5-HTTLPR s-allele (Smits *et al.* 2006; Homberg *et al.* 2007; Caspi *et al.* 2010), has been used. Genetic knockout of 5-HTT in these rats causes a complete absence of 5-HTT protein in the brain, as was shown previously using autoradiography (Homberg *et al.* 2007). Furthermore, behavior of 5-HTT$^{-/-}$ rats is strongly cue-dependent (Schipper, Kiliaan, & Homberg 2011; Nonkes *et al.* 2012). Moreover, 5-HTT$^{-/-}$ rats have been found to self-administer more cocaine in short and long access paradigms (Homberg *et al.* 2008; Verheij *et al.* In press), measuring regular and compulsive cocaine self-administration behavior, respectively (Ahmed & Koob 1998). These 5-HTT$^{-/-}$ rats also show decreased extinction of cocaine-seeking behavior (Homberg *et al.* 2008). On the other hand, 5-HTT$^{-/-}$ rats exhibit higher cognitive flexibility, as illustrated by improved reversal learning of sucrose (appetitive to appetitive switch), and faster fear extinction when also presented with a cue predicting food delivery (aversive to appetitive switch) (Nonkes, de Pooter, & Homberg 2012; Nonkes, Maes, & Homberg 2013). Based on these findings, it is our second hypothesis that 5-HTT$^{-/-}$ rats benefit more from appetitive to aversive counter-conditioning compared with their 5-HTT$^{+/+}$ counterparts.

Here, we present a touchscreen-based counterconditioning paradigm that was able to reduce the reinstatement of sucrose and cocaine-seeking behavior. We have compared the efficacy of counter-conditioning to that of typical extinction training in a within subject experimental design. Our touchscreen equipped behavioral cages (for review see: Talpos & Steckler 2013), enabled us to train rats to associate several distinct visual cues with reward or punishment within the same sensory dimension (sight), preventing bias. Two groups of rats were trained to self-administer sucrose or cocaine in response to several distinct visual discriminative cues, using the touch-screen itself as manipulandum. Afterwards, we counter-conditioned one of these visual cues while using typical extinction on the other cue, enabling us to measure the effectiveness of counter-conditioning in a within-subject design. After full extinction of reward-seeking behavior, we exposed the animals to either sucrose or cocaine reward and measured reinstatement of sucrose and cocaine-seeking behavior.

## MATERIALS & METHODS

### Animals

All experiments were approved by the Committee for Animal Experiments (DEC 2014-159) of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, and all efforts were made to diminish animal suffering and to reduce the number of rats used. The 5-HTT knockout rats (5-HTT$^{-/-}$, Slc6a41Hubr) were generated on a Wistar background by ENU-induced mutagenesis (Smits *et al.* 2004; Smits *et al.* 2006) and have been described previously (Homberg *et al.* 2007). In total, 16 5-HTT$^{+/+}$ and 13 5-HTT$^{-/-}$ rats receiving sucrose and 7 5-HTT$^{+/+}$ and 11 5-HTT$^{-/-}$ rats receiving cocaine completed the protocol. Rats were housed in Plexiglas cages in pairs in a temperature (21 ± 1°C) and humidity-controlled room (60% relative humidity), and had *ad libitum* access to water and food, except during testing. After surgery, rats were housed individually. A 12-hour light–dark cycle was maintained, with lights off at 08:00 a.m.

### Surgery

Rats used for cocaine self-administration were equipped with intravenous catheters implanted in the right jugular vein using isoflurane (initially 5%, during surgery 2–3%) as anesthesia. For more details on the procedure see

 

Verheij *et al.* (2016). After surgery, the rats were allowed to recover for a minimum of 1 week before testing started. Catheter patency was maintained by daily sterile saline-heparin (0.3 ml; 13.5 mg/ml, 140 units/mg) infusions.

### Apparatus

All the behavioral testing took place in modular operant rat touchscreen boxes (Med Associates). In short, the box measured 30 × 24 × 21 cm (l × d × h) and was located in a sound attenuating cubicle. Near the top of the cubicle, a fan was installed to push fresh air into the cabinet and to provide a steady background noise during the sessions. The floor of the touchscreen boxes consisted of 19 stainless steel rods connected to a shock scrambler (Med Associates). The right wall of the box contained all the typical operant equipment. The middle of this wall contained a pellet receptacle for sucrose delivery. On either side of this receptacle, a retractable lever was placed with a matching lever light placed above it. These levers, which were always retracted, and signal lights, which were always turned off, served no function during the experiments described in this manuscript. A house light was mounted near the top of the box above the pellet dispenser. The opposite wall consisted completely out of an infrared touch screen (Conclusive Solutions); the screen was covered with a black aluminum mask with two 10 × 10-cm holes placed 1 cm apart for stimulus presentation and served as the manipulandum during the experiments. The door, back wall and top of the box all consisted of clear Plexiglas. A spring-covered tube entered the box through the top and was connected to a syringe in a mechanical pump for cocaine solution delivery.

### Behavioral task

The counter-conditioning task was developed specifically for these experiments and consisted of a pretraining phase, allowing the animals to habituate to the experimental chamber and the brightly illuminated touchscreen, followed by four distinct experimental phases: acquisition, counter-conditioning, extinction and reinstatement (see below). An example of outcomes during the four main phases of the experiment can be found in figure 1. Furthermore, an overview of length of the phases and criteria for advancing to the next phase is available in Table S1.

#### *Pretraining*

Pretraining was divided into three phases: pellet training, screen training and cue training. Pellet training consisted of a single session during which one 45 mg sucrose pellet was delivered in the pellet receptacle. Upon retrieval, a 1-minute intertrial interval (ITI) initiated, after which another pellet was delivered for a maximum of five pellets. During this session, the touch screens were powered on and did not display any image (black screen), causing a slight illumination of the interior. The next day, animals were placed back into the same chamber for screen training. During screen training, the touchscreen constantly displayed a dark gray image in both response areas, causing a mediocre illumination of the cage interior. Touching either of the response areas lead to the delivery of a sucrose pellet after which the screen turned off for a 1-minute ITI. Rats were trained under these conditions for 1 hour for 4 days or until a minimum of five pellets were collected in a single session. Next, the rewarding visual cues that were used for typical conditioning/extinction (Discriminative stimulus, DS+) and counter-conditioning (DScc) were introduced in a single session, identical to screen training, but instead of a dark gray image, DS+ and DScc images were presented and reinforced upon response. No DS-cues were presented during this session. Cues are presented in figure S1 and were counter balanced across rats.

#### *Acquisition*

During the acquisition phase, a pair of cues was presented for a maximum of 30 seconds. When rats did not respond during this period, the screen turned off for a 30-second ITI after which another pair of cues was presented (missed trial). If rats responded to a rewarding cue (DS+ or DScc), a reward followed in the form of a 45-mg sucrose pellet or an infusion of 0.5-mg/kg cocaine solution depending on the cohort (correct trial). After delivery of the reward, the protocol proceeded to the ITI. If rats responded to a DS-cue, the protocol proceeded to a time-out period during which the house light was turned on for 30 seconds to signal unavailability of the reward followed by the ITI (incorrect trial). Therefore, if a rat responded to a DS-cue, the total duration of reward unavailability was 60 seconds (versus 30 seconds on a DS+, DScc or missed trial). After the ITI, the protocol presented a new set of images. Images were always presented as DS+/DS-, DS-/DS+, DScc/DS-, DS-/DScc or DS-/DS-pairs (and never as DS+/DScc or DScc/DS+ pairs, left cue/right cue) bringing chance responding level to 40% correct (note: chance responding during potentially rewarding trials, thus excluding DS-/DS-trials, was 50%). Acquisition sessions lasted for 1 hour for the sucrose group and 6 hours for the cocaine group. The latter was chosen to test the effectiveness of counter-conditioning in animals with a history of extended cocaine use. Primary outcomes during this phase were the number of rewards collected and the percent of correct responses during potentially rewarding trials, calculated by adding CS+ and CScc responses and dividing them

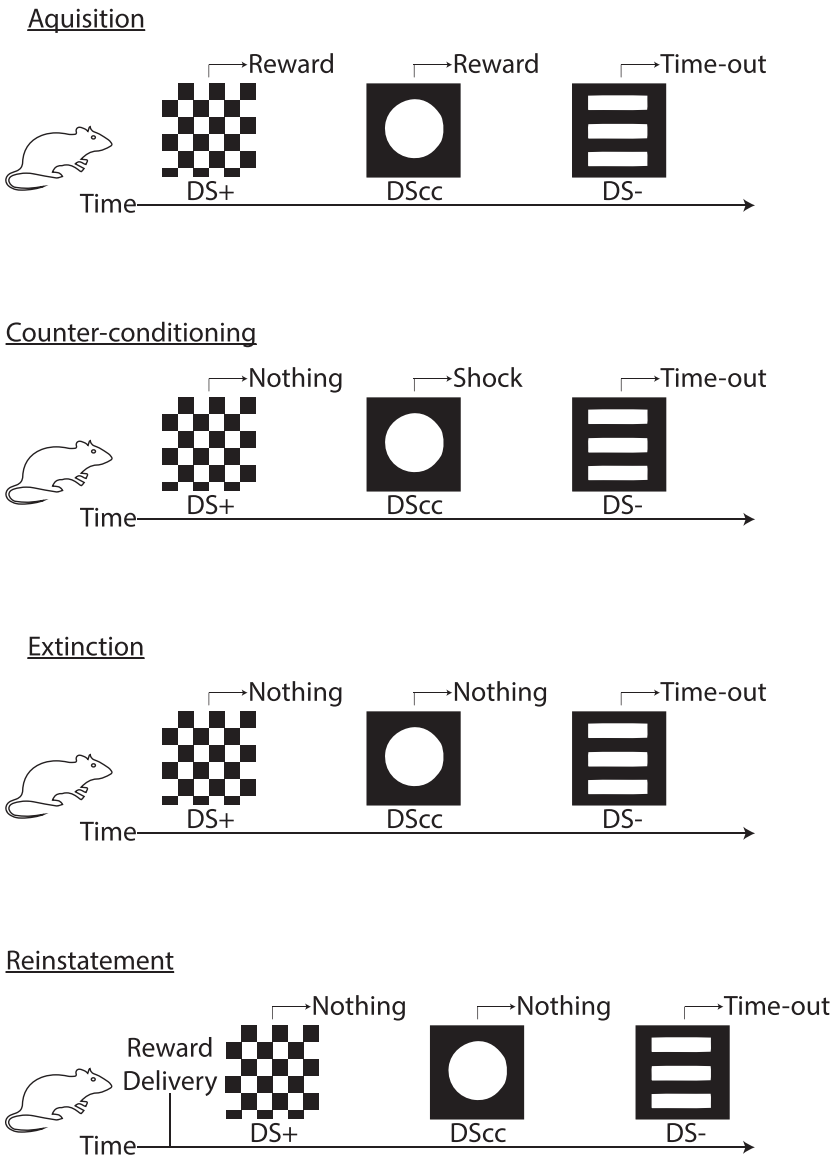


**Figure 1** Overview of cue response contingencies. Depicted is an overview of the contingencies of responses to DS+, DScc and DS- cues during the four main phases of the experiment. Note that visual cues were counter-balanced between rats

by the total number of responses during potentially rewarding trials. Animals were trained under this schedule for a minimum of 14 sessions and a maximum of 30 sessions, until 80% correct responding was reached for three consecutive sessions.

*Counter-conditioning*

After acquisition, the rats proceeded to the counter-conditioning phase. Counter-conditioning was identical to the acquisition phase with the exception that DS+ responding was no longer rewarded and DScc responding resulted in a 0.5 s, 0.25 mA footshock. The counter-conditioning phase consisted of a single session that lasted for 1 hour.

*Extinction*

During the extinction phase that followed, neither DS+ nor DScc delivered any rewards or shocks. Extinction lasted a minimum of 14 sessions and until there were less than 10 combined DS+/DScc responses per session for three consecutive sessions. The sessions lasted 1 hour for the sucrose group and 4 hours for the cocaine group.

*Reinstatement*

To determine reward-induced reinstatement, a baseline measurement of responding was recorded for 45 minutes under extinction conditions, followed by the delivery of a large reward (five sucrose pellets delivered in the pellet receptacle or a 10-mg/kg i.p. cocaine infusion, depending

 

on the cohort). Reinstatement was then measured as a fold increase compared with the baseline. No further reinforcement was delivered upon DS+ or DScc responding. For a detailed flow schedule of the paradigm, see figure S2.

### Statistics

All statistical analyses were performed by using IBM SPSS version 20 (IBM software). Note that rats were exposed to three cues (figure 1), allowing us to conduct *within-subject* analyses for cue effects. Data acquired during the acquisition of self-administration were analyzed using genotype * session repeated measures ANOVAs. The counter-conditioning and reinstatement phase were analyzed using cue * genotype repeated measures ANOVA. The number of cue responses during the extinction phase of the cocaine cohort was analyzed using cue * genotype repeated measures ANOVA. The same data of the sucrose cohort were analyzed using genotype * cue type * extinction block univariate analysis, because some of the repeated data points were missing due to hardware issues. Additionally, the amount of days to criterion during the acquisition and extinction phases were analyzed using the Mann–Whitney U tests. Effects were considered significant when $P < 0.05$ and nonsignificant effects are noted as $P$ = NS.

## RESULTS

### Acquisition

During acquisition, both DS+ and DScc responses resulted in the delivery of sucrose or cocaine, depending on the cohort. Figure 2a,b represents the percentage of correct trials during potentially rewarding trials. Repeated measures ANOVA of the data shown in figure 2a revealed that there was no significant genotype * session interaction or overall genotype effect in the sucrose cohort (interaction: $F_{(13,\ 351)} = 1.175$, $P$ = NS; genotype: $F_{(1,\ 27)} = 2.376$, $P$ = NS). Correct responding was above chance level after three sessions for all animals and stabilized around 90% after approximately 11 sessions. In line with this, there was no significant difference in days-to-

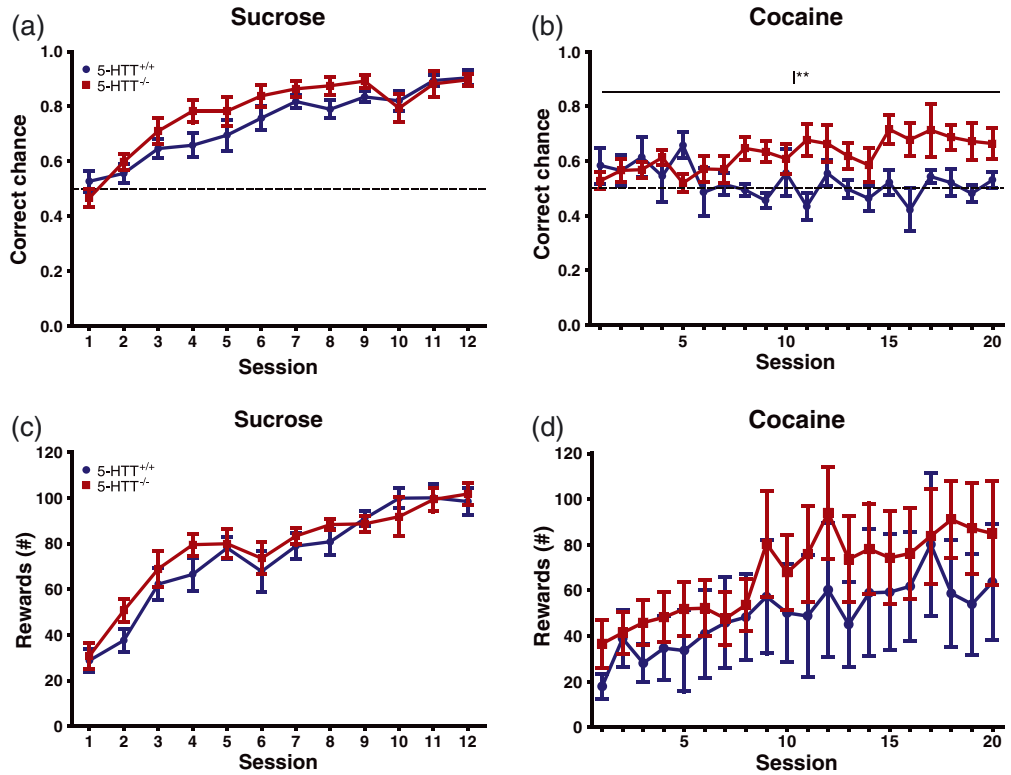


**Figure 2** Acquisition of sucrose and cocaine self-administration in a touchscreen-based set-up in 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats. All data are represented as mean ± SEM. The 5-HTT$^{+/+}$ rats are represented by blue lines and circles, and 5-HTT$^{-/-}$ rats are represented by red lines and squares. During this phase in the experiment, rats were able to self-administer 0.5-mg/kg cocaine or 45-mg sucrose pellets, depending on the cohort, in response to three visually distinct, counter-balanced, cues. Figure 2a,b represents the percentage of correct responding of the sucrose and cocaine cohort, respectively, while Figure 2c,d represents the amount of rewards collected by both cohorts. Figure 2a shows that no differences were observed in the accuracy of acquiring the task in the sucrose cohort. However, in the cocaine cohort (figure 2b), 5-HTT$^{-/-}$ rats were more accurate in their responding over time (I**). Figure 2c,d reveals that in neither the sucrose nor cocaine cohorts, the two genotypes showed differences in the amount of rewards collected

criterion (for criterion see Table S1) between 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats (data not shown. 5-HTT$^{+/+}$: 17.56 (±0.69) days, 5-HTT$^{-/-}$: 16.07 (±0.81) days. Mann–Whitney U test: U = 39.5, $P$ = NS). In contrast to these sucrose findings, repeated measures ANOVA for the cocaine cohort revealed a significant genotype * session interaction ($F_{(19,304)}$ = 2.165, $P < 0.01$; figure 2b), indicating that 5-HTT$^{-/-}$ rats were more accurate over the course of this phase compared with their 5-HTT$^{+/+}$ counterparts. However, this did not result in a difference in days-to-criterion (for criterion see Table S1) between genotypes (data not shown. 5-HTT$^{+/+}$: 27.57 (±0.68) days, 5-HTT$^{-/-}$: 25.18 (±1.07) days Mann–Whitney U test: U = 19, $P$ = NS). Figure 2c,d represents the pooled number of rewards collected by the sucrose and cocaine cohorts. We observed no differences between genotypes in either the number of sucrose pellets collected (figure 2c: interaction: $F_{(11,231)}$ = 0.804, $P$ = NS; genotype: $F_{(1,21)}$ = 0.589, $P$ = NS) or cocaine infusions received (figure 2d: interaction: $F_{(19,304)}$ = 0.545, $P$ = NS; genotype: $F_{(1,16)}$ = 0.545, $P$ = NS).

### Counter-conditioning

During a single counter-conditioning session, responses to the DScc cue were paired to a mild footshock while responding to DS+ had no programmed consequences. In the sucrose group, ANOVA analysis revealed a significant counter-conditioning effect ($F_{(1,27)}$ = 6.747, $P < 0.05$) caused by lower DScc responses in the 5-HTT$^{-/-}$ group, as well as an overall genotype effect ($F_{(1,27)}$ = 5.3000, $P < 0.05$) caused by the overall higher number of DS+ responses in the 5-HTT$^{-/-}$ compared with the 5-HTT$^{+/+}$ rats (figure 3a). 5-HTT$^{-/-}$ rats in the cocaine group had significantly more DS+/DScc responses (figure 3b, genotype effect: $F_{(1,16)}$ = 5.466, $P < 0.05$) responded significantly more compared with 5-HTT$^{+/+}$ rats, but we observed no counter-conditioning specific effects (counter-conditioning effect: ($F_{(1,\ 16)}$ = 0.865, $P$ = NS).

### Extinction

During the extinction phase, responding to neither DS+ nor DScc had programmed consequences. Figure 4a,b shows the number of responses per cue made during this phase while figure 4c,d shows the number of sessions required to reach the predefined extinction criterion (see Table S1). For visual clarity, the data of the extinction sessions were pooled into five blocks (sucrose: five blocks of three sessions, cocaine: five blocks of five sessions). Statistical analysis of the sucrose data revealed no differences between genotypes ($F_{(4,160)}$ = 1.077, $P$ = NS), effects of counter-conditioning ($F_{(4,160)}$ = 0.079, $P$ = NS) or interaction ($F_{(4,160)}$ = 0.14, $P$ = NS). Additionally, the data shown in figure 4c indicate that there was no difference between genotypes in reaching extinction criterion (U = 105, $P$ = NS). In rats with extended history of cocaine intake, statistical analysis revealed both a significant genotype * extinction block interaction ($F_{(4,128)}$ = 3.141, $P < 0.05$) as well as a significant overall genotype effect ($F_{(1,32)}$ = 6.418, $P < 0.05$). These data are indicative for an overall extinction deficit in the 5-HTT$^{-/-}$ rats, which was independent from counter-conditioning. In line with this, 5-HTT$^{-/-}$ rats needed more extinction sessions to reach extinction criterion (figure 4d, U = 15.5, $P < 0.05$).

### Reinstatement

To determine if reinstatement was successful, the number of DS+ and DScc responses were combined and compared during extinction and reinstatement conditions for both cohorts. Paired sample $t$-testing revealed that neither

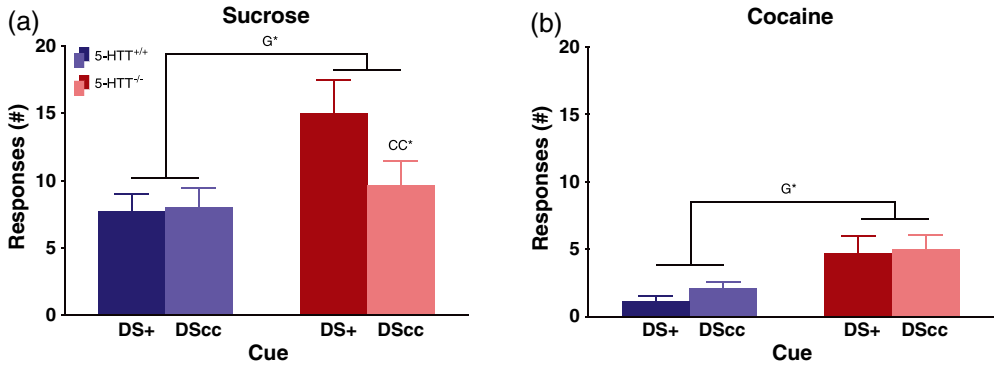


**Figure 3** Counter-conditioning after sucrose or extended access cocaine self-administration. All data are represented as mean ± SEM. The 5-HTT$^{+/+}$ responding is represented using blue bars and 5-HTT$^{-/-}$ responding using red bars. For both groups, the darker bars represent DS+ responding while the lighter bars represent DScc responding. During this single session phase, DScc responses are followed by a mild footshock, while DS+ responding is unrewarded. Figure 3a,b shows a higher response rate in 5-HTT$^{-/-}$ rats of both cohorts (G*). Additionally, in the sucrose cohort, analysis revealed a significant counter-conditioning, caused by the lower CScc responding in the 5-HTT$^{-/-}$ rats (CC*)

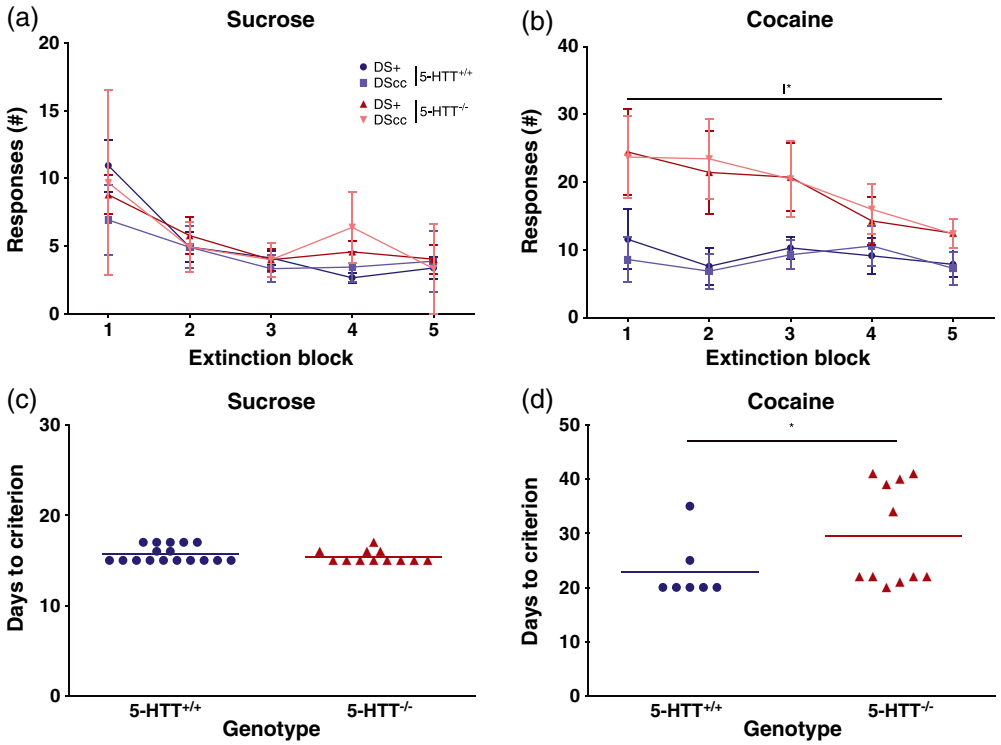


**Figure 4** Extinction after counter-conditioning in sucrose and cocaine cohorts. All data are represented as mean ± SEM. Figure 4a,b represents the number of responses to DS+ and DScc cues split by cohort (sucrose and cocaine, respectively). For visual clarity, the data are pooled into five blocks (sucrose (a): five blocks of three sessions, cocaine (b): five blocks of five sessions) and are represented as mean ± SEM. During this experimental phase, neither DS+ nor DScc had any programmed consequences. No differences in extinction were observed in the sucrose cohort (figure 4a), and genotypes did not differ in reaching extinction criterion (figure 4c). In contrast to this, the data in figure 4b indicate an overall deficit of extinction of cocaine seeking behavior in the 5-HTT$^{-/-}$ rats over time, which was independent from counter-conditioning (I*). In addition, these rats take longer to reach the extinction criterion (* Figure 4d)

5-HTT$^{+/+}$ ($t_{(12)} = -0.888$, $P$ = NS) nor 5-HTT$^{-/-}$ ($t_{(10)} = -0.687$, $P$ = NS) of the sucrose cohort increased responding significantly after reward administration (figure 5a). In contrast to this, reinstatement was significant for both genotypes in the cocaine cohort (figure 5b: 5-HTT$^{+/+}$: $t_{(6)} = -2.763$, $P < 0.05$; 5-HTT$^{-/-}$: $t_{(10)} = -2.296$, $P < 0.05$). ANOVA analysis revealed an effect of counter-conditioning on the fold increase of responding in both cohorts (sucrose, figure 5c: $F_{(1,27)} = 4.595$, $P < 0.05$; cocaine, figure 5d: $F_{(1,16)} = 6.500$, $P < 0.05$). This significant reduction in reward responding was observed in 5-HTT$^{+/+}$ rats only, although there were no genotype (sucrose: $F_{(1,27)} = 0.016$, $P$ = NS; cocaine: $F_{(1,16)} = 0.102$, $P$ = NS) and interaction (sucrose: $F_{(1,27)} = 2.597$, $P$ = NS; cocaine: $F_{(1,16)} = 1.401$, $P$ = NS) effects.

## DISCUSSION

Here we show that, in line with our first hypothesis, a single counter-conditioning session was able to reduce reinstatement of reward-seeking in 5-HTT$^{+/+}$ animals with a history of extended access to cocaine self-administration. However, contrary to our second hypothesis, reinstatement of reward-seeking behavior was not reduced in 5-HTT$^{-/-}$ rats by counter-conditioning. In addition, we demonstrated that 5-HTT$^{-/-}$ rats were more accurate during acquisition of cocaine self-administration compared with 5-HTT$^{+/+}$ rats. Furthermore, during the counter-conditioning session, 5-HTT$^{-/-}$ rats responded more persistently to previously rewarding cues and sucrose seeking was inhibited by footshocks in 5-HTT$^{-/-}$ rats. Lastly, 5-HTT$^{-/-}$ rats exhibited an extinction deficit after cocaine self-administration compared with 5-HTT$^{+/+}$ rats, with no effect of counterconditioning.

During the reinstatement session, 5-HTT$^{+/+}$ rats responded less to the DScc cues compared with DS+ cues in both the sucrose and cocaine cohorts. This indicates that the within-subject task we outline above is able to replicate results obtained in the past, using between-subject methods in humans and animals (Bouton & Peck 1992; Van Gucht *et al.* 2010; Tunstall *et al.* 2012; Kaag *et al.* 2016). Additionally, we show for the first time that counter-conditioning is capable of reducing reinstatement of rats with an extended access to cocaine self-administration. However counter-conditioning did not have an effect in 5-HTT$^{-/-}$ rats, in neither sucrose nor cocaine cohorts. It must be noted that there was no

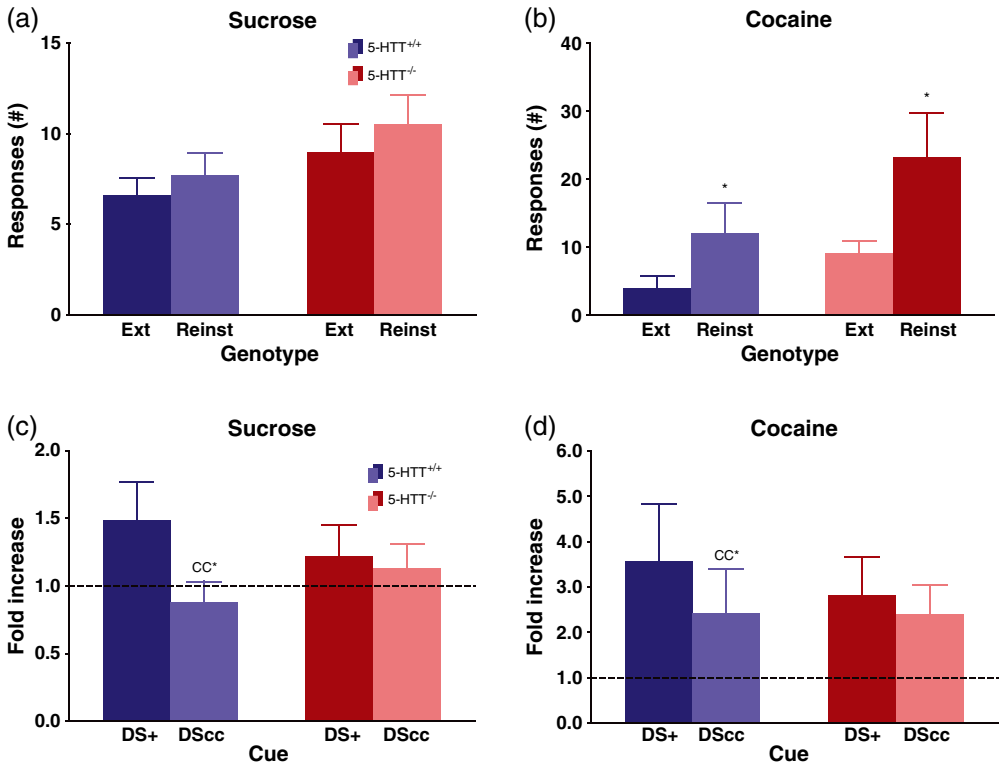


**Figure 5** Reinstatement of sucrose and cocaine-taking behavior after counter-conditioning and conventional extinction. All data are represented as mean ± SEM. To determine whether reinstatement took place, the DS+ and DScc responses were added together for extinction and reinstatement conditions for both cohorts. Analysis revealed that reinstatement of sucrose-seeking behavior (figure 5a) was not significant, while both genotypes reinstated cocaine-seeking behavior (* figure 5b). In figure 5c,d, reinstatement is expressed as a ratio of responding compared with a previous 45-minute session under extinction conditions. In both sucrose (c) and cocaine (d) cohorts, counter-conditioning reduced CScc responding in 5-HTT$^{+/+}$ rats only (CC*)

significant reinstatement in the sucrose cohort. Therefore, we should be careful in interpreting these results. In recent literature, sucrose seeking does not always lead to significant reinstatement and suggesting it may be very dependent on the experimental design (Kosten & Meisch 2013; Watterson *et al.* 2013). However, despite not having significant reinstatement overall, 5-HTT$^{+/+}$ rats still showed an effect in counter-conditioning in this cohort. Moreover, while reinstatement in the cocaine cohort is much more robust, the data follow the same pattern, suggesting a general insensitivity of 5-HTT$^{-/-}$ rats to counter-conditioning. As mentioned above, the existing literature on counter-conditioning is sparse, and the literature on the underlying mechanisms is virtually non-existent. A notable exception to this includes the study of Kaag *et al.* (2016) who demonstrated that shock-induced counter-conditioning of monetary rewards in humans (appetitive to aversive switch) was associated with attenuated activation of the ventral striatum and VTA. In line with this latter study and our results, humans carrying the s-allele of the 5-HTTLPR display increased ventral striatum activity during a simple appetitive conditioning task (Klucken *et al.* 2013). This suggests that low serotonin transporter availability may prevent top-down attenuation of striatal activity, thus preventing effective counter-conditioning. This is what we observed here for 5-HTT$^{-/-}$ rats.

During the acquisition phase, both 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats receiving sucrose were able to acquire a task based on visual discrimination despite their albino background (Prusky *et al.* 2002). This is illustrated by the observation that all rats acquired the task quickly and were able to maintain an average correct response rate of 90% throughout this phase. However, in the cocaine cohort, 5-HTT$^{+/+}$ rats were unable to maintain stable responding above chance levels during cocaine self-administration, caused by a higher number of errors (and thus still maintaining a stable cocaine intake), while 5-HTT$^{-/-}$ rats performed significantly above chance. This observation seems to be in line with the reputation of cocaine to reduce cognitive functioning and observations in our lab that 5-HTT$^{-/-}$ rats are more cognitively flexible compared with 5-HTT$^{+/+}$ rats even after exposure to cocaine self-administration (Nonkes *et al.* 2013). A reduction of cognitive functioning in 5-HTT$^{+/+}$ rats during the acquisition phase of the cocaine task may raise the question whether these animals were able to distinguish the visual cues during the following phases of the study. However,

 

during the reinstatement phase, 5-HTT$^{+/+}$ animals were still able to distinguish between the DS+ and DScc cues.

There are substantial differences between the experiments described in the preceding text and typical cocaine self-administration using levers. As discussed in the preceding text, the task is more complex, leading to more incorrect responding when cocaine (but not sucrose) is used as a reward. Incorrect responding leads to a time-out period of 60 seconds (30-second time-out plus 30-second ITI, figure S2). This feature, intended to force rats to make accurate responses, result in a lower number of cocaine infusions when compared with typical 1-lever self-administration. This large difference in methodology is most likely why we did not observe significant genotype differences in the number of cocaine infusions during acquisition as previously reported (Homberg *et al.* 2008; Verheij *et al.* In press). However, rats of both genotypes were still able to administer increasing amounts of cocaine during the experiment. Additionally, we were able to replicate the finding that 5-HTT$^{-/-}$ rats suffer impaired extinction after (typical) cocaine self-administration (Homberg *et al.* 2008).

In addition to this replication of extinction behavior, 5-HTT$^{-/-}$ rats were more persistent in their cocaine seeking when receiving shocks after DScc responses during the counter-conditioning session. This is illustrated by a higher amount of both DS+ and DScc responses during counter-conditioning compared with 5-HTT$^{+/+}$ rats and the inability of the shocks to reduce DScc responding like in the sucrose group. Several researchers have shown insensitivity to punishment in more typical cocaine self-administration set-ups. For instance, Pelloux, Everitt, & Dickinson (2007) showed that rats with an extended history of cocaine seeking persisted their reward-seeking behavior despite receiving footshocks at the time. In a subsequent study, they showed that the subgroup of rats that was resistant to punishment had reduced serotonergic signaling in the fronto-striatal circuitry, which they remediated by acute SSRI treatment (Pelloux *et al.* 2012). This suggests that there is a causal relationship between 5-HT and resistance to punishment in drug addiction. These reports and the data acquired in this study seem to imply that, while this experiment was not designed to measure this, 5-HTT$^{-/-}$ rats maintained drug seeking despite negative consequences, possibly due to reduced serotonin mediated top-down control. Further experiments, preferably using the seeking–taking chained schedule (Vanderschuren & Everitt 2004; Pelloux *et al.* 2007) should be conducted to confirm this observation.

Prefrontal cortex-mediated top-down control over limbic regions is essential for successful extinction of fear and addiction-related behaviors (Peters, Kalivas, & Quirk 2009). In the experiments described in the preceding text, 5-HTT$^{-/-}$ rats with a history of long access to cocaine self-administration showed a severe extinction deficit. This is in line with many previous observations where 5-HTT$^{-/-}$ rats and mice have shown extinction impairments in a variety of tests, including fear conditioning/extinction and short access cocaine self-administration paradigms (Homberg *et al.* 2008; Pang *et al.* 2011; Schipper *et al.* 2011; Nonkes *et al.* 2012). Additionally, there was no effect of counter-conditioning on the extinction phase. While most research, including this report, seems to focus on the effect of counter-conditioning on reinstatement, there are some indications that it could also facilitate extinction (Van Gucht *et al.* 2010; Tunstall *et al.* 2012). However, these studies observed this difference when the extinction group, as opposed to the counter-conditioning group, is not exposed to any aversive conditions (i.e. without yoked/shocked control). In our experiments, all rats received both DS+ (no shock) and DScc (shock) conditions, which may explain the lack of differences in the early extinction phase.

The current study has some limitations. Although we observed a reduction in reward-seeking during a single reinstatement session, we have not tested the long-term effects of counter-conditioning. In addition, counter-conditioning has been proven to be context-specific (Peck & Bouton 1990; Brooks *et al.* 1995). The current experiments all took place in the same context and thus may not be directly translatable to real world situations. Additional experiments will need to prove if counter-conditioning can be made context independent by, for instance, multiple-context training or pharmacological interventions.

In short, this work shows for the first time that counter-conditioning can be a valid therapy to reduce cocaine-induced reinstatement in individuals with an extended cocaine history. However, individuals with altered serotonin signaling may benefit less from this therapy.

### Acknowledgments

The work described in this paper was funded by The Netherlands Organization for Health Research and Development (ZonMw, project number 91211002 awarded to Dr. Homberg). ZonMw had no further role in the design of the study, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the paper for publication.

## AUTHOR CONTRIBUTIONS

P. K., J. R. H., A. M. K. and L. R. were responsible for study concept and design. P. K., A. A. and J. P. acquired animal behavior data. Data analysis and interpretation were performed by P. K. P. K. drafted the manuscript, and J. R. and

M. M. M. V. provided critical revision of the manuscript. All authors critically reviewed the content and approved the final version for publication.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**Table S1. Overview of experiment**. Described are the presented cues, and advancement criteria for pre-training and the 4 experimental phases.

**Figure S1. Visual stimuli.** The three used visual stimuli measured 10 × 10 cm and were counter-balanced across rats.

**Figure S2.** Flow diagram of a single session during the 4 experimental phases.