Increased cocaine self-administration in rats lacking the serotonin transporter: a role for glutamatergic signaling in the habenula

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Conflicts of Interest

The authors declare no conflict of interest in relation to the work herein described.

Abstract

Serotonin (5-HT) and the habenula (Hb) contribute to motivational and emotional states such as depression and drug abuse. The dorsal raphe nucleus, where 5-HT neurons originate, and the Hb are anatomically and reciprocally interconnected. Evidence exists that 5-HT influences Hb glutamatergic transmission. Using serotonin transporter knockout ($SERT^{-/-}$) rats, which show depression-like behavior and increased cocaine intake, we investigated the effect of SERT reduction on expression of genes involved in glutamate neurotransmission under both baseline conditions as well as after short-or long-access cocaine (ShA and LgA, respectively) intake. In cocaine-naïve animals, SERT removal led to reduced baseline Hb mRNA levels of critical determinants of glutamate transmission, such as *SLC1A2*, the main glutamate transporter and NMDA (*Grin1*, *Grin2A* and *Grin2B*) as well as AMPA (*Gria1* and *Gria2*) receptor subunits, with no changes in the scaffolding protein *Dlg4*. In response to ShA and LgA cocaine intake, *SLC1A2* and *Grin1* mRNA levels decreased in $SERT^{+/+}$ rats to levels equal of those of $SERT^{-/-}$ rats. Our data reveal that increased extracellular levels of 5-HT modulate glutamate neurotransmission in the Hb, serving as critical neurobiological substrate for vulnerability to cocaine addiction.

Key words: habenula; cocaine; serotonin transporter; glutamate; vulnerability.

Introduction

One of the major factors contributing to compulsivity in drug addiction involves a gradual decrease in drug-induced hedonic effects and the emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability, stress, depressive mood) when access to the drug is prevented (Koob and Le Moal, 2008). A key regulator of negative emotional states is serotonin (Andrews et al., 2015). Indeed, human studies have clearly shown that reduced expression and function of the plasmalemmal serotonin transporter (SERT), whose main function is the rapid uptake of released serotonin back into presynaptic terminals, is closely associated with an anxious and pro-depressive phenotype (Lesch et al., 1996). Less well-known is that reduction of SERT and changes in the extracellular levels of serotonin are also associated with an increased risk of psychostimulant addiction (Müller and Homberg, 2015). Notably, SERT knockout ($SERT^{-/-}$) rats, which display anxiety and depression-like behavior (Kalueff et al., 2010), also exhibit increased cocaine intake (Homberg et al., 2008; Karel et al., 2018; Nonkes et al., 2011; Verheij et al., 2018). Changes in serotonin may thus mediate negative reinforcement as driving force in compulsive drug intake. Indeed, SERT down-regulation in the dorsal raphe nucleus was found to increase both compulsive cocaine intake and anxiety during withdrawal (Verheij et al., 2018). The role of SERT in environmental sensitivity in general (Homberg and Lesch, 2011) appears to be critical for the trait 'sensory processing sensitivity' (SPS)' (Aron et al., 2012; Homberg et al., 2016; Lionetti et al., 2018). This trait is observed in humans and animals that are extremely vulnerable to both positive and negative environmental stimulation and may play a role in the co-presence of addictive states and pro-depressive phenotypes in subjects with inherited SERT down-regulation.

Raphe serotonergic neurons project to various brain regions implicated in anxiety, depression and drug addiction, including the habenula (Hb) (Metzger et al., 2017; Zhang et al., 2018): a serotonergic circuitry, in fact, exists from the dorsal raphe nucleus to the Hb, and from the Hb back to the dorsal raphe nucleus, through the rostral tegmental area and interpeduncular nucleus. The Hb is a small conserved epithalamic structure that has recently received increasing attention in addiction research(Gao et al., 2018; López et al., 2018; Meye et al., 2017; Meye et al., 2015; Zapata et al., 2017) and it contributes to the induction of negative emotional states in drug addiction (Batalla et al., 2017; Klein et al., 2018). Serotonergic receptors are densely expressed in Hb (Tchenio et al., 2016; Wagner et al., 2016), suggesting that SERT-reduction-induced changes of extracellular serotonin during both baseline conditions and after cocaine self-administration (Verheij et al., 2014) might influence whole Hb homeostasis. Notably, the rostral tegmental area and interpenduncular nucleus also innervate the ventral tegmental area where dopaminergic neurons are located. Since a cocaine challenge in $SERT^{-/-}$ rats only affects the central levels

of serotonin, and not the central levels of dopamine and norepinephrine (Verheij et al., 2014), it is plausible to hypothesize that a serotonergic circuitry is specifically implicated in the negative emotional states and increased cocaine self-administration as seen in $SERT^{-/-}$ rats.

Although Hb contains both glutamatergic and cholinergic neurons, recent research has mainly focused on the modulation of glutamate in the action of cocaine. Mouse experiments revealed that non-contingent cocaine administration enhanced glutamatergic transmission in neurons from the lateral Hb targeting the rostral tegmental area (Meye et al., 2015), which in turn influences serotonin signaling by inhibiting the dorsal raphe nucleus (Metzger et al., 2017). Disrupting this mechanism prevented development of depressive-like symptoms during withdrawal from repeated involuntary injections of cocaine (Meye et al., 2015). As to whether similar mechanisms occur in response to voluntary cocaine self-administration remains to be assessed.

Using $SERT^{-/-}$ rats, we here investigated the effect of SERT reduction on the baseline gene expression levels of critical determinants of glutamate neurotransmission in the whole Hb, and the possible additive effects of regular and compulsive cocaine self-administration. $SERT^{+/+}$ and $SERT^{-/-}$ rats were exposed to a short-access protocol (1h/day) that mimics regular (controlled) cocaine intake, or a long-access protocol (6h/day) in which rats lose their control over drug intake, mimicking the transition to compulsive drug-taking observed in dependent states(Ahmed and Koob, 1998; Koob and Volkow, 2010) . Across the three different conditions (cocaine-naive, short access and long access cocaine intake) we have investigated the effects of SERT deletion on the homeostasis of the glutamatergic system, by analyzing the mRNA expression of the main glutamate receptors (NMDA and AMPA), the scaffolding protein *Dlg4* that is critical for the regulation of glutamate signaling at the post-synaptic membrane, as well as the main glial glutamate transporter *SLC1A2*. Our findings reveal that serotonin regulates glutamate transmission in the Hb and it dynamically influences the response to cocaine self-administration.

Material and Methods

Animals. $SERT^{-/-}$ rats (SLC6A41Hubr) were generated by N-ethyl-N-nitrosurea (ENU) induced mutagenesis (Smits et al., 2006)and outcrossed with commercially available Wistar rats (Harlan, Ter Horst, the Netherlands) for at least ten generations (Homberg et al., 2007). Male $SERT^{-/-}$ rats and their wild-type ($SERT^{+/+}$) counterparts, both weighing 250-300 g at the beginning of the study, were equipped with jugular vein catheters and subjected to short and long access (ShA and LgA) cocaine self-administration according to the procedures described below (ShA: $SERT^{+/+}$: n=12, $SERT^{-/-}$: n=12 and LgA: $SERT^{-/-}$: n=12, $SERT^{-/-}$: n=14). All rats were housed in groups of 2-3 in Macrolon type III cages (42 x 26 x 15 cm) under a 12 h / 12 h reversed day/night cycle (lights off at 8:00 AM) in a temperature-controlled room (22±2 °C). Food pellets and water were available ad libitum, except during the cocaine self-administration sessions. All procedures were carried out in agreement with the current National Research Council Guide for the Care and Use of Laboratory Animals and were approved by local Institutional Animal Care and Use Committees. All efforts were made to reduce the number of animals used and their suffering.

Drug. Cocaine was provided by National Institute on Drug Abuse (NIDA), Rockville, MD, and was dissolved in saline 0.9%.

Intravenous catheterization. Rats were implanted with a micro Renathane catheter (0.3 mm i.d. × 0.64 mm o.d.; MRE037, Braintec scientific Inc, Braintree, MA) into the right external jugular vein according to previously reported procedures (for details: Wee et al., 2007). This aseptic surgery procedure was performed under isoflurane anesthesia (2-3%). After surgery, rats were given analgesics (Flunixin®, 2.5 mg/kg, s.c., Merck Animal Health, Madison, NJ) and antibiotics (Cefazolin®, 0.033 mg/0.1 mL, i.v., Sagent Pharmaceuticals, Schaumburg, IL) for at least one week. Catheter patency was maintained by daily infusion of 0.1 mL heparinized saline (30 USP, Hospira, Lake Forest, IL).

Self-administration chambers. Cocaine self-administration was performed in standard operant chambers (28 x 26 x 20 cm, Med Associates Inc., St Albans, VT) that were placed in a ventilated, light- and sound-attenuating cubicle. The cocaine self-administration chambers were equipped with a swivel system allowing rats to move freely during self-administration sessions. Cocaine was delivered by a 15 r.p.m. syringe pump (Razel Scientific Instruments, Georgia, VT). The start of a session was signaled by the

presentation of 2 retractable levers into the self-administration chamber. Pressing the right lever was programmed to deliver cocaine (volume: 0.1 mL in 4s) whereas pressing the left lever had no programmed consequences. During drug administration, a stimulus light above the active lever was illuminated and illumination lasted throughout a time-out period of 40s, during which operant responding was not reinforced.

Cocaine self-administration training. One week after surgery, rats were trained to self-administer cocaine (0.5 mg/kg/infusion) under a fixed ratio 1 (FR1) schedule of reinforcement (for details: Verheij et al., 2016; Verheij et al., 2018). Additional groups of cocaine-naive $SERT^{-/-}$ and $SERT^{+/+}$ rats also underwent intravenous catheterization, were handled daily, and received daily infusion of heparinized saline, but were not exposed to the self-administration chambers (Verheij et al., 2016; Verheij et al., 2018).

Short and long access cocaine self-administration. Two days after cocaine self-administration training, one group of rats was allowed to self-administer 0.5 mg/kg/infusion of cocaine in daily 1 h sessions (limited or Short Access (ShA) group of rats) whereas another group of rats self-administered this dose of the psychostimulant in daily 6 h sessions (extended or Long Access (LgA) group of rats), for a total of 15 days (Ahmed and Koob, 1998).

Progressive ratio responding. In order to test the effects of reduced SERT expression on the motivation to work for cocaine, rats were allowed to self-administer cocaine under a progressive ratio (PR) schedule of reinforcement (Hodos, 1961). The test was performed one day following cocaine self-administration session 15. The number of lever presses required to obtain a single infusion of cocaine exponentially increased according to the following equation: number of responses per infusion = (5 x e (injection number × 0.2)) – 5 (for details: Richardson and Roberts, 1996). When a rat failed to achieve this response requirement within a period of 60 min, the PR session ended and the breakpoint was recorded. Twenty-four and seventy-two h after the PR test, the animals were subjected to additional ShA or LgA cocaine self-administration sessions (see above).

Collection habenula tissue. Twenty-four h following the last cocaine self-administration session, rats were sacrificed by decapitation, brains were quickly collected, and stored at -80^0C. The Hb was located according to the rat brain atlas of Paxinos and Watson (2005) (coordinates between bregma -3.00 mm and

bregma -4.20 mm) from frozen brain sections of 200 µm using a sterile 1-mm-diameter needle. Hb tissue was stored at −80°C until being processed for molecular analysis (see below).

RNA Preparation and Real-Time Polymerase Chain Reaction

Total RNA of 6 randomly selected animals per genotype per treatment was isolated by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Segrate, Milan, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time reverse transcription polymerase chain reaction (real time RT-PCR) to assess mRNA levels, as previously described (Caffino et al., 2017). Briefly, an aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed by TaqMan qRT-PCR instrument (CFX384 real time system, Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 wells formats in triplicate as multiplexed reactions. Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (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 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reaction. Data were analyzed with the comparative threshold cycle ($\Delta\Delta$Ct) method using either 36B4 or β-actin as reference genes. The primer efficiencies were experimentally set up for each couple of primers. Primers and probes sequences are shown in table 1.

Statistical analysis

Data were collected in individual animals (independent determinations) and are presented as means $\pm$ standard errors. The effects of genotype on cocaine self-administration were analyzed using a two-way analysis of variance (ANOVA) with a correction for repeated measures followed by a Studen's t test. The effects of genotype on last session, mean, total and progressive ratio intake were analyzed using one-way analysis of variance (ANOVA) followed by a Student's t test

To enable visual comparisons across genotypes with different degrees of expression of glutamatergic molecular determinants, values are presented as percent of the control group, namely the $SERT^{+/+}$-naive group that was exposed to neither short- nor long-access to cocaine. Molecular changes produced by genotype and cocaine exposure alone as well as by their combination were analyzed using a two-way ANOVA, with genotype and cocaine self-administration as independent variables. When dictated by relevant interaction terms, Fisher's least significant difference (LSD) test was used to characterize

differences among individual groups of rats. However, when no interaction between genotype and cocaine self-administration was observed, only the main effects were reported. Pearson product-moment coefficients were calculated to study putative correlations between molecular and behavioural variables in the pooled group of SERT $SERT^{+/+}$ and $SERT^{-/-}$ rats. Prism 6.0 (GraphPad) was used to analyze all the data. Significance for all tests was assumed at $p < 0.05$.

Results

Figure 1a illustrates the experimental paradigm used in our experiments. Rats from cocaine short- and long-access (ShA and LgA, respectively) were sacrificed 24 hours after the last session. Naive rats were sacrificed the same day and at the same time of the day. Hb was dissected through punching as depicted in Fig. 1b.

Cocaine self-administration in SERT$^{+/+}$ and SERT$^{-/-}$ rats

Figure 1c and 1d show an increase in the voluntary daily intake of cocaine in SERT$^{-/-}$ versus SERT$^{+/+}$ rats under both ShA and LgA conditions (two-way ANOVA for repeated measures: genotype effect: ShA: $F_{(1,22)}$=11.11, p=0.003 and LgA: $F_{(1,24)}$=4.73, p=0.040; genotype x session effect: ShA: $F_{(14,308)}$=1.50, p=0.108 and LgA: $F_{(14,336)}$=1.95, p=0.021). Last session, mean, total and progressive ratio intake was also increased in SERT$^{-/-}$ versus SERT$^{+/+}$ rats (one-way ANOVA: genotype effect: ShA (table 2 and figure 1e): last session intake: $F_{(1,22)}$=5.46, p=0.029, mean intake: $F_{(1,22)}$=11.06, p=0.003, total intake: $F_{(1,22)}$=11.11, p=0.003, PR intake: $F_{(1,22)}$=6.06, p=0.022 and LgA (table 2 and figure 1e): last session intake: $F_{(1,24)}$=3.18, p=0.087, mean intake: $F_{(1,24)}$=4.67, p=0.041, total intake: $F_{(1,24)}$=4.73, p=0.040, PR intake: $F_{(1,24)}$=6.15, p=0.021).

Expression levels of the glial glutamate transporter in the Hb under basal conditions in SERT$^{+/+}$ and SERT$^{-/-}$ rats

We first evaluated the expression of critical determinants of glutamatergic neurotransmission in the Hb of SERT$^{-/-}$ and wild-type rats under naive conditions and exposed to the different paradigms of cocaine self-administration. Figure 2 shows *SLC1A2* mRNA levels, i.e. the main glial glutamate transporter responsible for the clearance of glutamate from the synaptic cleft. Two-way ANOVA revealed a main effect of treatment ($F_{(2,27)}$=9.05, p=0.001), genotype ($F_{(1,27)}$=6.54, p=0.017) and a treatment x genotype interaction ($F_{(2,27)}$=5.30, p=0.011). Examining the individual treatment effects, we found that SERT deletion reduced *SLC1A2* expression in naive rats (-21%, p=0.001 vs SERT$^{-/-}$-naive, Fisher's LSD test) whereas the LgA procedure significantly reduced *SLC1A2* expression in SERT$^{+/+}$ animals (-25%, p=0.001 vs SERT$^{+/+}$-naive; -13%, p=0.001 vs SERT$^{+/+}$-ShA) but not in SERT$^{-/-}$ rats (-4%, p=0.681 vs SERT$^{-/-}$-naive; -8%, p=0.427 vs SERT$^{-/-}$-ShA).

Expression levels of NMDA and AMPA receptor subunits in the Hb under basal conditions in SERT$^{+/+}$ and SERT$^{-/-}$ rats

Next, we investigated glutamate receptor expression analyzing the mRNA levels of the main subunits of N-methyl-D-aspartate (NMDA) receptor and of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

(AMPA) receptor (Fig. 3 and fig. 4, respectively). As shown in figure 3, the expression of the obligatory subunit, *Grin1*, and the two accessory subunits, *Grin2A* and *Grin2B*, of the NMDA receptor revealed a main effect of treatment (*Grin1*: $F_{(2,28)}$=8.48, p=0.001, panel a; *Grin2A*: $F_{(2,29)}$=26.14, p<0.0001, panel b; *Grin2B*: $F_{(2,28)}$=18.43, p<0.0001, panel c), genotype (*Grin1*: $F_{(1,28)}$=13.53, p=0.001, panel a; *Grin2A*: $F_{(1,29)}$=10.51, p=0.003, panel b; *Grin2B*: $F_{(1,28)}$=18.05, p=0.0002, panel c) and a treatment x genotype interaction (*Grin1*: $F_{(2,28)}$=14.97, p<0.0001, panel a; *Grin2A*: $F_{(2,29)}$=3.95, p=0.03, panel b; *Grin2B*: $F_{(2,28)}$=3.36, p=0.049, panel c). Further intergroup subtesting indicated that SERT deletion reduced *Grin1*, *Grin2A* and *Grin2B* mRNA levels in the Hb of naive rats (*Grin1*: -49% vs $SERT^{+/+}$-naive, p<0.0001, panel a; *Grin2A*: -41% vs $SERT^{+/+}$-naive, p= 0.0003, panel b; *Grin2B*: -36% vs $SERT^{+/+}$-naive, p= 0.0001, panel c). Interestingly, the duration of daily cocaine exposure differently influenced the expression of NMDA subunits in $SERT^{+/+}$ vs $SERT^{-/-}$ rats. In particular, only the LgA condition reduced *Grin1* mRNA levels in $SERT^{+/+}$ (-55% vs $SERT^{+/+}$-naive, p=0.0003; -48% vs $SERT^{+/+}$-ShA, p<0.0001; panel a) but not in $SERT^{-/-}$ rats (+14% vs $SERT^{-/-}$-naive, p=0.14; +20% vs $SERT^{+/+}$-LgA, p=0.048; panel a). While both ShA and LgA exposure reduced *Grin2A* expression in $SERT^{+/+}$ rats (ShA: -62% vs $SERT^{+/+}$-naive, p<0.0001; -39% vs $SERT^{+/+}$-LgA, p=0.0008; LgA: -23% vs $SERT^{+/+}$-naive, p=0.026; panel b), only the ShA procedure significantly reduced *Grin2A* in $SERT^{-/-}$ rats (-32% vs $SERT^{-/-}$-naive, p=0.003; -39% vs $SERT^{-/-}$-LgA, p<0.0001; panel b). Similarly to *Grin2A*, *Grin2B* expression was reduced in both ShA and LgA $SERT^{+/+}$ animals (ShA: -47% vs $SERT^{+/+}$-naive, p<0.0001; LgA: -35% vs $SERT^{+/+}$-naive, p=0.0001; panel c); however, only the ShA procedure in combination with the removal of SERT significantly reduced *Grin2A* levels (-21% vs $SERT^{-/-}$-naive, p=0.016; panel c).

Two-way ANOVA of *Gria1* and *Gria2* revealed a main effect of treatment ($F_{(2,29)}$=38.76, p<0.0001, fig. 4a) only for *Gria1*, and a significant genotype effect (*Gria1*: $F_{(1,29)}$=23.99, p<0.0001, fig. 4a; *Gria2*: $F_{(1,29)}$=72.28, p<0.0001, fig. 4b) and a treatment x genotype interaction (*Gria1*: $F_{(2,29)}$=3.54, p=0.042, fig. 3a; *Gria2*: $F_{(2,29)}$=5.05, p=0.013, fig. 4b) for both *Gria1* and *Gria2*. Examining the individual treatment effects we found that deletion of SERT reduced *Gria1* and *Gria2* mRNA levels in the Hb of naive rats (*Gria1*: -22% vs $SERT^{+/+}$-naive, p=0.0002, panel a; *Gria2*: -12% vs $SERT^{+/+}$-naive, p=0.014, panel b). In $SERT^{+/+}$ rats, *Gria1* mRNA levels were reduced in both ShA- and LgA-exposed animals (ShA: -42% vs $SERT^{+/+}$-naive, p<0.0001; -25% vs $SERT^{+/+}$-naive, p<0.0001; LgA: -17% vs $SERT^{+/+}$-naive, p=0.002, panel b), whereas *Gria2* was increased only after ShA procedure (+13% vs $SERT^{+/+}$-naive, p=0.011, panel b). In $SERT^{-/-}$ rats, cocaine self-administration, independently from the duration of the daily psychostimulant exposure, reduced the mRNA levels of both AMPA subunits (*Gria1* ShA: -23% vs $SERT^{+/+}$-naive, p<0.0001;

LgA: -14% vs SERT$^{+/+}$-naive, p=0.008; -19% vs SERT$^{+/+}$-LgA, p=0.0008, panel a; *Gria2* ShA: -33% vs SERT$^{+/+}$-ShA, p<0.0001; LgA: -23% vs SERT$^{+/+}$-LgA, p<0.0001).

Expression levels of postsynaptic density scaffold protein in the Hb under basal conditions in SERT$^{+/+}$ and SERT$^{-/-}$ rats

To further characterize the impact of the combination of SERT deletion and cocaine self-administration on the postsynaptic terminal, we evaluated *Dlg4* mRNA levels, an index of post-synaptic density integrity. As shown in figure 5, two-way ANOVA revealed a main effect of treatment ($F_{(2,29)}$=64.79, p<0.0001), genotype ($F_{(1,29)}$=5.62, p=0.025) and a treatment x genotype interaction ($F_{(2,29)}$=11.80, p=0.0002). At variance from the other glutamatergic markers evaluated, the deletion of SERT did not alter *Dlg4* mRNA levels in the Hb of naive rats (+13% vs SERT$^{+/+}$-naive, p=0.075). ShA cocaine self-administration reduced *Dlg4* expression in both SERT$^{+/+}$ (-44% vs SERT$^{+/+}$-naive, p<0.0001; -64% vs SERT$^{+/+}$-LgA, p<0.0001) and SERT$^{-/-}$ rats (-65% vs SERT$^{+/+}$-naive, p<0.0001; -35% vs SERT$^{+/+}$-LgA, p<0.0001), whereas LgA increased *Dlg4* levels in SERT$^{+/+}$ (+20% vs SERT$^{+/+}$-naive, p=0.011) while decreasing them in SERT$^{-/-}$ rats (-30% vs SERT$^{-/-}$-naive, p=0.0003; -39% vs SERT$^{+/+}$-LgA, p<0.0001).

Correlation between cocaine intake and expression levels of components of the glutamatergic synapse in the Hb

To investigate the potential relationship between the features of cocaine self-administration and the modulation of the glutamate synapse we performed a Pearson's product–moment correlation analysis. As shown in table 3, Pearson's correlation analysis revealed that cocaine intake during the last session, the mean cocaine intake over sessions and the total cocaine intake correlates negatively with *Grin1* and *SLC1A2* expression and positively with *Grin2A* and *Dlg4* expression.

Discussion

Hb controls 5-HT raphe function and vice versa (see introduction). It has been shown that direct, electrical stimulation of the Hb reduces 5-HT-firing in the raphe nucleus (Varga et al., 2003), and that 5-HT controls Hb function (Shabel et al., 2012; Shalem et al., 2015; Zuo et al., 2016). These observations are critical since accumulating evidence implicate Hb in the pathogenesis of serotonin-related disorders such as anxiety, depression and possibly drug addiction. $SERT^{-/-}$ rats self-administer much more cocaine than their wild-type littermates suggesting that changes in central extracellular 5-HT levels facilitate cocaine self-administration (Homberg et al., 2008; Karel et al., 2018; Nonkes et al., 2011; Verheij et al., 2018) . This increased cocaine self-administration is associated with increased anxiety-related behavior during withdrawal (Verheij et al., 2018). Given that the Hb may serve as anti-reward node (Batalla et al., 2017), glutamate in this region may play an important role in the behavioural pattern of these rats. Here we investigated whether the glutamate system in the Hb is implicated in the observed phenotypes of SERT KO rats. We replicated previously published data showing that $SERT^{-/-}$ rats exhibit increased cocaine self-administration under ShA and LgA conditions (Verheij et al., 2018) and found that reduced SERT availability modulates Hb expression of genes involved in glutamate neurotransmission under baseline conditions and after SHA and LgA to cocaine.

A key finding is that, in the Hb of $SERT^{-/-}$ rats, the basal level of the main glial glutamate transporter *SLC1A2* is reduced, suggesting increased glutamate levels in the synaptic cleft, in line with the evidence that blockade of SERT facilitates glutamate transmission (Xie et al., 2016). Increased firing of glutamate neurons and the related Hb hyper-excitability are linked to symptoms of depression (Cui et al., 2014). These results suggest that the herein shown reduction of *SLC1A2* in the Hb may contribute, at least in part, to the negative emotional state previously observed in $SERT^{-/-}$ rats (Olivier et al., 2008). Notably, in rats withdrawn from ethanol, depression- and anxiety-like behaviors were accompanied by reduced local *SLC1A2* expression (Kang et al., 2018), and the finding that restoring *SLC1A2* function alleviated these symptoms point to Hb *SLC1A2* as a critical target to alleviate drug withdrawal symptoms (Kang et al., 2018). Interestingly, LgA cocaine self-administration strongly reduced *SLC1A2* expression in $SERT^{+/+}$, an effect that was not observed in $SERT^{-/-}$ rats, suggesting that removal of SERT in cocaine-naïve animals leads to molecular features that are observed also following LgA cocaine self-administration. Taking into account that SLC1A2 reduction may promote cocaine seeking (see: Knackstedt et al., 2010), these findings reveal that the life-time deletion of SERT, by long-lastingly reducing Hb *SLC1A2* expression, may contribute

to make SERT$^{-/-}$ rats more prone to self-administer cocaine (Homberg et al., 2008; Karel et al., 2018; Nonkes et al., 2011; Verheij et al., 2018).

Next to *SLC1A2,* also the expression of NMDA/AMPA subunits were reduced in SERT$^{-/-}$ rats, perhaps as an adaptive mechanism to tone down the activation of glutamate neurotransmission. Interestingly, it has been shown by others that stress decreases the expression of *SLC1A2* and increases cocaine self-administration. This condition was found to be associated with increased central glutamate overflow, and changes in AMPA currents and dendritic spine density (Garcia-Keller et al., 2016). This makes it likely that the reduced *SLC1A2* expression we observed (see figure 2), along with reduced AMPA/NMDA receptor subunit expression (see figures 3 and 4), is due to increased glutamate signaling. In addition, Meye and co-workers (2015) have elegantly shown that cocaine evokes functional increases in glutamate signaling in the habenula. Our data add to previous electrophysiological analyses showing that, under physiological conditions, SERT contributes to the maintenance of a correct tone of the glutamate synapse in the Hb (Xie et al., 2016). SERT also modulates the response to cocaine self-administration, either following a ShA or LgA paradigm. In response to LgA cocaine self-administration, the expression of the NMDA subunit *Grin1* was reduced in wild-type rats, an effect that was not observed in SERT$^{-/-}$ rats. Again, as observed for SLC1A2, SERT ablation in cocaine naïve rats resembles the effect of LgA cocaine self-administration and the modulation of *Grin1* response is not reduced in SERT$^{-/-}$ rats. Gene expression patterns are somewhat different when examining the accessory subunits *Grin2A* and *Grin2B*, which are reduced in cocaine-naïve SERT$^{-/-}$ rats and similarly modulated in both genotypes following ShA to cocaine self-administration. With respect to AMPA receptors, despite the reduced basal levels of *Gria1* in SERT$^{-/-}$ rats, the dynamic response to ShA or LgA to cocaine is similar in both genotypes, whereas *Gria2* subunit is differently regulated in both genotypes following both modalities of cocaine self-administration. Together, these data suggest that increased glutamate signaling under baseline conditions may reduce NMDA and AMPA receptor subunit expression and shape a predisposition to cocaine-induced negative emotional states, potentially through increased activation of the rostral tegmental area and thereby inhibition of raphe serotonergic signaling. The data, furthermore, suggest that *Grin1* compromises adaptations to cocaine-induced increased glutamate signaling, contributing to increased LgA cocaine self-administration in SERT$^{-/-}$ rats. If this holds true, it is possible to hypothesize that hypoglutamatergic states in the Hb may influence the rate of cocaine self-administration (Allen et al., 2007).

A further critical finding involves the regulation of *Dlg4*, a protein playing a critical role in the structure of glutamate synapse. We did not observe any difference between cocaine-naïve genotypes. ShA to cocaine similarly and dramatically down-regulates *Dlg4* expression in SERT$^{-/-}$ and SERT$^{+/+}$ rats. However,

its regulation following LgA cocaine is opposite, as it is enhanced in wild-type rats while reduced in SERT$^{-/-}$ rats. Since *Dlg4* is highly enriched at excitatory synapses and it plays a role in shaping the dendritic arbor (Gardoni, 2008; Sweet et al., 2011), this result suggests that LgA to cocaine self-administration might increase formation of dendritic spines in wild-type rats, as it occurs for instance in the nucleus accumbens and medial prefrontal cortex (DePoy et al., 2014; Robinson and Kolb, 1999), while reducing their number in SERT$^{-/-}$ rats. These results highlight the structural impact of the combination of SERT removal with cocaine self-administration on the glutamate synapse in the Hb.

We found that the expression of specifically SLC1A2, Dlg4, and Grin2A genes correlated with cocaine intake behaviour. Although correlational analyses do not necessarily indicate involvement in mechanisms, our correlations may give some clues about glutamate contribution in cocaine self-administration. The negative correlation with SLC1A2 corresponds to the decrease in glial glutamate transporter levels as found by us (present manuscript) and others in association with cocaine self-administration (Knackstedt et al., 2010). The positive Grin2A correlation with cocaine intake is in line with the negative emotional state herein hypothesized, given that the administration of a GluN2A-preferring NMDA receptor antagonist produces an antidepressant effect (Gordillo-Salas et al., 2018). Also, Pearson's correlation suggests that the Grin2B may not be involved in the mechanisms of voluntary cocaine self-administration, indicating that its reduced expression might more likely be the result of intake-independent aspects of the self-administration procedure, such as drug memory-related plasticity (Shen et al., 2011).

We are aware that our study holds some potential limitations. We have analyzed the whole Hb, because we were unable to find reliable landmarks to differentiate between its medial and lateral components. Moreover, such dissection, because of the very small size of habenula, would not result in enough tissue to perform the numerous molecular analyses herein shown. However, the evidence that cocaine self-administration was found to increase c-fos expression in both the lateral and the medial habenula (Gao et al., 2018) suggests that the results obtained are due to cocaine-induced changes in both regions. Another limitation relies on the fact that we infer changes in glutamate levels based on the expression of various molecular determinants of the glutamate synapse. Future electrophysiological measurements are necessary to obtain direct evidence for the suggested changes.

Taken together, our data contribute to a better understanding of the basic mechanisms underlying the serotonergic control of Hb homeostasis. Our data suggest that the removal of SERT may shape motivational states via changes in critical determinants of glutamate neurotransmission and that it alters its subsequent response to different paradigms of cocaine self-administration. These changes may also contribute to the negative emotional states often observed in drug addicts, which has already been linked

to a dysfunctional glutamate synapse (Caffino et al., 2015). Finally, the results may have relevance for understanding the trait 'sensory processing sensitivity' (SPS) (Lionetti et al., 2018), since a link between SPS and the SERT gene has indeed been suggested (Homberg et al., 2016). In fact, increased glutamate signaling in the Hb could contribute to heightened sensitivity to adverse stimuli, like emotional states arising with compulsive drug self-administration, which in turn strongly drive continuation of drug use.

Table 1 Sequences of primers and probes for quantification of mRNA levels on the glutamatergic system.

Gene	Forward Primer	Reverse Primer	Probe
Gria1	5'-CCTCGAAGATCCTTACGTGATG-3'	5'-TCGCTGACAATCTCAAGTCG-3'	5'-ATAGCGGTCATTGCCCTCAAACTGG-3'
Gria2	5'-TGCTTGAAGGGAATGAGCGT-3'	5'-ATTTTGGTGTCGGCATCCCT-3'	5'-GGCTACTGTGTTGACTTAGCTG-3'
Grin1	5'-TCATCTCTAGCCAGGTCTACG-3'	5'-CAGAGTAGATGGACATTCGGG	5'-TGGGAGTGAAGTGGTCGTTGGG-3'
Grin2A	5'-GCACCAGTACATGACCAGATTC-3'	5'-ACCAGTTTACAGCCTTCATCC-3'	5'-CGTCCAACTTCCCGGTTTTCAAGC-3'
Grin2B	5'-TTCATGGGTGTCTGTTCTGG-3'	5'-GGATGTTGGAGTGGGTGTTG-3'	5'-TCATCACGGATTGGCGCTCCT-3'
Dlg4	5'-CAAGAAATACCGCTACCAAGATG-3'	5'-CCCTCTGTTCCATTCACCTG-3'	5'-TCAACACGGACACCCTAGAAGCC-3'
SLC1A2	5'-TTGCTGGCATTTTCCAAGC-3'	5'-TTAATGGTTGCTCCGACTGG-3'	5'-CAAGCGTGTGACCAGATTCGTCCT-3'
36b4	5'-CCTCGAAGATCCTTACGTGATG-3'	5'-TCGCTGACAATCTCAAGTCG-3'	5'-ATAGCGGTCATTGCCCTCAAACTGG-3'
β-actin	5'- TGCTTGAAGGGAATGAGCGT-3'	5'- ATTTTGGTGTCGGCATCCCT-3'	5'- GGCTACTGTGTTGACTTAGCTG-3'

Table 2. Effects of deletion of SERT on last session, mean and total cocaine intake during short- (ShA) and long-access (LgA) self-administration.

	$SERT^{+/+}$ ShA	$SERT^{-/-}$ ShA	$SERT^{+/+}$ LgA	$SERT^{-/-}$ LgA
Coc intake during last session	8.7±1.7	20.8±4.9$^{\#}$	78.8±13.3	102.9±5.3*
Avg coc intake over sessions	5.4±0.9	11.5±1.6$^{\#\#}$	55.5±10.5	80.6±5.8*
Total coc intake over sessions	81.4±13.4	172.3±23.7$^{\#\#}$	823.3±157.3	1209.2±87.5*

$^{\#}p<0.05$, $^{\#\#}p<0.01$ vs $SERT^{-/-}$-ShA rats; $^{*}p<0.05$ vs $SERT^{+/+}$-LgA

Table 3. Pearson's product–moment correlation analyses between different features of cocaine intake and mRNA levels of the glutamatergic markers in Hb.

		Grin1	*Grin2B*	*Grin2A*	*Gria1*	*Gria2*	*Dlg4*	*SLC1A2*
Coc intake during last session	r	-0,5406	0,2259	0,6232	0,4687	-0,3305	0,661	-0,4565
	P value	0,0139*	0,3249	0,0025*	0,0321*	0,1434	0,0011**	0,0375*
Avg coc intake over sessions	r	-0,5507	0,1117	0,5008	0,4284	-0,351	0,5398	-0,48
	P value	0,0119*	0,6298	0,0208*	0,0527	0,1187	0,0115*	0,0277*
Total coc intake over sessions	r	-0,5515	0,1117	0,5013	0,4293	-0,3525	0,5416	-0,4824
	P value	0,0117*	0,6298	0,0206*	0,0521	0,117	0,0112*	0,0268*

r= pearson correlation coefficient, coc= cocaine, avg= average.

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Author's contribution

Michel Verheij and Chao Guo performed behavioral experiments. Lucia Caffino and Lin Que performed the molecular analyses. Lucia Caffino and Michel Verheij did the statistical analyses. Lucia Caffino, Lin Que and Chao Guo managed the literature searches. Lucia Caffino, Michel Verheij, Judith Homberg and Fabio Fumagalli designed the study, wrote the protocol and interpreted the data. Lucia Caffino, Michel Verheij, Judith Homberg and Fabio Fumagalli wrote the manuscript.

All authors contributed to and have approved the final manuscript.

Figure legends

Figure 1. Schematic representation of the experimental paradigm performed in $SERT^{+/+}$ and $SERT^{-/-}$ rats (panel a) and specific coordinates of habenula dissection (panel b). Number of active lever presses during the 15 days of cocaine ShA (1 h/day, panel c) and LgA (6 h/day, panel d) in $SERT^{+/+}$ and $SERT^{-/-}$ rats. Progressive ratio (PR) responding in $SERT^{+/+}$ and $SERT^{-/-}$ rats that underwent ShA and LgA procedure, measured after 15 days of cocaine self-administration (panel f).

Panel a: One week after surgery, rats (12 x group) were trained to self-administer cocaine (0.5 mg/kg/infusion) under a fixed ratio 1 (FR1) schedule of reinforcement (for details: Verheij et al., 2016; Verheij et al., 2018). Additional groups of cocaine-naive $SERT^{-/-}$ and $SERT^{+/+}$ rats (12 x group) also underwent intravenous catheterization, were handled daily, and received daily infusion of heparinized saline, but were not exposed to the self-administration chambers.

$^{*}p<0.05$, $^{**}p<0.01$ vs $SERT^{+/+}$-ShA rats in panel c; $^{*}p<0.05$, $^{**}p<0.01$ vs $SERT^{+/+}$-ShA rats in panel d (two-way ANOVA for repeated measures followed by Student's t test). $^{*}p<0.05$ vs $SERT^{+/+}$-ShA rats and #p<0.05 vs $SERT^{+/+}$-LgA (panel f)

Figure 2. Interaction between SERT deletion and cocaine self-administration on the homeostasis of the glutamatergic system in the habenula: effects on glial glutamate transporter, *SLC1A2*, expression.

mRNA levels of *SLC1A2 in Hb* are expressed as percentages of $SERT^{+/+}$-naive rats. Histograms represent the mean ± SEM of five-six rats per group.

$^{**}p<0.01$, $^{***}p<0.001$ vs $SERT^{+/+}$-naive; $^{\#}p<0.05$ vs $SERT^{+/+}$-ShA rats (two-way ANOVA followed by Fisher's LSD test)

Figure 3. Interaction between SERT deletion and cocaine self-administration on the homeostasis of the glutamatergic system in the habenula: effects on NMDA receptor subunit gene expression. mRNA levels of *Grin1* (panel a), *Grin2A* (panel b) and *Grin2B* (panel c) are expressed as percentages of $SERT^{+/+}$-naive rats. Histograms represent the mean ± SEM of five-six rats per group.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs $SERT^{+/+}$-naive; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs $SERT^{+/+}$-ShA rats; £ p<0.05, £££ p<0.001 vs $SERT^{-/-}$-LgA; §§ p<0.01, §§§ p<0,001 vs $SERT^{-/-}$-naive (two-way ANOVA followed by Fisher's LSD test)

Figure 4. Interaction between SERT deletion and cocaine self-administration on the homeostasis of the glutamatergic system in the habenula: effects on AMPA receptor subunit gene expression. mRNA levels of

Gria1 (panel a) and *Gria2* (panel b) are expressed as percentages of $SERT^{+/+}$-naive rats. Histograms represent the mean ± SEM of five-six rats per group.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs $SERT^{+/+}$-naive; $^{\#\#\#}p<0.001$ vs $SERT^{+/+}$-ShA rats; $$$ p<0.001 vs $SERT^{+/+}$-LgA; §§ p<0.01, §§§ p<0,001 vs $SERT^{-/-}$-naive (two-way ANOVA followed by Fisher's LSD test)

Figure 5. Interaction between SERT deletion and cocaine self-administration on the homeostasis of the glutamatergic system in the habenula: effects on post-synaptic density 95, *Dlg4* gene expression. mRNA levels of *Gria1* (panel a) and *Gria2* (panel b) are expressed as percentages of $SERT^{+/+}$-naive rats. Histograms represent the mean ± SEM of five-six rats per group.

$^{*}p<0.05$, $^{***}p<0.001$ vs $SERT^{+/+}$-naive; $^{£££}p<0.001$ vs $SERT^{-/-}$-LgA rats; $^{\$\$\$}p<0.001$ vs $SERT^{+/+}$-LgA; $^{§§§}p<0,001$ vs $SERT^{-/-}$-naive (two-way ANOVA followed by Fisher's LSD test)

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