RESEARCH ARTICLE

Responsivity of serotonin transporter knockout rats to short and long access to cocaine: Modulation of the glutamate signalling in the nucleus accumbens shell

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Background and Purpose: It has been well established that glutamate in the nucleus accumbens (NAc) plays a critical role in the motivation to take drugs of abuse. We have previously demonstrated that rats with ablation of the serotonin transporter (SERT$^{-/-}$ rats) show increased cocaine intake reminiscent of compulsivity.

Experimental Approach: By comparing SERT$^{-/-}$ to SERT$^{+/+}$ rats, we investigated whether SERT deletion influences glutamate homeostasis under control conditions as well as after short access (ShA: 1 h per session) or long access (LgA: 6 h per session) to cocaine self-administration. Rats were killed at 24 h after the last self-administration session for ex vivo molecular analyses of the main determinants of the glutamate system, including transporters (vesicular and glial), receptors (main post-synaptic subunits of NMDA and AMPA receptors together with the metabotropic subunit mGLUR5), and scaffolding proteins (SAP102, SAP97, and GRIP) in the NAc shell (sNAc)

Key Results: In cocaine-naive animals, SERT deletion was associated with changes indicative for a reduction in glutamate signalling. ShA and LgA exposure led to a further dysregulation of the glutamatergic synapse.

Conclusion: SERT deletion may render the glutamatergic synapses of the NAc shell more responsive to both ShA and LgA intake of cocaine.

KEYWORDS
cocaine self-administration, glutamate, nucleus accumbens, serotonin transporter

1 | INTRODUCTION

One of the key neurotransmitters implicated in the transition from the recreative use of drugs to non-controlled compulsive drug use is serotonin (5-hydroxytryptamine, 5-HT). Serotonin is thought to mediate negative affect and thereby drive drug use for its temporary ability to relieve the subject from negative emotional states. Synaptic serotonin levels strictly depend upon the serotonin transporter (SERT), rendering SERT as a key factor in this transition. We have previously demonstrated an increase in cocaine intake in rats lacking SERT (SERT$^{-/-}$ rats); in particular, we found that cocaine intake was enhanced under conditions mimicking limited access to cocaine [the so-called short-access condition (ShA)] as well as conditions that lead to escalated intake of this psychostimulant [the so-called long-access condition (LgA)] (Homberg et al., 2008; Karel et al., 2018; Verheij et al., 2018).

Recently, we have thoroughly investigated the role of the glutamate transmission in different brain areas of SERT$^{+/+}$ and SERT$^{-/-}$ rats, given the primary role played by this neurotransmitter in the rewarding and craving-eliciting effects of the psychostimulant cocaine, and the anxiety correlated to cocaine use (Richard & Berridge, 2011). We revealed a previously unappreciated dysregulation of glutamate homeostasis in the medial prefrontal cortex, habenula, and nucleus accumbens core (cNAc) (Caffino, Mottarlini, Targa, et al., 2021; Caffino, Mottarlini, Van Reijmersdal, et al., 2021; Caffino et al., 2019) in SERT$^{-/-}$ rats, indicating that the interaction between serotonin and glutamate in several brain regions plays an important role in drug addiction.

It is widely established that the NAc, a structure located in the ventral striatum, is critical for the mesolimbic reward network. The NAc plays a prominent role in the (mal)adaptive changes that characterize the different facets of addiction: that is, reward, drug intake, withdrawal, and motivation. Indeed, cocaine has been widely shown to modulate glutamate neurotransmission in the NAc (Cornish & Kalivas, 2000; Kalivas, 2004; LaCrosse et al., 2016; Logan et al., 2018; Scofield et al., 2016; Stefanik et al., 2018). In addition, evidence exists to show that serotonin in the NAc regulates glutamate homeostasis in naive and cocaine-treated rats (Muramatsu et al., 1998; Zayara et al., 2011). We have recently shown that SERT-deletion altered glutamate homeostasis in the nucleus accumbens core primarily after long, but not short, access to cocaine (Caffino, Mottarlini, Targa, et al., 2021), adding further insights into the tight relationship between serotonin and glutamate in the NAc. Specifically, we found that SERT-deletion evoked changes indicative of a reduction in glutamate signalling, and altered glutamate homeostasis mainly after LgA, but not ShA, to cocaine (Caffino, Mottarlini, Targa, et al., 2021). Although the role of the cNAc appears to be related to the expression of motivation for cocaine (Di Chiara, 2002), the NAc *shell* (sNAc) is known to modulate the reinforcing properties of this psychostimulant (Di Chiara, 2002; McBride et al., 1999). Hence, serotonin-glutamate interactions may well differ between the sNAc and cNAc.

Here, we sought out the changes in the glutamatergic synapse in the sNAc of naive SERT$^{-/-}$ rats versus wild-type controls and in response to ShA and LgA cocaine self-administration. Under these

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What is already known

- Moderate and escalated cocaine self-administration is enhanced in SERT$^{-/-}$ rats.

What does this study adds

- SERT$^{-/-}$ rats show increased responsivity of accumbal shell glutamatergic neurons under both regimens.

What is the clinical significance

- Accumbal shell neurons may increase cocaine proneness in subjects with inherited SERT reduction.

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experimental conditions, we focused our attention on the glutamatergic synapse by investigating the expression of molecular markers of release (vGluT1) and reuptake (GLT-1) as well as the main subunits of the main NMDA (GluN1, GluN2A, and GluN2B) and AMPA (GluA1 and GluA2) glutamate receptors together with their respective scaffolding proteins (SAP102 for NMDA; SAP97 and GRIP for AMPA). We also evaluated the expression of the metabotropic receptor mGLUR5 whose modulation is known to influence the glutamate synapse (Sengmany & Gregory, 2016). Further, we measured the structural effects of the combination of SERT knockout and ShA/LgA procedures by evaluating the expression levels of the integral protein of the glutamate synapse PSD95, the adhesion molecule Neuroligin-1, and the cytoskeletal protein Arc/Arg3.1. Such analyses were performed using the whole homogenate of the sNAc, taking advantage of the availability of brain areas that had been collected from previous experiments and were not yet used for molecular determinations (Caffino et al., 2019).

2 | METHODS

2.1 | Animals

Our report of the experiments follows the ARRIVE guidelines (Kilkenny et al., 2010).

We employed rats in this study, because the rat is the preferred species for preclinical addiction research (Homberg et al., 2017). SERT$^{-/-}$ rats (SLC6A41Hubr) were generated by *N*-ethyl-*N*-nitrosurea (ENU) induced mutagenesis (Smits et al., 2006) outcrossed with commercially available Wistar rats (Harlan, Ter Horst, the Netherlands) for at least 10 generations (Homberg et al., 2007). Rats were housed in groups of two in enriched Macrolon type III cages (42 × 26 × 15 cm; Techniplast 1291H, Tecnilab-BMI) with corncobs

bedding (irradiated, SPPS COB12, Bio Services) under conventional conditions (no filtertops). The animals had access to food (dried pellets of standard chow food (Ssniff RM V1534-703 diet supplied by Bio-Services) and water ad libitum, except during test phases. The rats were housed under a reversed day and night cycle (lights off at 08:00 AM, lights on at 08.00 PM) in temperature (21 ± 1°C) and humidity (55 ± 5%) controlled rooms. Testing (cocaine self-administration) took place in the dark phase of the light/dark cycle. Male $SERT^{-/-}$ and $SERT^{+/+}$ rats were subjected to ShA and LgA cocaine self-administration according to the procedures described in Caffino et al. (2019). The number of animals analysed for protein expression levels was as follows: naive: $SERT^{+/+}$: $n = 7$, $SERT^{-/-}$: $n = 7$; ShA: $SERT^{+/+}$: $n = 6$, $SERT^{-/-}$: $n = 6$ and LgA: $SERT^{-/-}$: $n = 7$, $SERT^{-/-}$: $n = 7$). Behavioural testing was always done blindly, by an experimenter who was unaware of the genotype of the animals. Allocation of the rats to the treatment group (ShA and LgA) was random. The experimental procedures were performed under a project license from the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, The Hague, The Netherlands), in full compliance with the legal requirements of Dutch legislation on the use and protection of laboratory animals (Animal Testing Act). All efforts were made to reduce the number of animals used and their suffering. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

2.2 | Cocaine self-administration

Briefly, 1 week after surgery, rats were trained to self-administer cocaine (0.5 mg·kg^{-1} per infusion) under a fixed ratio 1 (FR1) schedule of reinforcement (for details, please see Caffino et al., 2019; Verheij et al., 2016, 2018). Two days after cocaine self-administration training, rats were allowed to self-administer cocaine during daily 6 h sessions (LgA group of rats), or 1 h sessions (ShA group of rats) for a total of 15 days (Ahmed & Koob, 1998). Additional groups of cocaine-naive $SERT^{-/-}$ and $SERT^{+/+}$ rats also underwent intravenous catheterization, were handled daily, and received daily infusion of heparinized saline. The animals were not exposed to the self-administration chambers to prevent boredom in the self-administration cages, which could potentially affect gene expression (Verheij et al., 2016, 2018).

2.3 | Tissue collection

Twenty-four hours following the last cocaine self-administration session, rats were killed by decapitation without anaesthesia. This procedure was used to avoid effects of anaesthetics on protein expression, in accordance with the Dutch legal regulations for killing rodents. Brains were quickly collected and stored at −80°C. These brains were collected in a previous study (Caffino et al., 2019). Using the rat brain atlas of Paxinos and Watson (2007),

the sNAc (from Bregma +2.76 mm to Bregma +0.84 mm) was punched from frozen brain sections of 220 μm using a sterile 1-mm-diameter needle (Giannotti et al., 2016). Punches from the right and left hemisphere were pooled. sNAc tissue was stored at −80°C until processed for molecular analysis (see below).

2.4 | Protein extraction and western blot analyses

The immuno-related procedures used comply with the recommendations made by the British Journal of Pharmacology (Alexander et al., 2018). Proteins were extracted as previously described with minor modifications (Caffino, Giannotti, et al., 2017).

Briefly, bilateral punches of sNAc were homogenized in a glass–glass potter in cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 0.1 mM PMSF, in the presence of commercial cocktails of protease (Roche, Monza, Italy) and phosphatase (Sigma-Aldrich, Milan, Italy) inhibitors and then sonicated. Total proteins were measured in the total homogenate according to the Bradford Protein Assay procedure (Bio-Rad, Milan, Italy), using BSA as calibration standard.

The experimental details for the western blotting conform the guidelines of the British Journal of Pharmacology (Alexander et al., 2018). Western blots were run as previously described (Caffino, Piva, et al., 2017). Briefly, 10 μg of proteins for each sample were run on a sodium dodecyl sulfate-8% polyacrylamide gel under reducing conditions and then electrophoretically transferred onto nitrocellulose membranes (GE Healthcare, Milan, Italy). Blots were blocked 1 h at room temperature with I-Block solution (Life Technologies Italia, Italy) in TBS + 0.1% Tween-20 buffer and then incubated with antibodies against the total proteins of interest.

The conditions of the primary antibodies were the following: Anti vGlut1 (1:1000, Cell Signaling Technology Inc., RRID:AB_2797887), anti GLT-1 (1:5000, AbCam, RRID:AB_1566262), anti GluN1 (1:1000, Invitrogen, RRID:AB_2533060), anti GluN2B (1:1000, Santa Cruz Biotechnology, RRID:AB_670229), anti GluN2A (1:1000, Invitrogen, RRID:AB_2536209), anti SAP102 (1:1000, Cell Signaling Technology Inc.), anti GluA1 (1:2000, Cell Signaling Technology Inc, RRID:AB_641040), anti GluA2 (1:2000, Cell Signaling Technology Inc., RRID:AB_10622024), anti SAP97 (1:1000, AbCam, RRID:AB_2091910), anti mGluR5 (1:1000, Millipore, RRID:AB_2295173), anti Neuroligin-1 (1:1000, Synaptic System, RRID:AB_2151646) and anti β-Actin (1:10,000, Sigma-Aldrich, RRID:AB_476697). Expression levels of every single protein was normalized using its own β-actin loading control, which was detected by evaluating the band density at 43 kDa. Optic density (OD) of immunocomplexes was visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories). Gels were run two times each and the results represent the average from two different runs. We used a correction factor to average the different gels: correction factor gel 2 = average of (OD protein of interest/OD β-actin for each sample loaded in gel 1)/(OD protein of interest/OD β-actin for the same sample loaded in gel 2) (Caffino et al., 2020).

2.5 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). All animals tested were treated as independent values, and there were no technical replicates.

The Kolmogorov–Smirnov test was employed to determine normality of residuals: no significant variance in homogeneity was found. Thus, the molecular changes produced by genotype and cocaine exposure alone as well as by their combination were analysed using a two-way ANOVA, with the factors genotype and type of cocaine access as independent variables. When dictated by relevant interaction terms, Tukey's multiple comparisons test was used to characterize differences among individual groups of rats. Two-way ANOVA analyses were performed using raw data (Table S1). Then, data were normalized as percentages of the cocaine-naive SERT$^{+/+}$ control rats that were not exposed to either cocaine ShA or LgA to enable visual comparisons across genotypes with different degrees of expression of glutamatergic molecular determinants. Values are presented as percentage of control rats. Subjects were eliminated from the final dataset if their data deviated from the mean by 2 SDs. Prism 6.0 (GraphPad) was used to analyse all the data. Data are shown as mean ± SEM and as percentage of baseline to control for unwanted sources of variation. Significance for all tests was assumed at $P < 0.05$.

2.6 | Materials

Macrolon type III cages were purchased from (Techniplast 1291H; Tecnilab-BMI). Corncobs bedding and standard chow food were provided from (Bio-Services). Cocaine was purchased from the (National Institute on Drug Abuse, Rockville, MD), and was dissolved in saline 0.9%. Commercial cocktails of protease and phosphatase used in the sucrose buffer during tissue homogenization were provided from (Roche, Monza, Italy) and (Sigma-Aldrich, Milan, Italy) respectively. Bradford Protein Assay to quantify total proteins in the total homogenate was purchased from (Bio-Rad, Milan, Italy). Nitrocellulose membranes were acquired from (GE Healthcare, Milan, Italy) and blocked with I-Block solution purchased from (Life Technologies Italia, Italy).

2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos, et al., 2021; Alexander, Kelly, et al., 2021; Alexander, Mathie, et al., 2021; Alexander et al., 2018).

3 | RESULTS

3.1 | Cocaine intake

As reported previously (Caffino et al., 2019), we did not observe genotype differences when the animals were trained to acquire cocaine self-administration. Under ShA conditions, SERT$^{-/-}$ rats, compared with SERT$^{+/+}$ rats, displayed a significant increase in cocaine intake (SERT$^{-/-}$ in mean ± SEM: 172 ± 24 infusions versus SERT$^{+/+}$ in mean ± SEM: 81 ± 13 infusions accumulated over all sessions). Likewise, under LgA conditions, SERT$^{-/-}$ rats, compared with SERT$^{+/+}$ rats, self-administered a higher amount of cocaine (SERT$^{-/-}$ in mean ± SEM: 1209 ± 88 infusions versus SERT$^{+/+}$ in mean ± SEM: 823 ± 157 infusions accumulated over all sessions). Under both ShA and LgA conditions, there were no differences in the number of inactive lever presses between genotypes.

3.2 | Expression levels of the vesicular glutamate transporter and the glial glutamate transporter in the homogenate of the sNAc following ShA and LgA to cocaine in SERT$^{+/+}$ and SERT$^{-/-}$ rats

We initially evaluated the expression of the vesicular glutamate transporter (vGluT1), which stores glutamate into presynaptic vesicles before release, in the homogenate of sNAc of SERT$^{-/-}$ and SERT$^{+/+}$ rats under naive conditions and cocaine ShA or LgA conditions. Two-way ANOVA (for details, see Table S1) revealed a genotype effect and a significant interaction between cocaine access and genotype (Figure 1a). Further intergroup sub-testing revealed reduced expression of vGluT1 in SERT$^{+/+}$ rats after LgA conditions, whereas vGluT1 was enhanced in SERT$^{-/-}$ rats after both conditions.

Next, we analysed the expression level of the main glial glutamate transporter, that is, GLT-1, which reuptakes glutamate from the synaptic cleft. Two-way ANOVA (for details, see Table S1) revealed a significant interaction between genotype and drug exposure. Interestingly, GLT-1 expression was reduced in SERT$^{-/-}$ naive rats, whereas cocaine administration reduced GLT-1 only in SERT$^{+/+}$ rats after both conditions (Figure 1b).

3.3 | Expression levels of PSD95, Neuroligin 1, and Arc/Arg3.1 in the homogenate of the sNAc following ShA and LgA to cocaine in SERT$^{+/+}$ and SERT$^{-/-}$ rats

To deepen the effects of the interaction between SERT and cocaine self-administration on the structural integrity of the glutamate synapse in the sNAc of SERT$^{-/-}$ and SERT$^{+/+}$ rats, we investigated the expression of three critical structural proteins of the synapse. In detail, we first examined the expression of PSD95, a structural protein of the glutamatergic postsynaptic density (Vickers et al., 2006). Two-way ANOVA (for details, see Table S1) revealed a significant cocaine
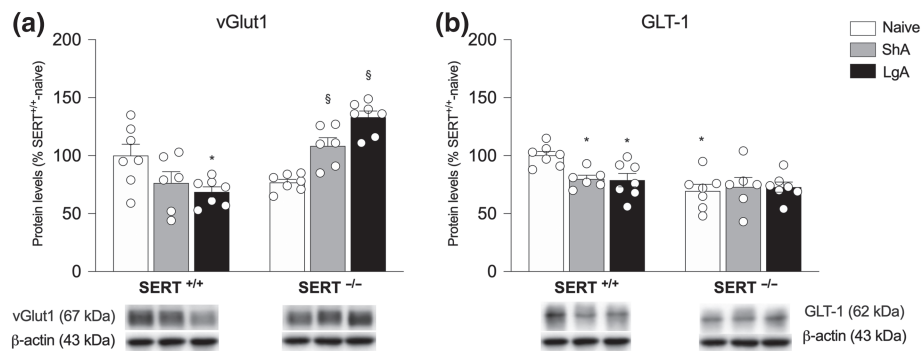
FIGURE 1 Interaction between SERT deletion and cocaine self-administration on the vesicular glutamate transporter 1 (vGlut1) and on the glial glutamate transporter 1 (GLT-1) in the sNAc. Protein levels of vGlut1 (a) and GLT-1 (b) in the sNAc are expressed as percentages of SERT$^{+/+}$-naive rats. Below the graphs, representative immunoblots are shown for vGlut1 (60 kDa), GLT-1 (62 kDa), and β-actin (43 kDa) proteins. Histograms represent the mean ± SEM of the following number of rats: naive (SERT$^{+/+}$ $n = 7$; SERT$^{-/-}$ $n = 7$), ShA (SERT$^{+/+}$ $n = 6$; SERT$^{-/-}$ $n = 6$), and LgA (SERT$^{+/+}$ $n = 7$; SERT$^{-/-}$ $n = 7$). *$P < 0.05$, versus SERT$^{+/+}$-naive; $^{§}P < < 0.05$, versus SERT$^{-/-}$-naive (Tukey's multiple comparisons test); LgA, cocaine long-access; N, naive; ShA, cocaine short-access
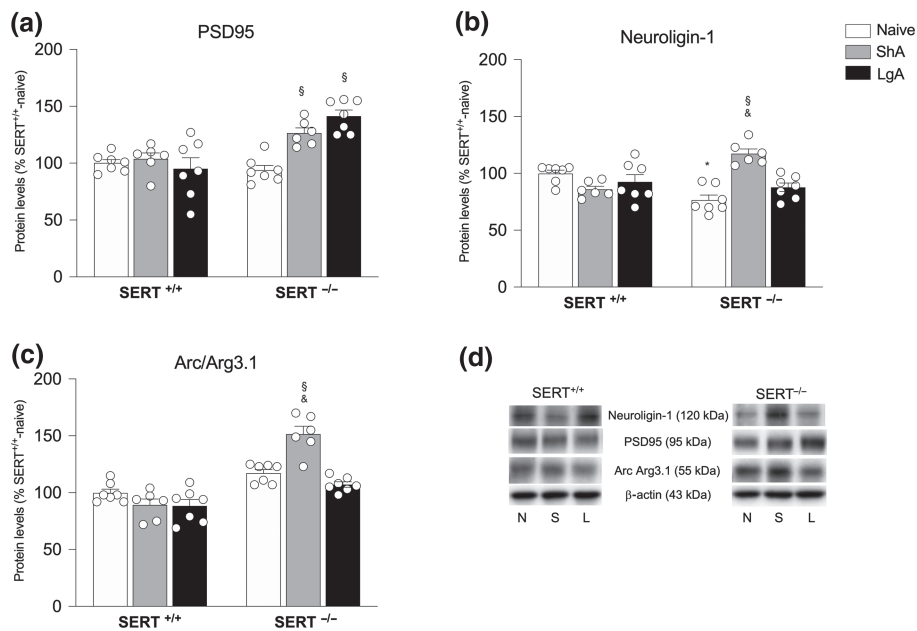

FIGURE 2 Interaction between SERT deletion and cocaine self-administration on the post-synaptic protein 95 (PSD95), Neuroligin-1 and arc/Arg3.1 in the sNAc. Protein levels of PSD95 (a), Neuroligin-1 (b), and arc/Arg3.1 (c) in the sNAc are expressed as percentages of SERT$^{+/+}$-naive rats. In panel (d), representative immunoblots are shown for PSD95 (95 kDa), Neuroligin-1 (132 kDa), arc/Arg3.1 (55 kDa), and β-actin (43 kDa) proteins. Histograms represent the mean ± SEM of the following number of rats: naive (SERT$^{+/+}$ $n = 7$; SERT$^{-/-}$ $n = 7$), ShA (SERT$^{+/+}$ $n = 6$; SERT$^{-/-}$ $n = 6$), and LgA (SERT$^{+/+}$ $n = 7$; SERT$^{-/-}$ $n = 7$). *$P < 0.05$, versus SERT$^{+/+}$-naive; $^{§}< 0.05$, versus SERT$^{-/-}$-naive; $^{\&}P < 0.05$, versus SERT$^{-/-}$-LgA (Tukey's multiple comparisons test). NLgA, cocaine long-access; N, naive; ShA, cocaine short-access

access, genotype and cocaine access x genotype interaction. Examining the individual treatment effects, we found that removal of SERT did not alter the expression of PSD95 in the sNAc of cocaine-naive rats, whereas it was significantly up-regulated under both the ShA and LgA conditions in SERT$^{-/-}$ rats. This was not observed for the wild-type rats (Figure 2a). Next, we measured the expression of Neuroligin-1, a protein that keeps pre- and postsynaptic sites close to ensure physiological neurotransmission (Luoet al., 2020). Two-way

ANOVA (for details, see Table S1) showed a significant cocaine access effect and a cocaine access x genotype interaction effect (Figure 2b). Post hoc testing of the main treatment effects indicated a significant reduction of Neuroligin-1 in SERT$^{-/-}$ naive rats. Furthermore, cocaine access enhanced Neuroligin-1 expression under the ShA condition with no changes as consequence of the LgA procedure in SERT$^{-/-}$ but not SERT$^{+/+}$ rats. Then, we measured the expression of activity-regulated cytoskeleton associated protein (Arc/Arg3.1), a cytoskeletal

protein involved in adaptive rearrangements following cocaine exposure (Caffino, Giannotti, et al., 2017). Two-way ANOVA (for details, see Table S1) revealed a significant cocaine access, genotype and cocaine access x genotype interaction effect (Figure 2c). Given the interaction of the two treatment paradigms, we made all intergroup comparisons. Similar to Neurologin-1, we found a significant up-regulation of Arc/Arg3.1 in the sNAc of $SERT^{-/-}$ but not $SERT^{+/+}$ rats under the ShA condition, with no effect under the LgA condition.

3.4 | Expression levels of NMDA receptor subunits in the homogenate of the sNAc following ShA and LgA to cocaine in $SERT^{+/+}$ and $SERT^{-/-}$ rats

We then moved our molecular analyses to the main glutamate receptors, starting with the measurement of the expression level of the obligatory subunit of the NMDA receptor, that is, GluN1. Two-way ANOVA (for details, see Table S1) revealed a significant genotype and a cocaine access x genotype interaction effect. Given the interaction of the two treatment paradigms, we made all intergroup comparisons. No effects were observed under cocaine-naive conditions. Of note, whereas in $SERT^{+/+}$ rats the LgA, but not ShA, condition reduced GluN1 expression, in $SERT^{-/-}$ rats the LgA condition was associated with increased GluN1 expression with no effect produced by the ShA

procedure (Figure 3a). Next, we investigated the expression level of two accessory subunits of the NMDA receptor: GluN2A and GluN2B (Figure 3b, c). Two-way ANOVA of GluN2A (for details, see Table S1) revealed a main effect of genotype, cocaine access and cocaine access x genotype interaction (Figure 3b). Therefore, we subdivided the data for further intergroup comparisons. Under cocaine-naive conditions, no changes were observed, whereas the LgA, but not ShA, procedure evoked a significant decrease of GluN2A levels in the sNAc of $SERT^{+/+}$ rats. This effect that was not observed in the knockout counterpart exposed to the same treatment condition (Figure 3b). With respect to GluN2B, two-way ANOVA (for details, see Table S1) revealed only a significant effect of cocaine access (Figure 3c) and, therefore, no further sub-testing was done. We then assessed the expression level of the main scaffolding protein of NMDA receptors, a protein whose function is to stably retain and stabilize these receptors in the post-synaptic membrane, SAP102. Two-way ANOVA (for details, see Table S1) showed a significant cocaine access and cocaine access x genotype interaction. Evaluating the individual treatment effects, we found that SERT ablation reduced the expression of SAP102 in cocaine-naive rats. In addition, we found that both LgA and ShA procedures enhanced the expression of SAP102 in the sNAc of $SERT^{-/-}$ rats, whereas, on the contrary, in the same subregion of $SERT^{+/+}$ rats the LgA, but not ShA, condition evoked a significant reduction (Figure 3d).
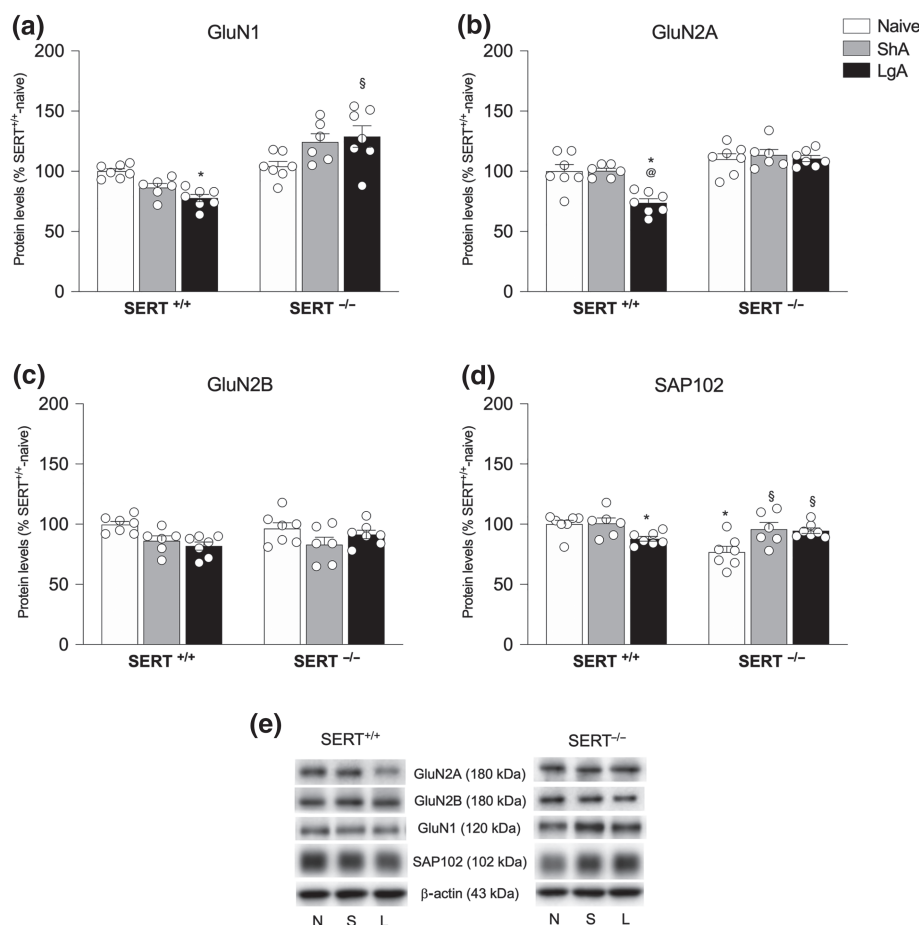

FIGURE 3 Interaction between SERT deletion and cocaine self-administration on the NMDA receptor subunits and the scaffolding protein SAP102 in the sNAc. Protein levels of GluN1 (a), GluN2A (b), GluN2B (c), and of the NMDA specific scaffolding protein SAP102 (d) in the sNAc are expressed as percentages of $SERT^{+/+}$-naive rats. In panel (e), representative immunoblots are shown for GluN2A (180 kDa), GluN2B (180 kDa), GluN1 (120 kDa), SAP102 (102 kDa), and β-actin (43 kDa) proteins. Histograms represent the mean ± SEM of the following number of rats. naive ($SERT^{+/+}$ $n = 7$; $SERT^{-/-}$ $n = 7$), ShA ($SERT^{+/+}$ $n = 6$; $SERT^{-/-}$ $n = 6$), and LgA ($SERT^{+/+}$ $n = 7$; $SERT^{-/-}$ $n = 7$). $*P < 0.05$, versus $SERT^{+/+}$-naive; $^{§}P < 0.05$, versus $SERT^{-/-}$-naive; $^{@}P < 0.05$, versus $SERT^{+/+}$-ShA (Tukey's multiple comparisons test); LgA, cocaine long-access; N, naive; ShA, cocaine short-access

3.5 | Expression levels of AMPA receptor subunits and related scaffolding proteins in the homogenate of the sNAc following ShA and LgA to cocaine in SERT$^{+/+}$ and SERT$^{-/-}$ rats

We then assessed the expression level of the two main subunits of the AMPA receptor GluA1 and GluA2, in the sNAc of SERT$^{-/-}$ and SERT$^{+/+}$ rats (Figure 4a and b, respectively).

As for the AMPA subunit GluA1, two-way ANOVA (for details, see Table S1) revealed a significant genotype effect and a cocaine access x genotype interaction effect (Figure 4a). Further analysis of the main treatment effects showed that the ablation of SERT altered the subsequent response to the ShA cocaine regimen, which led to a significant increase in GluA1 levels, with no effects after the LgA condition. No effects were observed in the sNAc of SERT$^{+/+}$ rats (Figure 4a). A different picture emerged when examining the GluA2 subunit. Two-way ANOVA (for details, see Table S1) revealed a significant genotype effect and a cocaine access x genotype interaction effect (Figure 4b). Again, SERT removal did not alter the expression of this AMPA subunit under cocaine naive conditions, whereas the two types of cocaine access differently dysregulated its expression in the sNAc of SERT$^{-/-}$ and SERT$^{+/+}$ rats. In fact, whereas no effects were found under the ShA condition in both genotypes, the

LgA procedure caused a significant reduction in the expression of the GluA2 subunit in SERT$^{+/+}$ rats and it up-regulated this subunit in SERT$^{-/-}$ rats.

Then we measured the expression of the GluA1 scaffolding protein SAP97. Two-way ANOVA (for details, see Table S1) revealed a significant genotype effect and a cocaine access x genotype interaction effect (Figure 4c). We found that SERT knockout reduced SAP97 expression under cocaine naive conditions. However, exposure to ShA or LgA caused effects that were dissimilar in the sNAc of SERT$^{-/-}$ and SERT$^{+/+}$ rats. In fact, the ShA regimen increased SAP97 in the sNAc of SERT$^{-/-}$ rats that were instead reduced in the same subregion of SERT$^{+/+}$ rats. At variance, no effect was observed in SERT$^{-/-}$ rats exposed to the LgA condition whereas a reduction was observed in SERT$^{+/+}$ rats under the same experimental conditions (Figure 4c).

Next, we assessed the protein levels of the GluA2 scaffolding protein GRIP. Two-way ANOVA (for details, see Table S1) revealed a significant cocaine access x genotype interaction effect (Figure 4d). The removal of SERT led to reduced expression of GRIP protein levels in cocaine naive animals. Of note, whereas no further changes were observed in both genotypes under the ShA regimen, LgA caused an increase in the expression of this scaffolding protein in SERT$^{-/-}$ rats that was instead decreased in SERT$^{+/+}$ rats.

FIGURE 4 Interaction between SERT deletion and cocaine self-administration on the AMPA receptor subunits and their specific scaffolding proteins in the sNAc. Protein levels of GluA1 (a) and GluA2 (b), SAP97 (c), and GRIP (d) in the sNAc are expressed as percentages of SERT$^{+/+}$-naive rats. In panel (e), representative immunoblots are shown for GluA1 (108 kDa), GluA2 (108 kDa), SAP97 (140 kDa), GRIP (122 kDa), and β-actin (43 kDa) proteins. Histograms represent the mean ± SEM of the following number of rats: naive (SERT$^{+/+}$ n = 7; SERT$^{-/-}$ n = 7), ShA (SERT$^{+/+}$ n = 6; SERT$^{-/-}$ n = 6), and LgA (SERT$^{+/+}$ n = 7; SERT$^{-/-}$ n = 7). *P < 0.05, versus SERT$^{+/+}$-naive; §P < 0.05, versus SERT$^{-/-}$-naive; $^{#}$P < 0.05, versus SERT$^{-/-}$-ShA (Tukey's multiple comparisons test); LgA, cocaine long-access; N, naïve; ShA, cocaine short-access
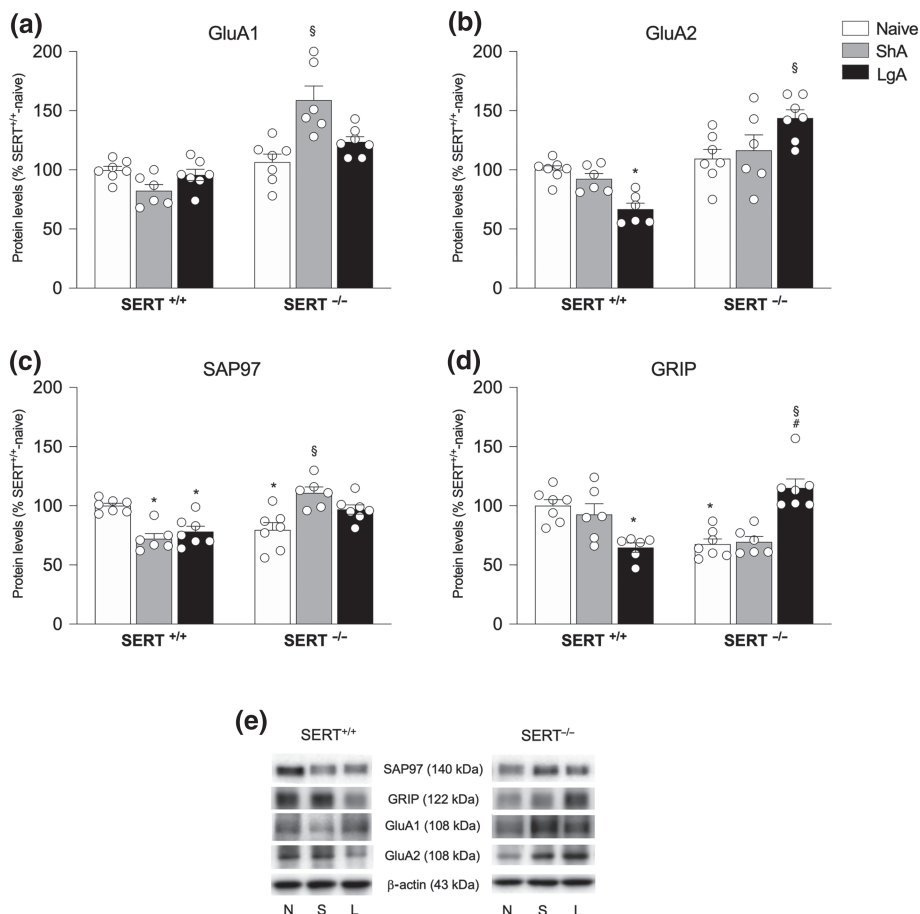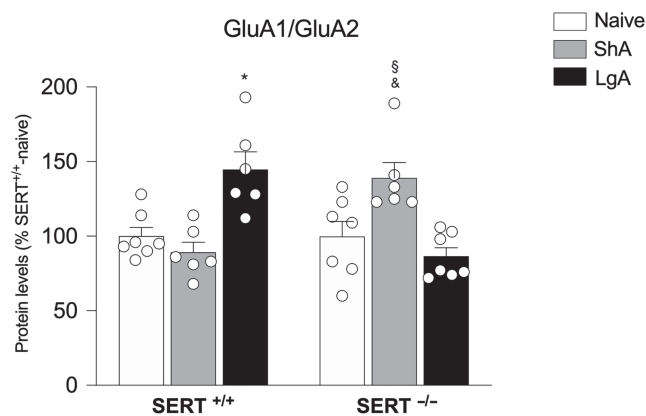
FIGURE 5 Interaction between SERT deletion and cocaine self-administration on the GluA1/GluA2 ratio in the sNAc. Protein levels of GluA1 and GluA2 AMPA receptor subunits expressed as GluA1/GluA2 ratio are shown as percentages of SERT$^{+/+}$-naive rats. Histograms represent the mean ± SEM of the following number of rats: naive (SERT$^{+/+}$ $n = 7$; SERT$^{-/-}$ $n = 7$), ShA (SERT$^{+/+}$ $n = 6$; SERT$^{-/-}$ $n = 6$), and LgA (SERT$^{+/+}$ $n = 7$; SERT$^{-/-}$ $n = 7$). *$P < 0.05$ versus SERT$^{+/+}$-naive; $^{§}P < 0.05$, versus SERT$^{-/-}$-naive; $^{&}P < 0.05$, versus SERT$^{-/-}$-LgA (Tukey's multiple comparisons test); LgA, cocaine long-access; N, naive; ShA, cocaine short-access
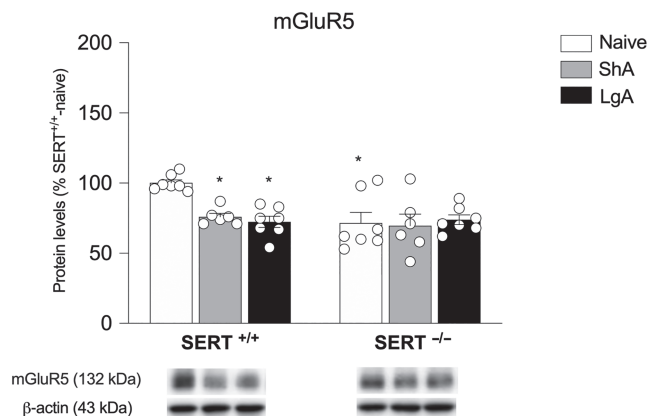

FIGURE 6 Interaction between SERT deletion and cocaine self-administration on the metabotropic receptor mGluR5 in the sNAc. Protein levels of mGluR5 in the sNAc are shown as percentages of SERT$^{+/+}$-naive rats. Below the graph, representative immunoblots are shown for mGluR5 (132 kDa) and β-actin (43 kDa) proteins. Histograms represent the mean ± SEM of the following number of rats: naive (SERT$^{+/+}$ $n = 7$; SERT$^{-/-}$ $n = 7$), ShA (SERT$^{+/+}$ $n = 6$; SERT$^{-/-}$ $n = 6$), and LgA (SERT$^{+/+}$ $n = 7$; SERT$^{-/-}$ $n = 7$). *$P < 0.05$, versus SERT$^{+/+}$-naive (Tukey's multiple comparisons test); LgA, cocaine long-access; N, naive; ShA, cocaine short-access

3.6 | GluA1/GluA2 ratio in the homogenate of the sNAc following ShA and LgA to cocaine in SERT$^{+/+}$ and SERT$^{-/-}$ rats

Because of the different changes in the expression of GluA1 and GluA2 AMPA receptors, we decided to investigate the GluA1/GluA2 ratio. Two-way ANOVA of GluA1/GluA2 ratio (for details, see Table S1) showed a significant effect of the interaction between genotype and cocaine self-administration (Figure 5). Further inter-group sub-testing revealed that, in the sNAc of SERT$^{+/+}$ rats, the GluA1/GluA2 ratio increased only in LgA-exposed animals, whereas, in their knockout counterparts, an up-regulation was observed only in ShA-exposed animals.

3.7 | Expression levels of metabotropic receptor mGluR5 in the homogenate of the sNAc following ShA and LgA to cocaine in SERT$^{+/+}$ and SERT$^{-/-}$ rats

In order to better understand the homeostasis of the glutamate synapse under our experimental condition, we evaluated the expression of the metabotrobic receptor mGluR5, that, by being mainly expressed at extrasynaptic sites, is able to alter the molecular composition of the synapse and has a critical role in cocaine-seeking behaviour (Niedzielska-Andres et al., 2021). Two-way ANOVA (for details, see Table S1) revealed a significant cocaine access, genotype and a cocaine access x genotype interaction effect (Figure 6). The removal of SERT reduced mGluR5 expression levels in cocaine naive animals. Exposure to cocaine under both ShA or LgA regimens reduced mGluR5 expression only in SERT$^{+/+}$ rats.

4 | DISCUSSION

The main finding of the present manuscript is that the removal of SERT alters the homeostasis of the glutamate synapse in the sNAc of naive rats and dysregulates the response to cocaine in SERT$^{-/-}$ animals following early withdrawal from both ShA or LgA cocaine self-administration compared with SERT$^{+/+}$ rats.

At first glance, it appears that the glutamatergic synapse is in a distressed situation in cocaine-naive SERT$^{-/-}$ rats. In fact, we observed that the expression of the main glutamate transporter GLT-1, which is chiefly responsible for glutamate reuptake, is significantly reduced in the sNAc of cocaine-naive SERT$^{-/-}$ rats. The consequent potential increase in the extracellular level of glutamate is expected to be associated with an increase in the extracellular level of serotonin in the synaptic milieu. Another finding in cocaine-naive SERT$^{-/-}$ rats is the reduced expression of the glutamate metabotropic receptor 5 (mGluR5). This observation is indeed interesting because mGluR5 is known to positively regulate the expression of GLT-1 (Vermeiren et al., 2005). Accordingly, we hypothesize that, in the sNAc of cocaine-naive SERT$^{-/-}$ rats, the reduced expression of mGluR5 leads to a reduction in GLT-1, which in turn causes an excess of extracellular glutamate levels. Thus, the excess of glutamate may spill over at the extrasynaptic sites and astrocytes, where mGluR5 is primarily expressed (Lujan et al., 1996), fuelling a vicious cycle that, in turn, reduces the expression of mGluR5 and, thereby, that of GLT-1. In line with this possibility, the expression of vGluT1 is reduced in cocaine-naive SERT$^{-/-}$ rats, an effect that may represent a compensatory response to counteract the increased glutamate neurotransmission, in line with previous data showing that serotonin inhibits synaptic glutamate currents in rat NAc neurons (Muramatsu et al., 1998).

Interestingly, we also investigated the expression of Neuroligin-1, a protein that has the function to keep the pre- and the post-synaptic glutamatergic terminals at close vicinity to warrant physiological neurotransmission (Muramatsu et al., 1998). The reduced Neuroligin-1 expression in the sNAc of cocaine-naive SERT$^{-/-}$ rats suggests reduced functional connectivity between pre-and post-synaptic terminals, which further strengthens the notion of compromised glutamatergic neurotransmission. We found that the expression of the main subunits of the NMDA and AMPA glutamate receptors was not altered in the sNAc of cocaine-naive SERT$^{-/-}$ rats, whereas the expression of their respective scaffolding proteins (SAP102 for NMDA and SAP97 and GRIP for AMPA) was reduced. This suggests that such receptors, despite their unaltered expression, are less retained at the post-synaptic membrane and, very likely, less functional. Taking these findings together, it appears that in the sNAc of cocaine-naive SERT$^{-/-}$ rats the glutamate synapse is impaired by the higher extracellular levels of serotonin, an effect that may underlie a different response to a challenge. It is possible to speculate that such a change in glutamate homeostasis as a consequence of SERT deletion may, at least partially, contribute to the higher intake of cocaine observed in SERT$^{-/-}$ rats (Homberg et al., 2008; Karel et al., 2018; Nonkes et al., 2011; Verheij et al., 2018). Notably, the reduction of GLT-1 expression, which is also seen in the cNAc of SERT$^{-/-}$ (Caffino, Mottarlini, Targa, et al., 2021), is extremely critical as it has been previously shown to favour cocaine seeking (Knackstedt et al., 2010).

Accordingly, our next step was to investigate the influence of SERT removal on the glutamate synapse in response to a condition characterized by short- (ShA) or long-(LgA) access to cocaine with killing early after withdrawal (24 h), taking into account that the cocaine self-administration data have been published elsewhere (Caffino et al., 2019) and show that SERT$^{-/-}$ rats self-administer higher amounts of cocaine compared with wild-type controls under both ShA and LgA conditions. At first sight, it appears that the glutamate synapse responds differently to ShA or LgA exposure, suggesting that the limited (as mimicked by ShA condition) and escalated (as resembled by LgA condition) cocaine access have indeed a different impact on the glutamate synapse in the sNAc of SERT$^{-/-}$ rats in comparison with the same brain subregion of SERT$^{+/+}$ rats. Firstly, we found a significant increase in the expression of vGluT1 following either ShA or LgA procedures, an effect that is opposite to that observed in the sNAc of SERT$^{+/+}$ rats. This suggests that, under cocaine treatment, the glutamate synapse of SERT$^{-/-}$ rats has lost the inhibitory control over glutamate release, at variance to SERT$^{+/+}$ rats. The dysregulated response observed in SERT$^{-/-}$ rats and the potential increase of glutamate release hypothesized above might explain the increased expression of PSD95 under both ShA or LgA conditions. PSD95 is an integral protein of the glutamate synapse, primarily expressed at the postsynaptic density (Vickers et al., 2006). The marked increase in the expression of PSD95 may reflect increased spine density, as previously demonstrated in the sNAc of rats exposed to cocaine self-administration (Wang et al., 2021), or increased size of the spine head, causing an enlargement of the postsynaptic density. Accordingly,

spines might be more rigid, that is, less plastic, following both ShA and LgA: again, this situation is not observed in the SERT$^{+/+}$ rats as PSD95 expression is unaltered under both cocaine regimens. In view of an enlarged post-synaptic density, the evidence that Neuroligin-1 expression is up-regulated in ShA, but not LgA, conditions in SERT$^{-/-}$ rats might represent an attempt of the synapse to maintain a physiological 'distance' between its pre and post terminals to guarantee a correct communication and transmission of the glutamatergic inputs, an effect that is lost in rats exposed to LgA. Notably, the same trend was observed when examining the expression of activity-regulated cytoskeleton associated protein (Arc/Arg3.1), suggesting that also the cytoskeleton is trying to adjust to the above mentioned post-synaptic changes in response to ShA exposure, an attempt that has failed in SERT$^{-/-}$ LgA rats.

It is interesting to point out that the relationship between mGluR5 and GLT-1 is maintained in both genotypes under the different procedures of cocaine self-administration. In fact, in the sNAc of SERT$^{+/+}$ rats, mGluR5 expression is significantly reduced following both ShA and LgA conditions, an effect that may drive the reduction of GLT-1 expression, which is observed in the same groups. Accordingly, in the sNAc of SERT$^{-/-}$ rats, cocaine self-administration did not change the baseline reduction of mGluR5 expression observed in cocaine-naive rats, an effect that is also observed for GLT-1 expression in the same cocaine-exposed groups. Again, these data point to a dysregulation of the glutamate synapse in the sNAc of SERT$^{-/-}$ rats.

Concerning the response of glutamate receptors, focusing on the NMDA receptor complex, the pattern of changes in GluN1 and GluN2A expression in SERT$^{+/+}$ rats exposed to cocaine mimics the reduced expression of vGluT1 in the same animals, raising the possibility that such reductions could be ascribed to decreased glutamate release. Such regulation is lost in the sNAc of SERT$^{-/-}$ rats, implying different receptor homeostasis of the synapse in these animals. In addition, in SERT$^{-/-}$ rats, the expression of the obligatory subunit GluN1 is increased under both ShA or LgA conditions, and this increase is paralleled by an increase in the expression of SAP102. Based on these data, we hypothesize that following the increased release of glutamate under both the ShA and LgA conditions, the GluN1 subunit is more exposed in the membrane, which we suppose is enlarged, and that such exposure is stabilized by the NMDA-specific scaffolding protein.

A different picture can be drawn when focusing our attention on AMPA receptors, with a modulation that appears to be subunit specific. In SERT$^{+/+}$ rats, no changes in the expression of the GluA1 subunit were observed following both cocaine regimens, whereas the expression of the GluA1 scaffolding protein SAP97 was significantly reduced. This suggests that this receptor is poorly retained at the membrane, as observed in SERT$^{-/-}$ naive rats. Conversely, such subunit expression is increased only after ShA, but not LgA, exposure in SERT$^{-/-}$ rats, an effect coupled with the increase of its scaffold SAP97. When examining the GluA2 subunit, in SERT$^{+/+}$ rats we found a significant reduction of GluA2 only following LgA, paralleled by a reduction of its scaffold GRIP. Instead, in SERT$^{-/-}$ rats, the GluA2 subunit expression was increased following the LgA procedure

only, an effect that was stabilized by the increased expression of its scaffolding protein GRIP. These results indicate a dysregulation caused by the interaction of high serotonin levels, due to SERT deletion, and cocaine in the two genotypes. Furthermore, the receptor subunit changes and the relation with their respective scaffolding proteins under the different experimental conditions appear extremely interesting because GluA1 and GluA2 receptors are expressed at different neurons: GluA1 is particularly associated with inhibitory neurons whereas GluA2 is coupled with excitatory neurons (Kerr et al., 1998). Thus, our data may reveal that, in the sNAc, glutamatergic neurons are differently affected by the combination of a hyperserotonergic tone and cocaine, with the ShA procedure affecting inhibitory neurons in SERT$^{-/-}$ rats and the LgA condition affecting excitatory neurons in SERT$^{+/+}$ rats, and SERT$^{-/-}$ rats in opposite direction. We also measured the formation of GluA2-lacking Ca^{2+}-permeable AMPA receptors, whose presence appears to favour conditioned place preference (Shukla et al., 2017) and incubation of cocaine craving (Conrad et al., 2008). The GluA1/A2 ratio is widely accepted as an indirect index of maladaptive plasticity (Wolf, 2016). Again, we found a different scenario between SERT$^{+/+}$ and SERT$^{-/-}$ rats. In the sNAc of SERT$^{+/+}$ rats we observed an increased formation of GluA2-lacking AMPA receptors under the LgA condition only, an effect that, however, is not stabilized given the reduction of the relative scaffolding protein SAP97. Conversely, in the sNAc of SERT$^{-/-}$ rats, the formation of GluA2-lacking AMPA receptors is promoted only in the ShA group. Because it has been hypothesized that the molecular composition of the excitatory synapses in the NAc and their functional state dictate the dynamic of cocaine-associative memories (Wright et al., 2020), the formation of these two distinct sets of AMPA receptors might be involved in the dysregulation of the excitatory neurotransmission, thereby enhancing the stabilization of cocaine experience-induced memories (Wang et al., 2021). This imbalance in AMPA receptor subunits, stabilized at the synapse by the increased expression of their specific anchoring proteins, coupled with increased formation of new synapses (suggested by increased expression of PSD95 and Neuroligin-1 in SERT$^{-/-}$ rats exposed to both ShA and LgA) suggests that SERT deletion in combination with cocaine exposure contributes to the formation of new connections encoding drug-related memory.

The origin of the glutamatergic changes in the sNAc in SERT$^{-/-}$ rats may be neurodevelopmental. Indeed, serotonin affects the migration of cortical glutamatergic neurons in the embryonic brain (Riccio et al., 2011), and SERT$^{-/-}$ rats show changes in glutamatergic markers at pre-weaning and juvenile stages (Brivio et al., 2019). For this reason, the rescue of the effects of SERT knockout and associated glutamatergic synaptic changes and vulnerability to cocaine addiction is challenging. Instead, up-regulation of GLT-1 through with *N*-acetylcysteine or **ceftriaxone** may offer opportunities to normalize the glutamatergic adaptations and help to reduce the vulnerability to cocaine, especially because these agents have been proposed as therapeutic interventions in correcting preclinical and clinical manifestations of drug addiction (Roberts-Wolfe & Kalivas, 2015).

The major limitation of our study relies on the fact that we have performed our analyses in the whole homogenate because the

subdivision in the two accumbal subregions did not allow us to make a more suitable cellular preparation for investigating the postsynaptic density; accordingly, we can only speculate when interpreting the data on synaptic versus extrasynaptic localization of glutamate receptors. Further, these studies were undertaken in male rats and we do not know if our findings can be extended to female rats. Finally, we could not determine whether the changes in glutamatergic markers are due to the last cocaine self-administration session, or the entire history of cocaine self-administration.

Taken together, these results suggest that the hyperserotonergic tone interacts with cocaine in the sNAc to unmask critical changes in the glutamate synapse, early after the exposure to the psychostimulant, which could be important for both moderate and escalated use of cocaine. This is an interesting result because we have recently published that such interaction involves only the LgA procedure in the cNAc. Thus, our findings also reveal a different sensitivity of these two closed subregions under the same experimental conditions, despite their close vicinity (Caffino, Mottarlini, Targa, et al., 2021). These findings indicate that deletion of SERT might influence the adult response to cocaine pointing to serotonin-glutamate interaction as a critical factor in the negative emotional state that can be observed in drug addicts after drug cessation (Gawin, 1991; Perrine et al., 2008). Further, our data are of translational value as recent literature reported a general reduction of the homeostasis of the glutamate system not only in the NAc (Engeli et al., 2021) but also in other human brain regions (Hulka et al., 2014, 2016; Martinez et al., 2014) of drug addicts, suggesting that such system may represent a valuable target for novel therapeutic interventions in humans.

AUTHOR CONTRIBUTIONS

L.C. conducted the western blot experiments, analysed data and contributed to the writing of the manuscript. F.M. conducted the western blot analyses, made the graphic abstract, overviewed figures and made literature research. G.T. contributed to sample preparation and conducted the western blot analyses and contributed to overview figures and literature research. M.V. conducted the self-administration experiment and contributed to the writing of the manuscript. F.F. conceived and planned the experiments, supervised the molecular analyses, contributed to the interpretation of the results, contributed to the writing of the manuscript and supervised the project. J.R.H. conceived and planned the experiments, contributed to the interpretation of the results and the writing of the manuscript. All authors discussed the results and contributed to the final manuscript.

CONFLICT OF INTEREST

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

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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