Addiction Biology

SSA SOCIETY FOR THE STUDY OF ADDICTION

ORIGINAL ARTICLE

doi:10.1111/adb.12483

D-Cycloserine enhanced extinction of cocaine-induced conditioned place preference is attenuated in serotonin transporter knockout rats

Peter Karel[1], Francesca Calabrese[2], Marco Riva[2], Paola Brivio[2], Bas Van der Veen[1], Liesbeth Reneman[3], Michel Verheij[1] & Judith Homberg[1]

Department of Cognitive Neuroscience, Centre for Neuroscience, Donders Institute for Brain, Cognition and Behaviour, Radboudumc, The Netherlands[1], Department of Pharmacological and Biomolecular Sciences, Universita'degli Studi di Milano, Italy[2] and Department of Radiology, Academic Medical Center, University of Amsterdam, The Netherlands[3]

ABSTRACT

D-Cycloserine (DCS), a partial NMDA receptor agonist, has been proposed as a cognitive enhancer to facilitate the extinction of drug-related memories. However, it is unknown whether there are individual differences in the efficacy of DCS. Here, we set out to investigate the influence of serotonin transporter (5-HTT) genotype on DCS treatment outcome and the underlying neural mechanism. To that end, we first determined the mRNA levels of several NMDA receptor subunits and observed a reduction in NR1/NR2C receptors in the ventromedial prefrontal cortex and nucleus accumbens of 5-HTT$^{-/-}$ compared with 5-HTT$^{+/+}$ rats. Based on this finding, we hypothesized a lower sensitivity to DCS in the 5-HTT$^{-/-}$ rats. To test this, rats were trained in a cocaine-induced conditioned place preference (CPP) paradigm. A significant extinction of CPP was observed in 5-HTT$^{+/+}$ rats receiving 1 mg/kg i.v. DCS, while a similar effect was found in the 5-HTT$^{-/-}$ rats only after 5 mg/kg. Following CPP, we tested if DCS were able to reduce FosB/ΔFosB protein expression, a molecular switch for cocaine-seeking behaviour. We observed an overall lower number of FosB/ΔFosB positive cells in 5-HTT$^{-/-}$ ventromedial prefrontal cortex and amygdala and an overall effect of DCS treatment on the number of positive cells in the nucleus accumbens. In conclusion, in this study, we show that the dosing of DCS to facilitate the extinction of cocaine-seeking behaviour is, at least partially, determined by 5-HTT genotype.

Keywords ΔFosB, addiction, cocaine, D-cycloserine, NMDA, serotonin transporter.

Correspondence to: Judith Homberg, Donders Institute for Brain, Cognition and Behaviour, Department of Cognitive Neuroscience, Behavioural Neurogenetics Group, Radboudumc, Kapittelweg 29, 6525 EN Nijmegen, The Netherlands. E-mail: judith.homberg@radboudumc.nl

INTRODUCTION

Carriers of the short (s)-allele of the serotonin transporter-linked polymorphic region (5-HTTLPR) exhibit a lower level of serotonin transporter (5-HTT) mRNA transcription and are hypothesized to have higher levels of extracellular brain serotonin (Heils *et al.* 1996). These individuals may have an increased risk to become addicted to different types of drug (Feinn, Nellissery & Kranzler 2005; Gokturk *et al.* 2008; Cao, Hudziak & Li 2013). An animal model mimicking the 5-HTTLPR s-allele is the serotonin transporter knockout (5-HTT$^{-/-}$) rat (Smits *et al.* 2006; Homberg *et al.* 2007). The 5-HTT$^{-/-}$ rats self-administer more cocaine in long and short access paradigms (Homberg *et al.* 2008; Verheij *et al.* 2012), measuring regular and compulsive cocaine self-administration behaviours, respectively (Ahmed & Koob 1998). The 5-HTT$^{-/-}$ rats furthermore exhibit increased cocaine-induced place preference (CPP) compared with wild-type (5-HTT$^{+/+}$) controls (Homberg *et al.* 2008). Importantly, these rats also display a difficulty in extinguishing cocaine and fear-associated memories (Homberg *et al.* 2008; Schipper, Kiliaan & Homberg 2011; Nonkes, de Pooter & Homberg 2012).

A potential mechanism underlying this extinction deficit involves decreased top-down control of the ventromedial prefrontal cortex (vmPFC) over limbic structures such as the amygdala (Amy) and the nucleus accumbens

(NAc; Peters, Kalivas & Quirk 2009). Projections from the vmPFC to these areas are glutamatergic and are essential for the consolidation of extinction learning (Burgos-Robles *et al.* 2007; LaLumiere, Niehoff & Kalivas 2010). Furthermore, the glutamatergic system is implicated in reward-related learning (Popik, Wrobel & Bisaga 2006). It has been previously observed that 5-HTT$^{-/-}$ rodents exhibit a lower vmPFC and higher Amy activation during fear extinction (recall; Pang *et al.* 2011). Additionally, morphological changes of glutamatergic pyramidal neurons in the PFC and Amy associated with reduced fear extinction recall (Wellman *et al.* 2007) have suggested a comparable neural hallmark in 5-HTT$^{-/-}$ rodents. These data suggest that the neural circuit implicated in the extinction of fear and reward-related memories exhibits changes in glutamatergic neurotransmission in 5-HTT$^{-/-}$ rats.

Stimulating glutamatergic neurotransmission, using the partial *N*-methyl-D-aspartate (NMDA) receptor agonist D-cycloserine (DCS), may provide a good basis for remediation of reduced extinction of cocaine-related memories in 5-HTT$^{-/-}$ rodents. In 2006, Botreau *et al.* inspired addiction research by showing that DCS, which was previously found to facilitate fear extinction in humans and rodents (Walker *et al.* 2002; Ledgerwood, Richardson & Cranney 2003), reduced drug-seeking in a cocaine-induced CPP test when administered systematically (15 mg/kg i.p.) or locally into the Amy (10 μg/side; Botreau, Paolone & Stewart 2006). This DCS effect was replicated in rats self-administrating cocaine by using a higher dose of DCS (30 mg/kg i.p.; Thanos *et al.* 2011) and later translated to humans responding to smoking or alcohol-related cues (Santa Ana *et al.* 2009; MacKillop *et al.* 2015). Notably, not all subsequent human studies were able to replicate these findings for cocaine (Kennedy *et al.* 2012; Santa Ana *et al.* 2015). A possible explanation is the presence of individual variability in responsivity to DCS, which may mask the effect of DCS in a subgroup of addicts who may benefit from treatment. To illustrate this, De Kleine *et al.* (2014) identified personality traits and demographic factors that predicted DCS treatment efficiency in a group of post-traumatic stress disorder patients (De Kleine *et al.* 2014).

In the present study, we set up experiments to investigate if 5-HTT genotype influences the efficacy of DCS to facilitate the extinction of cocaine-seeking behaviour. Firstly, we show altered mRNA expression of specific NMDA receptor (NMDAR) subunits in the vmPFC and Amy of naïve 5-HTT$^{-/-}$ rats. Accordingly, it is our first hypothesis that DCS less effectively facilitates the extinction of cocaine-induced CPP in 5-HTT$^{-/-}$ compared with 5-HTT$^{+/+}$ rats. To confirm this, we demonstrate that 5-HTT$^{-/-}$ rats required a higher DCS dose to facilitate extinction of cocaine-seeking behaviour in a cocaine-induced CPP test. Finally, to delineate the neuronal correlates of the DCS effects on extinction, we assessed neuronal activity by using FosB/ΔFosB immunohistochemistry. The remarkably stable isoform of the FOSB gene, ΔFosB, accumulates in several brain areas after repeated administration of cocaine. Because ΔFosB induction induces cocaine seeking in the CPP and self-administration paradigms (Kelz *et al.* 1999; Nestler, Barrot & Self 2001), it is our second hypothesis that DCS-facilitated reduction of cocaine-seeking behaviour as function of 5-HTT genotype is accompanied by a change in ΔFosB expression in the vmPFC, NAc and Amy.

MATERIALS AND METHODS

Animals

All experiments were approved by the Committee for Animal Experiments (DEC 2011–147) of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, and all efforts were made to minimalize 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 *N*-ethyl-*N*-nitrosourea-induced mutagenesis (Smits *et al.* 2004) and have been described previously (Homberg *et al.* 2007). Heterozygous 5-HTT knockout (5-HTT$^{+/-}$) rats, which had been outcrossed for at least 15 generations with wild-type Wistar rats obtained from Charles River Laboratories (Köln, Germany), were crossed to derive experimental male 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats. The rats started testing at the age of postnatal day 80–90. After weaning at the age of 21 days, ear punches were taken for genotyping. Genotyping was performed by Kbioscience (Hoddesdon, UK), and the procedure of genotyping has been described elsewhere (Homberg *et al.* 2007). The rats were housed in Plexiglas cages in pairs in a temperature (21 ± 1°C) and humidity-controlled room (60 ± 15 percent relative humidity) and had *ad libitum* access to water and food, except during testing. After surgery, the rats were housed individually. A 12-hour light–dark cycle was maintained, with lights on at 08:00 AM. Researchers analysing behavioural data or animal tissues were blinded to the experimental condition and/or genotype.

N-Methyl-D-aspartate receptor mRNA extraction and quantitative real-time polymerase chain reaction

For NMDAR mRNA analysis, naïve adult rats (postnatal day 80–90) were decapitated, and the brains were frozen in aluminium foil on dry ice. The brains were stored at −80°C and cut into 200-μm slices by using a Microm

HM500O cryostat (GMI Inc, USA). The vmPFC, NAc and Amy were then excised according the Paxinos and Watson rat brain atlas by using Harris Uni-CoreTM 2.0 (vmPFC) or Uni-CoreTM 1.2 (NAc and Amy) punches (Jed Pella Inc, USA) and stored at −80°C until further processing (Paxinos & Watson 2006). Total RNA was isolated by a single step of guanidinium isothiocyanate/phenol extraction by using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy), according to the manufacturer's instructions and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time polymerase chain reaction (RT-PCR) to assess NR1, NR2A, NR2B, NR2C and NR2D mRNA levels. An aliquot of each sample was treated with DNase (Tebu-bio, Italy) to avoid DNA contamination. RNA was analysed by a TaqMan quantitative RT-PCR instrument (CFX384 real time system, Bio-Rad Laboratories, Italy) by using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories, Italy). The samples were run in 384-well formats in triplicate as multiplexed reactions with a normalizing internal control (β-actin). The primer probes were purchased from Eurofins MWG-Operon. The primer sequences can be found in Table S1 and have been reported previously (Calabrese *et al.* 2012).

Thermal cycling was initiated with an incubation at 50°C for 10 minutes (RNA retrotranscription) and then at 95°C for 5 minutes (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 seconds to enable the melting process and then for 30 seconds at 60°C for the annealing and extension reactions. A comparative cycle threshold method was used to calculate the relative target gene expression.

Surgery

We chose to apply DCS through the intravenous route for alignment of the present CPP study with future intravenous cocaine self-administration and pharmacological magnetic resonance experiments. Intravenous catheters were implanted in the right jugular vein of 56 rats under isoflurane anaesthesia (initially 5 percent, during surgery 2–3 percent; for more details, see De Vries *et al.* 1998). 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 (0.1 ml; 13.5 mg/ml, 140 units/mg) infusions.

Apparatus

The CPP experiments took place in two, three-compartment place conditioning boxes comprising two identically sized conditioning chambers (27.0 × 27.0 × 26.0 cm, l × w × h) and a neutral middle compartment (10.0 × 27.0 × 26.0 cm, l × w × h). For contextual discrimination, one of the conditioning chambers had black and white checkered walls and a stainless steel grid floor. The grid floor consisted out of 18 stainless steel bars (ø = 6 mm) which were separated (centre–centre) by 8 mm. The other conditioning chamber had black and white striped walls and a black fine wire mesh floor. A reservoir with black bedding was located underneath each grid for waste collection. The middle compartment contained white walls and a grid floor of 10 steel bars (ø = 4 mm) which were separated (centre–centre) by 6 mm. The steel bars were situated along the length of the compartment, at a 90° angle of the rods of the checkered conditioning chamber. Removable inserts between the chambers allowed the rats to either explore the entire apparatus or be confined to one conditioning chamber. A camera (Sony, Japan) connected to a PC running EthoVision 3.1 software (Noldus, the Netherlands) was mounted near the ceiling, enabling live position tracking of the rats.

Conditioned place preference

The CPP experiments comprised three phases which spanned a total of 11 days, pretest (1 day), conditioning (8 days), and posttests (2 days). During the pretest, the rats (5-HTT$^{+/+}$ n = 31; 5-HTT$^{-/-}$ n = 25) were placed in the middle compartment and were then allowed to explore all three chambers freely for 15 minutes in order to determine the initial preference of the rats. Cocaine-paired chambers (CS+) were assigned in a counterbalanced fashion, meaning that half the rats were conditioned in their initially preferred compartment and half in their initially non-preferred compartment (effort was made to attain a 50–50 distribution of checked and striped CS+ compartments). During the conditioning phase, the rats were injected with either cocaine (CS+, 10 mg/kg, 1 ml/kg) or saline (CS−, 1 ml/kg) on alternating days (Homberg *et al.* 2008). The rats were then confined the corresponding conditioning chamber for 40 minutes. Twenty-four hours after the last conditioning session, a 15-minute posttest was performed, in which the rats were placed in the middle compartment and allowed to freely explore all compartments in a cocaine-free state. Directly after this first posttest, 0, 1 or 5 mg/kg of DCS (Sigma-Aldrich, USA) was administered intravenously. Twenty-four hours later, the effects of DCS on CPP were tested by performing an additional 15-minute posttest. End variable (ΔCS+) was calculated by subtracting time spent in CS+ compartment during pretest from time spent in CS+ compartment during each of the posttests (Homberg *et al.* 2008).

Tissue preparation

For ΔFosB staining, the rats that performed CPP were anesthetized 24 hours after the final posttest by using 120 mg/kg pentobarbital after which they were transcardially perfused by using 0.1 M phosphate buffered saline (PBS, pH = 7.2) and 4 percent paraformaldehyde (PFA, pH = 7.3) to fix the brains. The brains were then removed from the skulls and stored overnight in 4 percent PFA at 4°C for post fixation. The next day, the PFA was replaced with 0.1 M PBS containing 0.05 percent sodium azide. Prior to sectioning, the brains were transferred to 30 percent sucrose solution at room temperature for 2 days, and a freezing microtome was used to slice sections of 40 μm. The slices were stored in PBS containing azide until immunostaining.

FosB/ΔFosB immunohistochemistry

Because no reliable commercially available antibody for ΔFosB exists, we used an antibody that labels both FosB and ΔFosB and sacrificed animals 24 hours after performing the last posttest. Therefore, any differences found in FosB/ΔFosB expression are likely caused by ΔFosB (Hope *et al.* 1994; Perrotti *et al.* 2008; El Rawas *et al.* 2012).

Slices were taken from storage and washed three times for 10 minutes in 0.1 M PBS (pH = 7.2). Endogenous peroxidase activity was blocked by incubating the slices in 0.1 M PBS containing 1 percent hydrogen peroxide. After three washing steps, the tissue was pre-incubated for 1 hour with 0.1 M PBS containing 0.1 percent bovine serum albumin (Sigma-Aldrich) and 0.5 percent Triton X-100 (Sigma-Aldrich, PBS-BT). After this pre-incubation period, a rabbit anti-FosB antibody (sc-48, Santa Cruz, 1:2000) was added and left to incubate overnight (El Rawas *et al.* 2012). The slices were then washed three times by using 0.1 M PBS and incubated with a donkey antirabbit biotin-conjugated antibody (Jackson ImmunoResearch Laboratories, 1:1500 in PBS-BT) for 90 minutes. After three more washing steps, the slices were incubated with ABC vector elite (1:800 in PBS-BT). Following three more washing steps, immunolabelling was visualized by 3,3-diaminobenzedine tetrahydrochloride. To this end, sections were incubated for 10 minutes in a solution containing 0.02 percent 3,3-diaminobenzedine tetrahydrochloride and 0.03 percent nickel–ammonium sulphate in 0.05 M Tris buffer (pH = 7.6) after which 0.006 percent hydrogen peroxide was added for 10 minutes. The reaction was then stopped by washing three times in PBS. Subsequently, the slices were mounted on gelatin chrome alum-coated glass slides, dried overnight at 37°C, dehydrated in ascending alcohol concentrations, cleared in Xylol (Merck), embedded in Entellan (Merck) and coverslipped.

Statistical analysis

All statistical analyses were performed by using IBM SPSS version 20.0 (IBM software, USA). All data were checked for outliers and normality (using the Shapiro–Wilk statistic). NMDAR subunit mRNA expression was compared by using genotype (two levels) × subunit (five levels) univariate analysis of variance (ANOVA). Post-hoc independent sample *t*-tests were performed where applicable. To assess whether cocaine-induced CPP induction was successful, the data from posttest 1 were pooled per genotype and one sample *t*-test was performed. The overall effects of genotype and DCS treatment on CPP and locomotor activity were analysed by using a genotype (two levels) × dose (three levels) × time (two levels) repeated measures ANOVA. Post-hoc paired sample *t*-tests were performed when appropriate. ΔFosB immunoreactivity was analysed by using genotype (two levels) × dose (three levels) univariate ANOVAs. Level of significance was set at $P < 0.05$, and non-significant effects are indicated by P = NS.

RESULTS

N-Methyl-D-aspartate receptor subunit expression

Relevant NMDAR subunits were analysed in the vmPFC, NAc and Amy. As is shown in Figure 1, there was a significant genotype * subunit effect in the vmPFC [$F_{(4,109)} = 30.695$, $P < 0.001$] and NAc [$F_{(4,110)} = 3.726$, $P < 0.01$]. Post-hoc analysis revealed that, compared with 5-HTT$^{+/+}$ rats, 5-HTT$^{-/-}$ rats had a higher expression of NR2A [$t_{(22)} = 8.62$, $P < 0.001$] and lower expression of NR1 [$t_{(21)} = -3.02$, $P < 0.01$] in the vmPFC and a lower expression of NR2C [$t_{(22)} = -5.13$, $P < 0.001$] in the NAc. All other genes did not differ significantly.

Conditioned place preference

Data acquired during posttest 1 were pooled per genotype as displayed in Figure 2. Both 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats were able to acquire CPP [one sample *t*-test, 5-HTT$^{+/+}$: $t_{(30)} = 3.027$, $P < 0.05$; 5-HTT$^{-/-}$: $t_{(22)} = 3.607$, $P < 0.05$]. No difference between genotypes was observed [independent sample *t*-test, $t_{(1,52)} = 0.459$, P = NS].

For visualizing the effects of DCS on the extinction of CPP, data acquired during posttests 1 and 2 were split per genotype as seen in Figure 3a and b. Repeated measures ANOVA revealed a significant dose * time interaction [$F_{(2,48)} = 3.198$, $P < 0.05$]. Post-hoc paired sample

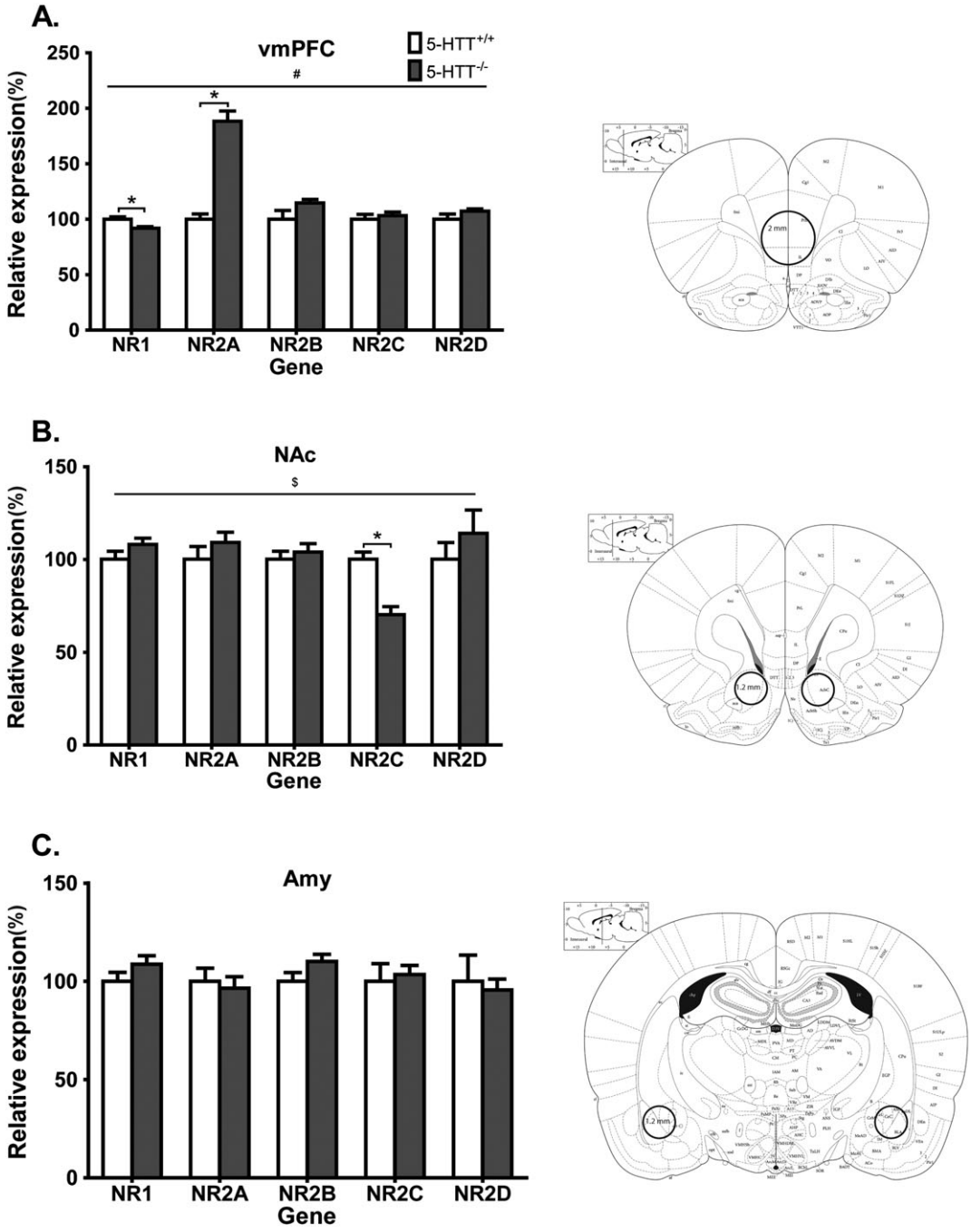

Figure 1 *N*-methyl-D-aspartate receptor subunit mRNA expression. Relative expression of NR1 and NR2 subunit mRNAs in (a) ventromedial prefrontal cortex, (b) amygdala and (c) nucleus accumbens (NAc), including diagrams representing where tissue was collected (adapted from Paxinos & Watson brain atlas). Wild-type serotonin transporter rats are represented by white bars and serotonin transporter knockout rats by grey bars; all data represent mean (±SEM). Overall genotype * subunit interaction was found in the ventromedial prefrontal cortex [$^{\#}F_{(4,109)} = 30.695$, $P < 0.001$] and NAc [$^{\$}F_{(4,110)} = 3.726$, $P < 0.01$]. Post-hoc independent sample *t*-test analysis revealed that serotonin transporter knockout rats show a lower expression of NR1 [$^{**}t_{(21)} = -3.017$, $P < 0.01$] and higher expression of NR2A in the ventromedial prefrontal cortex [$^{***}t_{(22)} = 8.620$, $P < 0.001$] as well as a lower expression of NR2C in the NAc [$^{***}t_{(22)} = -5.129$, $P < 0.001$]

t-tests between CPPs measured on posttest I and II revealed that vehicle-treated rats had no significant extinction of their CPP [5-HTT$^{+/+}$: $t_{(9)} = -0.490$, P = NS and 5-HTT$^{-/-}$: $t_{(7)} = -0.866$, P = NS]. However, CPP was significantly reduced in 5-HTT$^{+/+}$ [$t_{(10)} = 3.575$, $P < 0.01$] but not in 5-HTT$^{-/-}$ [$t_{(6)} = 0.414$, P = NS] rats that received 1 mg/kg DCS. In contrast, CPP was significantly reduced in 5-HTT$^{-/-}$ [$t_{(7)} = 2.465$, $P < 0.05$] but

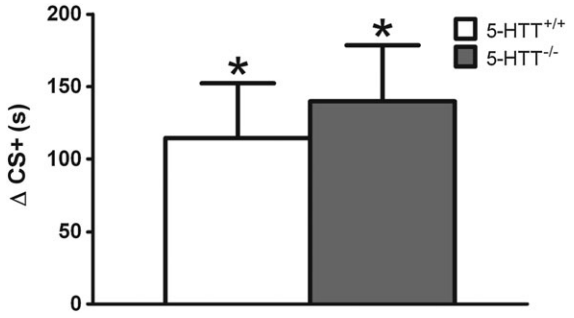

Figure 2 Posttest 1 place preference induction in wild-type serotonin transporter (5-HTT$^{+/+}$) and serotonin transporter knockout (5-HTT$^{-/-}$) rats. 5-HTT$^{+/+}$ rats are represented by white bars and 5-HTT$^{-/-}$ rats by grey bars; ΔCS+ was calculated by subtracting time spent in CS+ compartment during pretest from time spent in CS+ compartment during the first posttest. All data are represented as mean (±SEM). (a) ΔCS+ representing cocaine-induced conditioned place preference in 5-HTT$^{+/+}$ versus 5-HTT$^{-/-}$ rats during posttest 1. Both genotypes were able to acquire conditioned place preference [one sample *t*-test, 5-HTT$^{+/+}$: $t_{(30)} = 3.027$, $^{*}P < 0.05$; 5-HTT$^{-/-}$: $t_{(22)} = 3.607$, $^{*}P < 0.05$]. No difference between genotypes was observed [independent sample *t*-test, $t_{(1,52)} = 0.459$, P = NS]

not in 5-HTT$^{+/+}$ [$t_{(9)} = 1.225$, P = NS] rats that received 5 mg/kg DCS. Place preference behaviour can be divided into several components (Huston *et al.* 2013). In Figure S1A and B, we show that the number of CS+ compartment entries and the time per CS+ entry did not differ between genotypes, nor were they influenced by DCS treatment [entries: repeated measures ANOVA $F_{(2,48)} = 1.373$, P = NS, time per entry: repeated measures ANOVA: $F_{(2,48)} = 1.453$, P = NS]. General locomotor activity (expressed as distance moved) during both posttests is shown in Figure S1C. No significant difference in locomotor activity was observed by using a repeated measures ANOVA [$F_{(2,48)} = 0.407$, P = NS].

Immunohistochemistry

Twenty-four hours after, the last posttest animals were transcardially perfused and slices were stained for FosB/ΔFosB. Figure 4a–c shows the density of positive cells that was assessed in vmPFC, NAc and Amy. The 5-HTT$^{-/-}$ rats showed lower overall expression of ΔFoSB in the vmPFC [$F_{(1,46)} = 6.433$, $P < 0.05$]. Additionally, the density of ΔFosB positive cells in the Amy was significantly lower in 5-HTT$^{-/-}$ [$F_{(1,46)} = 4.665$, $P < 0.05$] rats, while there was a trend towards significance caused by the DCS treatment [$F_{(2,46)} = 3.018$, $P = 0.059$]. The most robust treatment effect was observed in the NAc where DCS-treated animals showed an overall lower density of ΔFosB positive cells when compared with the vehicle control [$F_{(2,47)} = 7.469$, $P < 0.01$].

DISCUSSION

In the experiments in the preceding text, we show that naïve 5-HTT$^{-/-}$ rats display changes in NMDAR subunit mRNA expression. More specifically, when compared with 5-HTT$^{+/+}$ rats, NR1 subunit mRNA is reduced and NR2A subunit mRNA expression is increased in the vmPFC and NR2C subunit expression is reduced in the NAc of 5-HTT$^{-/-}$ rats. In line with this observation and our first hypothesis, we found that 5-HTT$^{-/-}$, compared with 5-HTT$^{+/+}$, rats required a higher dose of DCS to potentiate the extinction of cocaine-induced CPP. This suggests that 5-HTT$^{-/-}$ rats are less responsive to DCS. Contrary to our second hypothesis, these behavioural differences were not mirrored by reduced ΔFosB expression in the same groups. Nonetheless, we did observe significant genotype effects in the vmPFC and Amy and an effect of DCS treatment in the NAc.

N-Methyl-D-aspartate receptors are functional heterotetramers most often formed by combining two NR1 subunits and two NR2 subunits. The NR1 subunits present in the protein contain the glycine binding site, and thus the binding site for DCS (Hood, Compton & Monahan 1989). Interestingly, it is the NR2 subunit that determines the efficacy of DCS on the receptor complex. As such, it has been shown that DCS exerts its function mostly through receptors consisting of NR1 and NR2C subunits (NR1/NR2C receptors) and less through NR1/NR2A and NR1/NR2B receptors (Sheinin, Shavit & Benveniste 2001; Dravid *et al.* 2010). We showed that the 5-HTT$^{-/-}$ rats have lower NR1 and higher NR2A subunit mRNA expression in the vmPFC. These results imply that there is, relative to NR1/NR2A and NR1/NR2B receptors, a lower number of NR1/NR2C receptors available for DCS to exhibit its beneficial effects during extinction consolidation. A similar shift in receptor ratio is seen in the NAc, although it is caused by a direct downregulation of NR2C subunit mRNA. Functionally, this is reflected by the higher DCS dose that was required in our 5-HTT$^{-/-}$ rats to facilitate extinction retention.

Here, we reproduced the finding reported by others (Botreau *et al.* 2006) that DCS increases the extinction of cocaine-induced CPP in wild-type rats and show that this is also possible in 5-HTT$^{-/-}$ rats at a higher dose. As mentioned in the Introduction, some human studies reported beneficial effects of DCS on the extinction of cue-induced drug craving (Santa Ana *et al.* 2009; MacKillop *et al.* 2015). However, multiple human studies have found no effect of DCS on extinction of cocaine-associated cues (Kennedy *et al.* 2012; Santa Ana *et al.* 2015) or even enhanced reactivity after treatment (Price *et al.* 2013; Prisciandaro *et al.* 2013). Although these contrasting outcomes may be related to methodological choices, it is also possible that these differences are

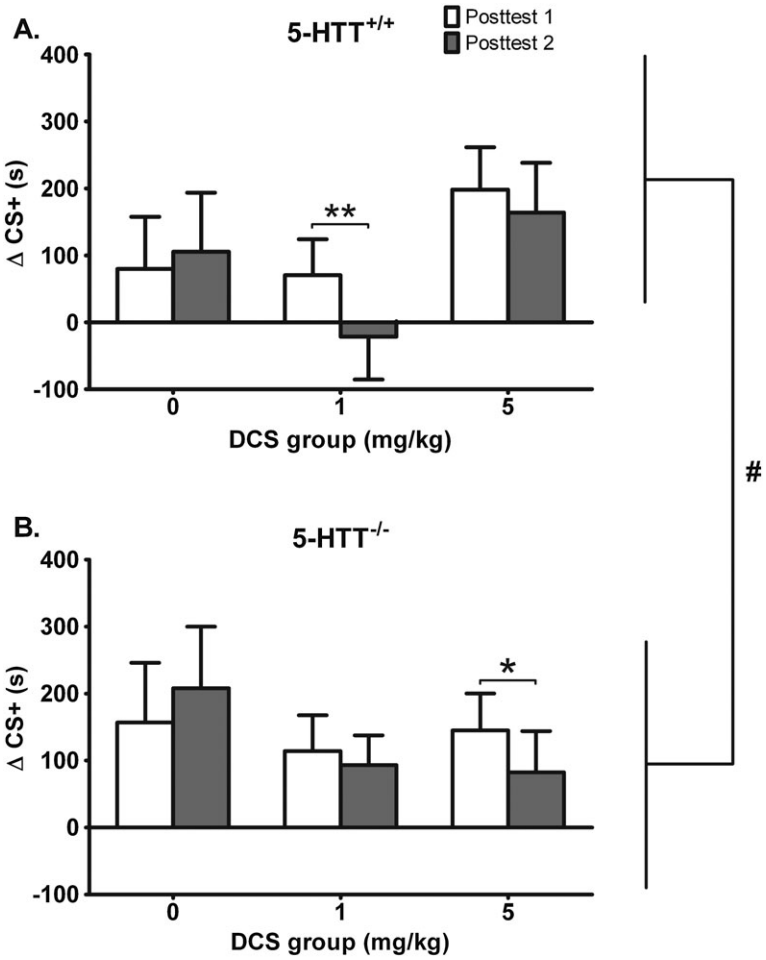

Figure 3 Effects of single i.v. D-cycloserine (DCS) injection on conditioned place preference (CPP) behaviour of wild-type serotonin transporter (5-HTT$^{+/+}$) and serotonin transporter knockout (5-HTT$^{-/-}$) rats. For visual clarity: (a) 5-HTT$^{+/+}$ rats and (b) 5-HTT$^{-/-}$; all data represented mean of ΔCS+ (±SEM), and both genotypes were analysed together. ΔCS+ was calculated by subtracting time spent in CS+ compartment during pretest from time spent in CS+ compartment during each of the posttests. Data acquired during posttest I are represented by white bars and posttest II by grey bars. Repeated measures ANOVA revealed a significant DCS dose * posttest interaction [$^{\#}F_{(2,48)} = 3.198$, $P < 0.05$]. Post-hoc paired samples *t*-tests revealed a significant lower CPP during posttest II in 5-HTT$^{+/+}$ rats treated with 1 mg/kg DCS [$n = 11$, $^{**}t_{(10)} = 3.575$, $P < 0.01$] and 5-HTT$^{-/-}$ rats treated with 5 mg/kg DCS [$n = 8$, $^{*}t_{(7)} = 2.465$, $P < 0.05$]. No reduction of CPP was observed in control rats (0 mg/kg, 5-HTT$^{+/+}$ $n = 10$, 5-HTT$^{-/-}$ $n = 8$), 5-HTT$^{-/-}$ rats treated with 1 mg/kg ($n = 7$) DCS and 5-HTT$^{+/+}$ rats treated with 5 mg/kg DCS ($n = 10$)

dependent on the nature of the study population. In this light, we show here that the 5-HTT genotype may influence the efficacy of DCS to potentiate the extinction of cocaine-related memories. As the 5-HTT$^{-/-}$ rat model has higher levels of trait anxiety before and after cocaine administration (Homberg *et al.* 2008; Verheij *et al.* 2014), our results are in line with the findings by Ho *et al.*, showing that the effectiveness of DCS is lowered in a rat model specifically bred for high anxiety compared with low anxiety counterparts (Ho *et al.* 2005). It is also noteworthy that the 5-HTT$^{+/+}$ rats did not benefit from increased (5 mg/kg) DCS treatment, suggesting an inverted U-shape functionality of DCS on behaviour. The human studies mentioned in the preceding text all used the same dose of DCS (50 mg), and all highlight the importance of dosing when discussing their results. In this light, our results confirm that there are individual differences in the effectiveness of as both genotypes only responded to a single (different) dose.

ΔFosB is a transcription factor that has been theorized to be a molecular switch for addiction behaviour, where high levels of the protein in the NAc trigger cocaine seeking (Nestler 2001). Here, we hypothesized that ΔFosB immunoreactivity would go down as a result of DCS treatment. DCS is thought to reduce CPP through actions in the NAc (Torregrossa *et al.* 2010). In line with this, we observed a significant DCS dose effect (independent of genotype) in this brain area. Additionally, the NAc is also the brain area to which most drug-seeking effects of ΔFosB are attributed (Nestler 2008). We also observed the overall lower expression of ΔFosB in 5-HTT$^{-/-}$ rats (irrespective of DCS dose) in the vmPFC and Amy. Even though it has been shown that, like in the NAc, repeated drug administration induces ΔFosB in these areas (Perrotti *et al.* 2008), the role of ΔFosB in cocaine-seeking behaviour in these areas has not been extensively researched. Interestingly, the finding that ΔFosB expression is reduced in the vmPFC of 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats is in line with previous observations of reduced vmPFC activity and immediate early gene expression in 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats (Molteni *et al.* 2009; Pang *et al.* 2011), as well as reduced medial prefrontal activity in human 5-HTTLPR s-allele carriers exposed to emotion provoking stimuli (Pezawas *et al.* 2005; Munafo, Brown, & Hariri 2008). The lower FosB/ΔFosB immunoreactivity in the Amy of 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats seems to contradict previous studies reporting increased baseline Amy (re)activity in 5-HTT$^{-/-}$ rodents and humans carrying

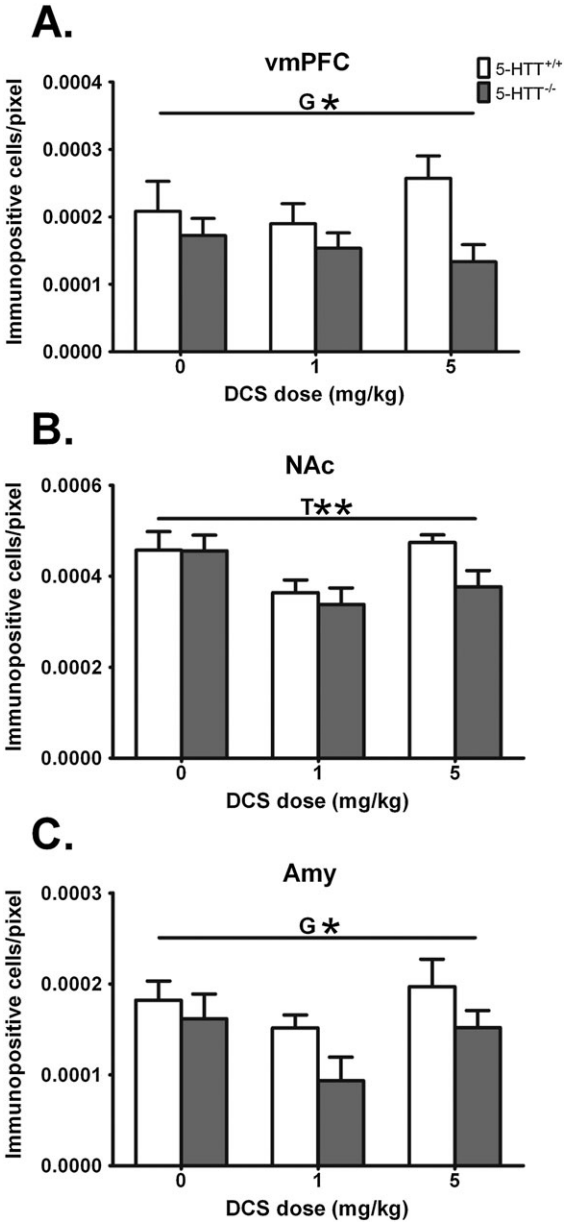

Figure 4 FoSB/ΔFosB immunoreactivity in serotonin transporter knockout (5-HTT$^{-/-}$) rats after D-cycloserine (DCS)-assisted conditioned place preference. Relative expression of immunopositive cells in the (a) ventromedial prefrontal cortex, (b) nucleus accumbens (B) and (c) amygdala. Wild-type serotonin transporter rats are represented as white bars and 5-HTT$^{-/-}$ as grey bars. All data are represented as mean of immunopositive cellular density (±SEM). The statistical outcome of the ANOVA is designated with (G) for overall genotype effect and (T) for overall DCS treatment effect. 5-HTT$^{-/-}$ rats displayed an overall lower number of FosB positive cells in the ventromedial prefrontal cortex [$^{G*}F_{(1,50)} = 4.688$, $P < 0.05$) and amygdala [$^{G*}F_{(1,46)} = 4.665$, $P < 0.05$]. Additionally, DCS treatment seemed to lower the number of FosB positive cells in the nucleus accumbens [$^{T**}F_{(2,49)} = 5.625$, $P < 0.01$]

the 5-HTTLPR s-allele (Hariri *et al.* 2002; Pang *et al.* 2011). Yet, because we do not know which neurons expressed FosB/ΔFosB, additional studies are needed to properly interpret these findings in terms of area activity levels. Even though these past observations in vmPFC and Amy were made under drug-free conditions and therefore may not reflect the same neurobiological state, FosB/ΔFosB immunoreactivity is lowered in the vmPFC and NaC of animals that are susceptive to develop anxiety and depression-like behaviours after social defeat stress (Vialou *et al.* 2014). Because 5-HTT$^{-/-}$ rodents show depression and anxiety-like behaviours (Holmes, Murphy & Crawley 2003; Olivier *et al.* 2008), which are also more exaggerated after social defeat stress (Jansen *et al.* 2010), it is possible that the lower FosB/ΔFosB expression observed here reflects an anxious state and is not (or not exclusively) related to DCS treatment.

There are some shortcomings in the present study. Differences in mRNA expression do not always translate to differences in protein expression. However, the effect size of especially the NR2A subunit in vmPFC is quite considerable and as such is likely to translate into differences at the protein level. To further determine the respective roles of different NMDARs in the working mechanism of DCS, it could be crucial for future studies to examine the NMDAR composition in the projection terminals of the vmPFC (e.g. Amy and NAc) to confirm these mRNA results. Furthermore, we were unable to reproduce our finding that cocaine-induced CPP is increased in the 5-HTT$^{-/-}$ rats (Homberg *et al.* 2008). This discrepancy may be attributed to major differences in methodology. In the present study, the animals required surgery and single housing which may have altered their sensitivity to cocaine (Nader *et al.* 2012). Additionally, we only used a single injection of DCS to extinguish CPP-seeking behaviour and are therefore unaware what repeated exposure may reveal.

In conclusion, in the preceding texts, we show that a genetic variation in serotonin transporter expression can be a predictor for the efficiency of DCS. It is possible that this reduction in sensitivity to DCS treatment is caused by a lower expression of NR1/NR2C NMDAR. Future studies should focus on confirming this difference in the expression on the protein level and the underlying mechanisms causing this shift.

Acknowledgments

The work described in this paper was funded by The Netherlands Organisation 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.

AUTHORS CONTRIBUTION

PK, JRH and LR were responsible for the study concept and design. FC and PB acquired the mRNA data, PK acquired the animal behaviour data, and PK and BV acquired the immunohistochemical data. Data analysis and interpretation were performed by PK. PK drafted the manuscript, and JRH, MV and MR provided the critical revision of the manuscript. All authors critically reviewed the content and approved the final version for publication.

References

Ahmed SH, Koob GF (1998) Transition from moderate to excessive drug intake: change in hedonic set point. Science 282:298–300.

Botreau F, Paolone G, Stewart J (2006) D-Cycloserine facilitates extinction of a cocaine-induced conditioned place preference. Behav Brain Res 172:173–178.

Burgos-Robles A, Vidal-Gonzalez I, Santini E, Quirk GJ (2007) Consolidation of fear extinction requires NMDA receptor-dependent bursting in the ventromedial prefrontal cortex. Neuron 53:871–880.

Calabrese F, Guidotti G, Molteni R, Racagni G, Mancini M, Riva MA (2012) Stress-induced changes of hippocampal NMDA receptors: modulation by duloxetine treatment. PLoS One 7: e37916.

Cao J, Hudziak JJ, Li D (2013) Multi-cultural association of the serotonin transporter gene (SLC6A4) with substance use disorder. Neuropsychopharmacology 38:1737–1747.

De Kleine RA, Hendriks GJ, Smits JA, Broekman TG, van Minnen A (2014) Prescriptive variables for D-cycloserine augmentation of exposure therapy for posttraumatic stress disorder. J Psychiatr Res 48:40–46.

De Vries TJ, Schoffelmeer AN, Binnekade R, Mulder AH, Vanderschuren LJ (1998) MK-801 reinstates drug-seeking behaviour in cocaine-trained rats. Neuroreport 9:637–640.

Dravid SM, Burger PB, Prakash A, Geballe MT, Yadav R, Le P, Vellano K, Snyder JP, Traynelis SF (2010) Structural determinants of D-cycloserine efficacy at the NR1/NR2C NMDA receptors. J Neurosci 30:2741–2754.

El Rawas R, Klement S, Salti A, Fritz M, Dechant G, Saria A, Zernig G (2012) Preventive role of social interaction for cocaine conditioned place preference: correlation with FosB/DeltaFosB and pCREB expression in rat mesocorticolimbic areas. Front Behav Neurosci 6:8.

Feinn R, Nellissery M, Kranzler HR (2005) Meta-analysis of the association of a functional serotonin transporter promoter polymorphism with alcohol dependence. Am J Med Genet B Neuropsychiatr Genet 133B:79–84.

Gokturk C, Schultze S, Nilsson KW, von Knorring L, Oreland L, Hallman J (2008) Serotonin transporter (5-HTTLPR) and monoamine oxidase (MAOA) promoter polymorphisms in women with severe alcoholism. Arch Womens Ment Health 11:347–355.

Hariri AR, Mattay VS, Tessitore A, Kolachana B, Fera F, Goldman D, Egan MF, Weinberger DR (2002) Serotonin transporter genetic variation and the response of the human amygdala. Science 297:400–403.

Heils A, Teufel A, Petri S, Stober G, Riederer P, Bengel D, Lesch KP (1996) Allelic variation of human serotonin transporter gene expression. J Neurochem 66:2621–2624.

Ho YJ, Hsu LS, Wang CF, Hsu WY, Lai TJ, Hsu CC, Tsai YF (2005) Behavioral effects of d-cycloserine in rats: the role of anxiety level. Brain Res 1043:179–185.

Holmes A, Murphy DL, Crawley JN (2003) Abnormal behavioral phenotypes of serotonin transporter knockout mice: parallels with human anxiety and depression. Biol Psychiatry 54:953–959.

Homberg JR, De Boer SF, Raaso HS, Olivier JD, Verheul M, Ronken E, Cools AR, Ellenbroek BA, Schoffelmeer AN, Vanderschuren LJ, De Vries TJ, Cuppen E (2008) Adaptations in pre- and postsynaptic 5-HT1A receptor function and cocaine supersensitivity in serotonin transporter knockout rats. Psychopharmacology (Berl) 200:367–380.

Homberg JR, Olivier JD, Smits BM, Mul JD, Mudde J, Verheul M, Nieuwenhuizen OF, Cools AR, Ronken E, Cremers T, Schoffelmeer AN, Ellenbroek BA, Cuppen E (2007) Characterization of the serotonin transporter knockout rat: a selective change in the functioning of the serotonergic system. Neuroscience 146:1662–1676.

Hood WF, Compton RP, Monahan JB (1989) D-cycloserine: a ligand for the *N*-methyl-D-aspartate coupled glycine receptor has partial agonist characteristics. Neurosci Lett 98:91–95.

Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, Duman RS, Nestler EJ (1994) Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. Neuron 13:1235–1244.

Huston JP, Silva MA, Topic B, Muller CP (2013) What's conditioned in conditioned place preference? Trends Pharmacol Sci 34:162–166.

Jansen F, Heiming RS, Lewejohann L, Touma C, Palme R, Schmitt A, Lesch KP, Sachser N (2010) Modulation of behavioural profile and stress response by 5-HTT genotype and social experience in adulthood. Behav Brain Res 207:21–29.

Kelz MB, Chen J, Carlezon WA Jr, Whisler K, Gilden L, Beckmann AM, Steffen C, Zhang YJ, Marotti L, Self DW, Tkatch T, Baranauskas G, Surmeier DJ, Neve RL, Duman RS, Picciotto MR, Nestler EJ (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. Nature 401:272–276.

Kennedy AP, Gross RE, Whitfield N, Drexler KP, Kilts CD (2012) A controlled trial of the adjunct use of D-cycloserine to facilitate cognitive behavioral therapy outcomes in a cocaine-dependent population. Addict Behav 37:900–907.

LaLumiere RT, Niehoff KE, Kalivas PW (2010) The infralimbic cortex regulates the consolidation of extinction after cocaine self-administration. Learn Mem 17:168–175.

Ledgerwood L, Richardson R, Cranney J (2003) Effects of D-cycloserine on extinction of conditioned freezing. Behav Neurosci 117:341–349.

MacKillop J, Few LR, Stojek MK, Murphy CM, Malutinok SF, Johnson FT, Hofmann SG, McGeary JE, Swift RM, Monti PM (2015) D-Cycloserine to enhance extinction of cue-elicited craving for alcohol: a translational approach. Transl Psychiatry 5:e544.

Molteni R, Calabrese F, Maj PF, Olivier JD, Racagni G, Ellenbroek BA, Riva MA (2009) Altered expression and modulation of activity-regulated cytoskeletal associated protein (Arc) in serotonin transporter knockout rats. Eur Neuropsychopharmacol 19:898–904.

Munafo MR, Brown SM, Hariri AR (2008) Serotonin transporter (5-HTTLPR) genotype and amygdala activation: a meta-analysis. Biol Psychiatry 63:852–857.

Nader J, Chauvet C, Rawas RE, Favot L, Jaber M, Thiriet N, Solinas M (2012) Loss of environmental enrichment increases

vulnerability to cocaine addiction. Neuropsychopharmacology 37:1579–1587.
Nestler EJ (2008) Transcriptional mechanisms of addiction: role of ΔFosB. Philosophical Transactions of the Royal Society of London B: Biological Sciences 363:3245–3255.
Nestler EJ, Barrot M, Self DW (2001) DeltaFosB: a sustained molecular switch for addiction. Proc Natl Acad Sci U S A 98:11042–11046.
Nonkes LJ, de Pooter M, Homberg JR (2012) Behavioural therapy based on distraction alleviates impaired fear extinction in male serotonin transporter knockout rats. J Psychiatry Neurosci 37:224–230.
Olivier JD, Van Der Hart MG, Van Swelm RP, Dederen PJ, Homberg JR, Cremers T, Deen PM, Cuppen E, Cools AR, Ellenbroek BA (2008) A study in male and female 5-HT transporter knockout rats: an animal model for anxiety and depression disorders. Neuroscience 152:573–584.
Pang RD, Wang Z, Klosinski LP, Guo Y, Herman DH, Celikel T, Dong HW, Holschneider DP (2011) Mapping functional brain activation using [14C]-iodoantipyrine in male serotonin transporter knockout mice. PLoS One 6:e23869.
Paxinos G, Watson C (2006) The Rat Brain in Stereotaxic Coordinates, 6th edn. Elsevier Academic Press: Amsterdam; Boston.
Perrotti LI, Weaver RR, Robison B, Renthal W, Maze I, Yazdani S, Elmore RG, Knapp DJ, Selley DE, Martin BR, Sim-Selley L, Bachtell RK, Self DW, Nestler EJ (2008) Distinct patterns of DeltaFosB induction in brain by drugs of abuse. Synapse 62:358–369.
Peters J, Kalivas PW, Quirk GJ (2009) Extinction circuits for fear and addiction overlap in prefrontal cortex. Learn Mem 16:279–288.
Pezawas L, Meyer-Lindenberg A, Drabant EM, Verchinski BA, Munoz KE, Kolachana BS, Egan MF, Mattay VS, Hariri AR, Weinberger DR (2005) 5-HTTLPR polymorphism impacts human cingulate-amygdala interactions: a genetic susceptibility mechanism for depression. Nat Neurosci 8:828–834.
Popik P, Wrobel M, Bisaga A (2006) Reinstatement of morphine-conditioned reward is blocked by memantine. Neuropsychopharmacology 31:160–170.
Price KL, Baker NL, McRae-Clark AL, Saladin ME, Desantis SM, Santa Ana EJ, Brady KT (2013) A randomized, placebo-controlled laboratory study of the effects of D-cycloserine on craving in cocaine-dependent individuals. Psychopharmacology (Berl) 226:739–746.
Prisciandaro JJ, Myrick H, Henderson S, McRae-Clark AL, Santa Ana EJ, Saladin ME, Brady KT (2013) Impact of DCS-facilitated cue exposure therapy on brain activation to cocaine cues in cocaine dependence. Drug Alcohol Depend 132:195–201.
Santa Ana EJ, Prisciandaro JJ, Saladin ME, McRae-Clark AL, Shaftman SR, Nietert PJ, Brady KT (2015) D-Cycloserine combined with cue exposure therapy fails to attenuate subjective and physiological craving in cocaine dependence. Am J Addict 24:217–224.
Santa Ana EJ, Rounsaville BJ, Frankforter TL, Nich C, Babuscio T, Poling J, Gonsai K, Hill KP, Carroll KM (2009) D-Cycloserine attenuates reactivity to smoking cues in nicotine dependent smokers: a pilot investigation. Drug Alcohol Depend 104:220–227.
Schipper P, Kiliaan AJ, Homberg JR (2011) A mixed polyunsaturated fatty acid diet normalizes hippocampal neurogenesis and reduces anxiety in serotonin transporter knockout rats. Behav Pharmacol 22:324–334.
Sheinin A, Shavit S, Benveniste M (2001) Subunit specificity and mechanism of action of NMDA partial agonist D-cycloserine. Neuropharmacology 41:151–158.
Smits BM, Mudde J, Plasterk RH, Cuppen E (2004) Target-selected mutagenesis of the rat. Genomics 83:332–334.
Smits BM, Mudde JB, van de Belt J, Verheul M, Olivier J, Homberg J, Guryev V, Cools AR, Ellenbroek BA, Plasterk RH, Cuppen E (2006) Generation of gene knockouts and mutant models in the laboratory rat by ENU-driven target-selected mutagenesis. Pharmacogenet Genomics 16:159–169.
Thanos PK, Bermeo C, Wang GJ, Volkow ND (2011) D-Cycloserine facilitates extinction of cocaine self-administration in rats. Synapse 65:938–944.
Verheij MMM, Contet C, Karel P, Latour J, Van der Doelen RHA, Kozicz T, Homberg JR, Koob GF (2014) Dorsal raphe serotonergic projections play a critical role in cocaine escalation. In: NIDA International Forum: San Juan, Puerto Rico.
Verheij MM, Contet C, Nonkes L, Latour J, Hanisch J, Vendruscolo L, Edwards S, Koob GF, Homberg JR (2012) Serotonin transporter gene variation and compulsive cocaine self-administration in rats. In: Society for Neuroscience 42nd annual meeting: New Orleans, USA.
Vialou V, Bagot RC, Cahill ME, Ferguson D, Robison AJ, Dietz DM, Fallon B, Mazei-Robison M, Ku SM, Harrigan E, Winstanley CA, Joshi T, Feng J, Berton O, Nestler EJ (2014) Prefrontal cortical circuit for depression- and anxiety-related behaviors mediated by cholecystokinin: role of DeltaFosB. J Neurosci 34:3878–3887.
Walker DL, Ressler KJ, Lu KT, Davis M (2002) Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats. J Neurosci 22:2343–2351.
Wellman CL, Izquierdo A, Garrett JE, Martin KP, Carroll J, Millstein R, Lesch KP, Murphy DL, Holmes A (2007) Impaired stress-coping and fear extinction and abnormal corticolimbic morphology in serotonin transporter knock-out mice. J Neurosci 27:684–691.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. NMDAR subunit qPCR primers and probes
Figure S1. CPP CS+ entries and locomotion behaviour. 5-HTT$^{+/+}$ rats are represented by white bars and 5-HTT$^{-/-}$ rats by grey bars, all data represent mean (±SEM). A) CS+ entries of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats during posttest 1 and 2. CS+ entries did not change by treatment or genotype (repeated measures ANOVA $F(2,48) = 1.373$, p = NS). B) Time spent in CS+ compartment per visit was neither influenced by DCS treatment nor by DCS treatment (repeated measures ANOVA $F(2,48) = 1.453$, p = NS). C) Locomotion of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ did not change between the posttests and did not differ between genotypes (repeated measures ANOVA, $F(2, 48) = 0.407$, p = NS)