

Hypersensitivity to amphetamine's psychomotor and reinforcing effects in serotonin transporter knockout rats: glutamate in the nucleus accumbens

Lucia Caffino^{1*}, Michel M. M. Verheij^{2*}, Karine Roversi², Giorgia Targa¹, Francesca Mottarlini¹, Piotr Popik³, Agnieszka Nikiforuk³, Joanna Golebiowska³, Fabio Fumagalli^{1,#} and Judith R. Homberg^{2,#}

¹Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Italy; ²Department of Cognitive Neuroscience, division of Molecular Neurogenetics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen Medical Centre, the Netherlands; ³Maj Institute of Pharmacology, Polish Academy of Sciences, Department of Behavioural Neuroscience and Drug Development, Krakow, Poland.

Corresponding author: Prof. Judith Homberg, Department of Cognitive Neuroscience, division of Molecular Neurogenetics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen Medical Centre, the Netherlands; email: Judith.Homberg@radboudumc.nl

*Lucia Caffino and Michel M.M. Verheij contributed equally to this manuscript and can be both considered first author

#Fabio Fumagalli and Judith R. Homberg share the senior authorship

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Conflict of interest

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

Abstract

Background and purpose: Amphetamine use disorder is a serious health concern, but surprisingly little is known about the vulnerability to the moderate and compulsive use of this psychostimulant and its underlying mechanisms. Previous research showed that inherited serotonin transporter (SERT) down-regulation increases the motor response to cocaine, as well as moderate (as measured during daily 1-hr self-administration sessions) and compulsive (as measured during daily 6-hr self-administration sessions) intake of this psychostimulant. Here we sought to investigate whether these findings generalize to amphetamine and the underlying mechanisms in the nucleus accumbens.

Experimental Approach: In serotonin transporter knockout (SERT^{-/-}) and wild-type control (SERT^{+/+}) rats we assessed the locomotor response to acute amphetamine (AMPH) and intravenous AMPH self-administration under short access (ShA: 1-hr daily sessions) and long access (LgA: 6-hr daily sessions) conditions. 24 hrs after AMPH self-administration we analysed the expression of glutamate system components in the nucleus accumbens shell and core.

Key results: We found that SERT^{-/-} animals displayed an increased AMPH-induced locomotor response and increased AMPH self-administration under LgA, but not ShA conditions. Further, we observed changes in the vesicular and glial glutamate transporters, NMDA and AMPA receptor subunits and their respective postsynaptic scaffolding proteins as function of serotonin transporter genotype and AMPH exposure (baseline, ShA and LgA), specifically in the nucleus accumbens shell.

Conclusion and implications: We demonstrate that SERT gene deletion increases the psychomotor and reinforcing effects of AMPH, and that the latter is potentially mediated, at least in part, by homeostatic changes in the glutamatergic synapse of the nucleus accumbens shell and/or core.

Key words: serotonin transporter, amphetamine, locomotor activity, self-administration, nucleus accumbens, glutamate.

Abbreviations

5-HTTLPR: serotonin transporter-linked polymorphic region

AMPA receptor: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AMPH: amphetamine

FR1: fixed ratio 1

GG: Greenhouse-Geisser

GLT-1: glial glutamate transporter

GluN1/2A/2B: glutamate NMDA receptor 1/2A/2B subunit

GluA1/A2: glutamate AMPA receptor 1/2 subunit

GRIP: glutamate receptor interacting protein; AMPA receptor scaffolding protein

LgA: long access (6 hr daily self-administration sessions)

NAc: nucleus accumbens

cNAc: core region of the nucleus accumbens

sNAc: shell region of the nucleus accumbens

NMDA receptor: N-methyl-D-aspartate receptor

PFC: prefrontal cortex

SERT^{-/-}: serotonin transporter knockout

SERT^{+/+}: wild-type counterpart of serotonin transporter knockout rat

SAP97: synapse associated protein 97; AMPA receptor scaffolding protein

SAP102: synapse associated protein 2013; NMDA receptor scaffolding protein

ShA: short access (1 hr daily self-administration sessions)

vGlut1: vesicular glutamate transporter 1

Bullet point summary**What is already known:**

- Rats lacking the serotonin transporter show increased moderate and compulsive cocaine self-administration.

What this study adds:

- Serotonin transporter knockout rats show also increased compulsive, but not moderate, amphetamine self-administration.
- This is associated with glutamatergic synaptic changes in the nucleus accumbens shell and core.

Clinical significance:

- We provide a mechanism for amphetamine addiction proneness in individuals with inherited serotonin transporter down-regulation.

Introduction

Amphetamine (AMPH) is a psychostimulant substance widely used across the world because of its euphoric effects, making it a remaining public health issue (World Drug Report, 2018). One in ten AMPH users develop dependency on the drug (Anthony et al., 1994). However, up to date, it is not clear which factors shape vulnerability to AMPH dependency and the underlying mechanisms are still elusive. This would be critical as such insight ultimately would help to inform early interventions or treatments.

In humans, the short (s) low expression allelic variant of the serotonin transporter-linked polymorphic region (5-HTTLPR) has been considered as risk allele for AMPH and cocaine addiction (Cao et al, 2013). Accordingly, we and others previously observed that serotonin transporter knockout ($SERT^{-/-}$) rats, which may model the 5-HTTLPR s-allele (Holmes et al., 2010; Schipper et al., 2019) in humans, are more sensitive to the psychomotor and reinforcing effects of these psychostimulants (Homberg et al., 2008; Oakly et al., 2014; Pettie et al., 2019; Verheij et al., 2018). However, as to whether these observations generalize to amphetamine is currently not known.

Mechanistically, psychostimulants increase extracellular levels of dopamine, serotonin and noradrenaline via an action on their respective transporter, although with varying sensitivity for the different psychostimulants. In particular, cocaine and AMPH are thought to mediate their reward-related effects by increasing dopamine levels (Koob, 1992; Natarajan & Yamamoto, 2011; Rocha et al., 1998; Rothman & Glowa, 1995; Woolverton & Johnson, 1992; Lile & Nader, 2003). Yet, we observed that cocaine, more strongly increased locomotor activity in $SERT^{-/-}$ versus their wild-type $SERT^{+/+}$ counterparts (Homberg et al., 2008). In addition, on top of high baseline levels of extracellular serotonin (Homberg et al., 2007), the cocaine-induced increase of

extracellular serotonin, but not of extracellular dopamine and noradrenaline, was reduced in the nucleus accumbens (NAc) of SERT^{-/-} rats (Verheij et al., 2014). This suggests that SERT and accumbal serotonin may play a critical role in the psychomotor and reinforcing effects of psychostimulants. The role of SERT, however, may differ between psychostimulants, since AMPH has a lower sensitivity for SERT than cocaine (Howell and Kimmel, 2008). Given the previously observed SERT-dependent changes in the psychomotor and reinforcing effects of cocaine, we have now analyzed the SERT dependent psychomotor and reinforcing effects of AMPH. To this end, the acute locomotor response to various doses of AMPH, as well as AMPH self-administration under short access (ShA, 1 h/day) conditions (modeling moderate drug use), and long access (LgA, 6 h/day) conditions (modeling uncontrolled compulsive drug use) (Ahmed & Koob, 1998) has been obtained in SERT^{-/-} and their wild-type control rats.

From a neurobiological point of view, the transition from goal-directed drug intake toward addictive states has been proposed to result from a shift of control of motivated behavior from the prefrontal cortex (PFC) to the striatum via glutamatergic projections (Everitt & Robbins, 2016; Kalivas & Volkow, 2005). We have recently shown that the interaction between serotonin and cocaine intake, as well as the transition from hedonic to compulsive use of the psychostimulant, dysregulates glutamatergic synapses in the habenula and PFC (Caffino et al., 2020; Caffino et al., 2019), pointing to serotonin-glutamate interactions as a neurobiological substrate of heightened vulnerability to drug dependence. Yet, surprisingly, little is known about the AMPH-induced neuroadaptations on glutamate signalling and the serotonergic control on glutamatergic homeostasis in the transition from hedonic to compulsive AMPH use (Faraone, 2018). Interestingly, it has been demonstrated that AMPH reduced striatal protein expression of metabotropic mGluR5 receptor in rats (Shaffer et al., 2010),

suggesting that altered glutamate signalling can contribute to altered sensitivity to the psychomotor and reinforcing effects of amphetamine.

The NAc is a brain region critical for the neuroadaptive changes sub-serving drug intake, withdrawal and motivation to search for drugs. This brain region is characterized by the above-mentioned SERT-dependent changes in the serotonin response to psychostimulants and a strong glutamatergic signalling. On this basis, we sought to determine the SERT-dependent effects of AMPH self-administration on the accumbal glutamatergic synapse. To this end, we deeply analyzed the glutamate synapse in the NAc. Specifically, because the NAc core subregion (cNAc) is selectively involved in incubation of drug seeking, whereas the NAc shell (sNAc) is involved in escalation of drug taking (Guillem et al., 2014), we investigated glutamate homeostasis in both subregions and focused on: 1) the mechanisms regulating the storage of glutamate in presynaptic vesicles, which is mediated by the vesicular glutamate transporter (vGlut1) (El Mestikawy et al., 2011), 2) the clearance of the neurotransmitter from the synaptic cleft by the glutamate transporter (GLT-1) (Roberts-Wolfe & Kalivas, 2015), as well as 3) the main glutamate post-synaptic receptors (NMDA and AMPA) (Traynelis et al., 2010) and 4) the main scaffolding proteins of NMDA (SAP102) and AMPA (SAP97, GRIP) glutamate receptors, whose job is to firmly anchor these receptors to the membrane (Oliva et al., 2012).

Materials and Methods

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

Animals

We employed rats in this study because the rat is the preferred species for preclinical addiction research (Homberg et al., 2017). We used SERT^{-/-} rats as model for the human SERT gene polymorphism as has been established previously (Caspi et al., 2010). SERT^{-/-} rats (SLC6A4^{1Hubr}) were generated by N-ethyl-N-nitrosurea induced mutagenesis (Homberg et al., 2007; Smits et al., 2006) and outcrossed with commercially available Wistar rats (Harlan, Ter Horst, the Netherlands) for at least 15 generations. Male SERT^{-/-} and SERT^{+/+} offspring were used for experiments as in human psychostimulant addiction has a clear male preponderance (Degenhardt et al., 2014). The rats were bred in Nijmegen and either tested in Nijmegen or transported to Poland. In Nijmegen, rats were housed in groups of 2 rats in enriched Macrolon type III cages (42 x 26 x 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 BioServices) 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) in temperature (21±1°C) and humidity (55±5%) controlled rooms. Testing (AMPH self-administration) took place in the dark phase of the light/dark cycle. In Poland, rats were group-housed 4 rats per cage (380 × 200 × 590 mm, Ehret Labor- und Pharmatechnik GMBH & Co.KG, Germany) with aspen litter (MIDI LTE E-002 Abedd, AnimaLab, Poland) and environmental enrichment. The rats had free access to food (VRF1 (P) Special Diets Services, England) and water. Animals were housed in a temperature-

controlled ($21 \pm 1^\circ\text{C}$) and humidity-controlled (40–50%) colony room under a 12/12 h light/dark cycle (lights on at 06:00 AM). Behavioural testing (locomotor activity test) was performed during the light phase of the light/dark cycle. Testing was always done blindly by an experimenter who was unaware of the genotype of the animals. **Animals were not randomized, as their genotype determined the group in which the animals were placed.** All procedures were carried out in agreement with the current Directive 2010/63/EU and Dutch and Polish Research Council Guide for the Care and Use of Laboratory Animals and were approved by local and national Institutional Animal Care and Use Committees. The experimental procedures at Radboudumc (Nijmegen, The Netherlands) 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). The experimental procedures in Poland were performed at the Maj Institute of Pharmacology, Polish Academy of Science, Krakow, Poland. All efforts were made to reduce the number of animals used and their suffering both in the Netherlands and in Poland.

Drugs

Dex(d)-Amphetamine was provided by Spruyt Hillen (IJsselstein, the Netherlands) and was dissolved in saline 0,9%.

Behaviour

AMPHETAMINE-induced locomotor activity

The psychomotor effects of AMPH were tested in a grey plywood open field of 50 x 50 x 50 cm, which was illuminated by ~ 20 Lux. After 30 mins of habituation, rats,

weighing between 500-600 g (age: 6-7 months), were treated with 0.5 mg/kg amphetamine (SERT^{+/+}: n=11; SERT^{-/-}: n=12), 1 mg/kg amphetamine (SERT^{+/+}: n=13; SERT^{-/-}: n=13) or vehicle (SERT^{+/+}: n=24; vehicle SERT^{-/-}: n=25) according to a standard Latin square design using the same animals with a break of 4 weeks between the various treatments. Distance moved was recorded for 120 mins (Fig 1a), and analyzed afterwards using the ANY-maze video tracking system (version 4.82) from Stoelting.

Amphetamine self-administration

Intravenous catheterization

Under isoflurane anaesthesia, SERT^{+/+} and SERT^{-/-} rats, weighing between 300 and 450 g (age: 3 - 5 months), received a micro Renathane catheter into the right external jugular vein (for details, see: (Wee et al., 2007)). The tubing (0.037" o.d. x 0.023" i.d., Braintree Scientific Inc., USA) was guided subcutaneously to a stainless-steel cannula (22G, Plastics One Inc., USA) at the back of the rat. After surgery, rats were given Flunixin (analgesic drug: 2.5 mg/ml/kg, s.c.) and Cefazolin (antibiotic drug: 15 mg/ml/kg) daily for respectively 3 and 7 days. After surgery, rats were singly housed to prevent damage to the cannula due to cage mates. These rats received a wooden block and nest material as cage enrichment, but not a shelter to prevent collisions with the cannula. The body weight of the animals was monitored daily and full recovery of bodyweight happened within 3 to 5 days. During recovery, no unexpected changes in animal behavior (e.g. no self-grooming, no water intake) were observed. Catheter patency was maintained by daily flushing with 0.2 ml heparinized saline. In case of obstruction of the catheter, the animal was removed from the study.

Self-administration chambers

After, at least, one-week of recovery, AMPH self-administration was performed in standard operant boxes (28 x 26 x 20 cm, Med Associates Inc., USA). The cannula was connected to a swivel system that allowed the animal to move freely. Once the animals were connected to the system, 2 retractable levers were presented. Pressing the active (correct) lever resulted in i.v. infusion of AMPH (volume: 0.1 ml in 3 s). Furthermore, a light cue above the active lever indicated a timeout period of 20 s during which no AMPH infusion was available. Pressing the inactive (incorrect) lever had no programmed consequences.

Intravenous amphetamine self-administration training

Rats were exposed to 1-hour daily training sessions (0.03 mg/kg/infusion) under a fixed ratio 1 (FR1) schedule over the course of 10 days. During training, the maximum number of infusions was set at 20 to prevent an early overdose.

Short-access and long-access amphetamine self-administration

After training, rats were divided into 2 groups with an equal number of AMPH injections during the last 3 training session. One group continued to self-administer AMPH (0.03 mg/kg/infusion) for 1 hour/day (the short (ShA) access group: SERT^{+/+} n=14, SERT^{-/-} n=13), and the other group self-administered the same dose for 6 hours/day (the long (LgA) access group: SERT^{+/+} n=14, SERT^{-/-} n=13) [see also: (Gipson & Bardo, 2009)]. Protein expression data of these groups were compared to a new group of animals that was subjected to surgery, but were not exposed to AMPH (the AMPH-

naive group: SERT^{+/+} n=6, SERT^{-/-} n=6; Fig 1b). The animals were tested daily between 8 am and 6 pm, for 18 consecutive days [see also: (Verheij et al., 2018)] and the order of testing ShA and LgA groups was changed every day. Due to a limited number of self-administration chambers, self-administration was performed in 2 cohorts of animals (age-matched rats of both genotypes were always tested simultaneously and in equal numbers). The intake did not differ between batches of animals.

Collection of nucleus accumbens tissue

Twenty-four hours after the last ShA or LgA session, rats were decapitated without anesthesia. This procedure was used to avoid effects of anesthetics on gene expression and is in accordance of the Dutch legal regulations for sacrificing rodents. Brains were collected, freshly frozen and stored at -80°C. Nucleus accumbens core (cNAc) and shell (sNAc) punches were collected from 220 um brain slices, as previously described (Giannotti et al., 2016). The punches were collected according to coordinates as described by Paxinos and Watson (from Bregma +2.76 to Bregma +0.84 mm) (Paxinos and Watson, 2007), using a punching needle with a diameter of 1 mm (Fig 1c). For both regions, the two hemispheres were pooled.

Protein extraction and Western blot analyses

The experimental details for the Western Blotting conforms the guidelines of the British Journal of Pharmacology (Alexander et al., 2018).

Bilateral punches of cNAc and sNAc of 63 animals were sonicated using a cold buffer containing 0.32 M sucrose, 1 mM Hepes solution, 0.1 mM EGTA, 0.1 mM PMSF, pH = 7.4, in presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. Total proteins have been measured in the whole homogenate by the Bio-Rad

Protein Assay (Bio-Rad Laboratories). Western blots were run as previously described (Caffino *et al.* 2016). Briefly, ten micro-grams 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 GLT1 (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 GRIP (1:2000, Synaptic system, RRID: AB_887728) and anti β -Actin (1:10000, 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 43kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories). Due to the high number of samples, they were divided across 3 gels, that were run simultaneously and analysed. Aliquots of samples collected from SERT^{+/+}-naïve rats were distributed across the various gels (A-C) and used as reference values. We used the following correction factor the different gels:

Correction factor gel B = average of (OD protein of interest/OD β -actin for each sample

loaded in gel A) / (OD protein of interest/OD β -actin for the same sample loaded in gel B). Correction factor gel C = average of (OD protein of interest/OD β -actin for each sample loaded in gel A) / (OD protein of interest/OD β -actin for the same sample loaded in gel C). OD= optical density. By calculating this correction factor, we were able to evaluate genotype and AMPH exposure as independent variables despite the high number of samples. Gels were run 2 times each and the results represent the average from 2 different runs.

Data and Statistical Analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Previous self-administration studies in cocaine-treated SERT^{-/-} and SERT^{+/+} animals have revealed a significant genotype x self-administration session effect with an effect-size of about 0.4 η^2 (see a.o. (Verheij et al., 2018)). Entering this effect size (based on the previously obtained standard deviation), together with an alpha-value of 0.05 and a power of 0.80, into G*power (version 3.1), revealed a minimum number of 13 to 14 rats per genotype. Because of the substantially smaller standard deviation typically observed in our molecular studies (e.g. Caffino et al., 2019, 2020), the number of animals in the AMPH-naive groups that were not exposed to self-administration were reduced to 6. All animals tested were treated as independent values, there were no technical replicates.

Behavioural data

The Kolgomorov-Smirnov test was employed to determine normality of residuals and no significant variance in homogeneity was found. The psychomotor data were checked for outliers using the Grubbs' method (data points with $P < 0.05$ were considered

outliers). 2 outliers were removed from the analysis (1 SERT^{+/+} and 1 SERT^{-/-} rat). Because of their longitudinal nature, over 28 daily sessions, the amphetamine self-administration data were not subjected to an outlier test. To analyze the AMPH - induced psychomotor responses, a three-way ANOVA with the factors genotype, AMPH dose and time (for repeated measures) was used. To analyze the AMPH self-administration data, number of rewards were analyzed using a three-way ANOVA with the factor genotype, access type and self-administration session (for repeated measures). Sphericity of the variance matrix was checked using the Mauchly test. Data analysis was Greenhouse-Geisser (GG) corrected when sphericity was violated. Statistics were performed in IBM SPSS statistics version 23. Student's t-test was conducted as post-hoc test when allowed. **Significance for all tests was assumed at $p < 0.05$. P values are given in the results section below. F values can be found in supplementary table 1.**

Molecular data

The Kolmogorov-Smirnov test was employed to determine normality of residuals and no significant variance in homogeneity was found. Thus, the molecular changes produced by genotype and AMPH exposure alone as well as by their combination were analyzed using a two-way ANOVA, with the factors genotype and type of AMPH access as independent variables. When dictated by significant ($p < 0.05$) interaction terms, Tukey's multiple comparisons test was used to characterize differences among individual groups of rats. Two-way ANOVA analyses were performed using absolute data. Then, data were normalised as percentages of the AMPH-naïve SERT^{+/+} control rats that were not exposed to either AMPH 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

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eliminated from the final dataset if their data deviated from the mean by 2 SDs. Prism 6.0 (GraphPad) was used to analyze all the data. Data are shown as mean \pm S.E.M and as % of baseline to control for unwanted sources of variation. Significance for all tests was assumed at $p < 0.05$. P values are mentioned in the figure legends. F values can be found in supplementary table 1.

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Results

The psychomotor effects of amphetamine in SERT^{+/+} and SERT^{-/-} rats.

AMPH resulted in a dose-dependent increase in locomotor activity over time (Fig 2: amphetamine dose x time effect (GG corrected): $p < 0.0001$). This increase in locomotor activity was stronger in SERT^{-/-} compared to SERT^{+/+} rats (Fig 2: genotype x amphetamine dose x time effect (GG corrected): $p < 0.01$).

ha eliminato: $F_{(3.72,169.63)}=36.678,$

ha eliminato: $F_{(3.72,169.63)}=3.668,$

Amphetamine self-administration in SERT^{-/-} and SERT^{+/+} rats.

As expected, the escalation of AMPH intake over the daily self-administration sessions was higher in LgA than ShA rats (Fig 3A vs Fig 3B: access x self-administration session effect (GG corrected): $p < 0.001$), and influenced by SERT genotype (Fig 3A and Fig 3B: genotype x access x self-administration session effect (GG corrected): $p < 0.01$). More specifically, SERT^{-/-} rats were found to increase the daily intake of AMPH under LgA (Fig 3B: genotype x self-administration session effect (GG corrected): $p < 0.01$), but not under ShA conditions (Fig 3A: genotype x self-administration session effect (GG corrected): not significant: n.s.). No differences between SERT^{-/-} and SERT^{+/+} rats were found for the number of incorrect lever presses (supplementary Fig 1A and supplementary Fig 1B: genotype (x self-administration session) effect: n.s.) and timeout responses (supplementary Fig 1C and supplementary Fig 1D: genotype (x self-administration session) effect (GG corrected): n.s.).

ha eliminato: $F_{(3.500,175.012)}=15.663,$

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Effect of ShA and LgA amphetamine self-administration on the glutamate presynaptic terminal in SERT^{+/+} and SERT^{-/-} rats.

We first focused our attention on the presynaptic terminal by analyzing the protein expression of the vesicular glutamate transporter (vGluT1) that is responsible of

glutamate storage in presynaptic vesicles and it is considered an indirect index of glutamate release from the presynaptic neuron to the extracellular space (El Mestikawy et al., 2011). In the cNAc, two-way ANOVA revealed a main effect of AMPH access, genotype and an AMPH access x genotype interaction (Fig 4a). Further intergroup sub-testing revealed increased expression of vGluT1 in SERT^{-/-} rats exposed to either ShA or LgA with no effects in SERT^{+/+} rats following the same procedures.

In the sNAc, two-way ANOVA revealed a main effect of AMPH access, genotype and an AMPH access x genotype interaction (Fig 4b). In this sub region of the NAc, vGluT1 expression was reduced in SERT^{-/-}-naïve rats. The lack of SERT influenced the response to the different regimens of AMPH exposure. In fact, while vGluT1 protein levels in SERT^{-/-} were increased following ShA but not LgA to AMPH, in SERT^{+/+} rats, the expression of vGluT1 was increased independently from the ShA or LgA procedure.

Effect of ShA and LgA amphetamine self-administration on glial cells in SERT^{+/+} and SERT^{-/-} rats.

We next investigated the protein expression of the glial glutamate transporter (GLT-1) that is responsible of the reuptake of glutamate back into the glial cells from the extracellular space (Roberts-Wolfe & Kalivas, 2015). In the cNAc two-way ANOVA revealed a significant AMPH access effect and an effect of genotype, whereas in the sNAc an effect of AMPH access, but no genotype effect was found. In both cNAc and sNAc (Fig 5a and b, respectively), two-way ANOVA revealed a significant AMPH access x genotype interaction. Examining the individual treatment effects, we found that SERT deletion in both cNAc and sNAc led to a significant increase of GLT-1 expression in response to the LgA, but not to the ShA procedure, whereas no significant effect was observed in SERT^{+/+} rats.

Effect of ShA and LgA amphetamine self-administration on NMDA receptor subunits and the respective scaffolding proteins in the post-synaptic terminal of SERT^{+/+} and SERT^{-/-} rats.

The next step was to investigate the protein expression of the main subunits of the NMDA receptors. In the cNAc, two-way ANOVA of GluN1 levels, the obligatory subunit of NMDA receptors revealed an effect of genotype and a significant AMPH access x genotype interaction (Fig 6a). Post-hoc testing revealed that the expression of this subunit of the NMDA receptor is significantly increased following the LgA, but not the ShA procedure in SERT^{-/-} rats. Neither ShA, nor LgA, affected GluN1 expression in the cNAc of in SERT^{+/+} rats. A different situation was, instead, observed in the sNAc. Two-way ANOVA revealed a main effect of AMPH access, genotype and a significant AMPH access x genotype interaction (Fig 6b). Upon sub-testing we found an increased expression of GluN1 in SERT^{-/-} naïve-rats, which was not further enhanced by the ShA and the LgA procedure. Conversely, both the ShA and LgA conditions caused an enhancement in GluN1 protein levels in SERT^{+/+} rats.

We then investigated the expression of the accessory subunits of the NMDA receptor, i.e. GluN2A and GluN2B. In the cNAc, two-way ANOVA of GluN2A levels revealed a main effect of AMPH access, and an AMPH access x genotype interaction effect (Fig 6c); in the same subregion, for the GluN2B subunit, two-way ANOVA revealed a main effect of genotype and an AMPH access x genotype interaction (Fig 6e). GluN2A subunit expression was reduced in SERT^{-/-} naïve-rats; further, the post-hoc intergroup comparisons revealed that the removal of SERT caused a different response to both the ShA and LgA conditions. In fact, GluN2A protein levels were reduced following both ShA and LgA AMPH self-administration in SERT^{+/+} rats, whereas in

SERT^{-/-} rats no changes were found for ShA while GluN2A protein levels were up-regulated following LgA. GluN2B levels were not altered in AMPH-exposed SERT^{+/+} rats. In SERT^{-/-} rats similarly to GluN2A, we found that the LgA, but not the ShA procedure significantly up-regulated the expression of this subunit.

In the sNAc, two-way ANOVA revealed only a significant genotype effect (Fig 6d) for GluN2A, and a significant AMPH access effect (Fig 6f) for GluN2B.

Following the analysis of the NMDA receptor subunits, we investigated the expression of the main scaffolding protein of NMDA receptors, SAP102 (Won et al., 2017). In the cNAc, two-way ANOVA revealed a main effect of AMPH access and an AMPH access x genotype interaction (Fig 7a). Post-hoc testing of the main treatment effects showed that both ShA and LgA AMPH self-administration resulted in reduced SAP102 expression in SERT^{+/+} rats, whereas no changes were observed under both experimental conditions in SERT^{-/-} rats. In the sNAc, no significant changes in SAP102 levels were observed (Fig 7b).

Effect of ShA and LgA amphetamine self-administration on AMPA receptor subunits and their respective scaffolding proteins in the post-synaptic terminal of SERT^{+/+} and SERT^{-/-} rats.

We then moved to the analysis of the expression of the main subunits of the AMPA receptor, i.e. GluA1 and GluA2. In the cNAc, for both GluA1 and GluA2 subunits (Fig 8a and b, respectively), two-way ANOVA revealed a significant AMPH access x genotype interaction. For GluA2 two-way ANOVA revealed also a main effect of genotype. SERT knock out reduced GluA1, but not GluA2, levels. The LgA condition reduced GluA1 levels in SERT^{+/+} while increasing it in SERT^{-/-}. GluA2 levels were reduced following both ShA and LgA AMPH self-administration in SERT^{+/+}, while

deletion of SERT increased GluA2 levels following both ShA and LgA AMPH self-administration. In the sNAc, two-way ANOVA of both GluA1 and GluA2 AMPA subunits revealed a main effect of AMPH access, genotype and an AMPH access x genotype interaction effect (GluA1: Fig 8b; GluA2: Fig 8d). Both ShA and LgA conditions resulted in an elevation in GluA1 and GluA2 protein levels in SERT^{+/+} rats whereas, in SERT^{-/-} rats, no changes were observed with the exception of the increased expression of GluA1 following LgA.

We then examined the expression of the scaffolding proteins specific for AMPA receptor subunits GluA1 and GluA2, i.e. SAP97 and GRIP, respectively (Won et al., 2017). In the cNAc, for both SAP97 and GRIP (Fig 9a and c, respectively), two-way ANOVA revealed a main effect of AMPH access, and an AMPH access x genotype interaction. In SERT^{+/+} rats, we found a significant reduction of SAP97 under LgA AMPH self-administration conditions only, whereas GRIP levels were reduced under both ShA and LgA conditions. Conversely, under both experimental conditions, no significant changes were shown in SERT^{-/-} rats. In the sNAc, two-way ANOVA of both SAP97 and GRIP levels revealed a main effect of AMPH access and an AMPH access x genotype interaction (SAP97: Fig 9b; GRIP: Fig 9d). Post-hoc testing of the main treatment effects in SERT^{+/+} rats revealed reduced expression of SAP97 following LgA, but not ShA, procedures whereas GRIP was similarly reduced by both experimental conditions. Conversely, in SERT^{-/-} rats, we found an increase in the expression of SAP97 following the LgA, but not the ShA condition, whereas GRIP expression was decreased in SERT^{-/-} naïve-rats without further changes following both ShA and LgA AMPH self-administration.

Discussion

We found that SERT^{-/-} rats displayed increased AMPH intake under LgA, but not ShA conditions. Furthermore, the psychomotor response to AMPH was significantly higher in SERT^{-/-} rats. Finally, we found that, after 24 hours of withdrawal from AMPH self-administration, the glutamatergic synapse was affected differently in SERT^{-/-} versus SERT^{+/+} rats, in a way that heavily relies on the subregion of the NAc (shell or core) and the type of AMPH access (ShA or LgA) (Fig 10).

Amphetamine's psychomotor and reinforcing effects

Because of the pronounced genotype differences in the psychomotor effects of AMPH, one could argue that locomotor activity had an influence on the observed genotype differences after LgA to AMPH self-administration. However, the lack of genotype differences in AMPH intake under ShA condition and in the number of incorrect responses suggests that the increased AMPH intake under LgA condition in SERT^{-/-} versus SERT^{+/+} rats did not result from increased AMPH-induced locomotor activity. Interestingly, while AMPH self-administration was only increased in SERT^{-/-} rats under LgA but not ShA conditions, cocaine self-administration was increased in these animals under both conditions (Verheij et al., 2018). While we did not conduct a direct comparison study, this implies that the serotonin influence on AMPH and cocaine self-administration is different. A potential explanation is that AMPH has a lower affinity for SERT than cocaine (Howell & Kimmel, 2008), and that serotonin, next to dopamine, contributes to psychostimulant's reinforcing effects too. Given the lack of a genotype difference in the cocaine- (Verheij et al., 2014) and amphetamine-induced (unpublished data) dopamine release in the NAc, the observed differences in the psychomotor

response to amphetamine and amphetamine self-administration shown by SERT^{+/+} vs. SERT^{-/-} rats are not likely related to the dopamine system.

Amphetamine effects in Nucleus Accumbens shell (sNAc)

Elevated serotonin levels can alter neurodevelopmental processes (Homberg et al., 2009; Witteveen et al., 2013, Garcia et al., 2019) and induce changes in neurotransmission (Guidotti et al., 2012; Calabrese et al., 2013; Brivio et al., 2019) through e.g. altered stimulation of serotonin receptors (e.g. Hall et al., 2014). Both routes can lead to alterations in the glutamate signaling in the NAc. Twenty-four hours into withdrawal from AMPH self-administration, we found that vGluT1 expression was reduced at baseline in the sNAc of SERT^{-/-} rats, compared to SERT^{+/+} rats, suggesting reduced vesicular release of glutamate in SERT^{-/-} rats with consequently lower extracellular levels in the synapse. Of note, SERT^{+/+} rats showed increased vGluT1 expression after both ShA and LgA, whereas SERT^{-/-} rats exhibited an increase of the vesicular transporter following ShA and no change following LgA. Since the extracellular levels of glutamate are determined by a balance between its release and reuptake, the evidence that the accumbal expression of the plasmalemmal glutamate transporter GLT-1 is not altered by AMPH in SERT^{+/+} rats might be indicative of an increased AMPH-induced overflow of glutamate in the sNAc of these animals. Instead, GLT-1 expression is increased in SERT^{-/-} LgA rats, possibly reducing the extracellular levels of glutamate, whereas it is not changed following ShA. This suggests that SERT deletion may be associated with an increased glutamate overflow as a consequence of a ShA-induced increase of vGluT1. On the other hand, SERT deletion may reduce glutamate overflow as a consequence of a LgA-induced increase of GLT-1 expression. Since the sNAc is critical for the reinforcing properties of psychostimulants (Guillem et

al., 2014), the possibility exists that altered extracellular glutamate levels in this brain subregion of SERT^{-/-} rats may affect drug seeking.

The analysis of the expression of glutamate receptors in the sNAc revealed differences between NMDA and AMPA receptors. At baseline, a significant change was represented by the increased expression of GluN1 in SERT^{-/-} rats, perhaps resulting from the reduced glutamate release caused by the decreased expression of vGluT1. Following both ShA and LgA AMPH self-administration, we found increased expression of GluN1 in SERT^{+/+} rats, suggesting that the proposed higher release of glutamate in SERT^{+/+} rats is not compensated by a significant reduction in the main NMDA subunit. Conversely, in SERT^{-/-} rats, neither ShA nor LgA are able to further enhance the expression of GluN1, suggesting that SERT deletion-induced changes in GluN1 expression may be maximal. Notably, the observed increase in GluN1 expression were not accompanied by an increase in the levels of the accessory subunits GluN2A and GluN2B and of its anchoring protein SAP102, suggesting receptor instability following both ShA and LgA.

Regarding AMPA receptors, we found no significant changes at baseline between the two genotypes. GluA1 and GluA2 expression was up-regulated following either ShA or LgA in SERT^{+/+} rats. However, when analyzing the main scaffolding proteins for these two AMPA subunits, we found reduced expression of both SAP97 and GRIP in SERT^{+/+} rats. These findings suggest that GluA1 and GluA2 receptors lose their stability following both drug regimens in SERT^{+/+} rats. In SERT^{-/-} rats, both GluA1 and SAP97 expression was significantly increased following LgA drug exposure, suggesting that LgA to AMPH increased the stability of at least the GluA1 receptor in these animals.

In SERT^{+/+} rats, the proposed AMPH-induced increased release of glutamate, not compensated by changes in the expression of GLT-1, leads to an increased expression of the main glutamate receptors. Instead, the localization and, presumably, the functionality of these receptors is attenuated by the uncoupling of their respective auxiliary proteins independently from the duration of the AMPH exposure. Conversely, in SERT^{-/-} rats, the proposed reduced glutamate release is accompanied by enhanced reuptake only following LgA drug exposure, a combination that leads to a functional compensation only for GluA1 but not for GluA2 AMPA subunit nor for the different NMDA receptor subunits. Of note, the increased expression of GluA1, but not GluA2, AMPA subunit following LgA may lead to the formation of GluA2-lacking and Ca²⁺-permeable AMPARs, a mechanism [reported](#) to drive addiction (Wolf, 2016).

Amphetamine effects in Nucleus Accumbens core (cNAc)

A different situation was observed in the cNAc. In fact, neither expression of protein regulating release, nor expression of protein regulating reuptake of glutamate were significantly changed at baseline in SERT^{-/-} rats compared to their wild-type counterpart. In addition, no significant changes in these proteins were observed in the cNAc of SERT^{+/+} rats exposed to either regimen of AMPH. Conversely, both ShA and LgA may have promoted a significant higher release of glutamate that was accompanied by a higher uptake in the LgA-, but not ShA, exposed in SERT^{-/-} rats. The up-regulation of GLT-1 may represent an adaptive mechanism to buffer the increased release of glutamate, which is absent in ShA-exposed rats. This may indicate that changes in GLT-1 expression reflect compensation in the core, and synergism in the shell (see above). Interestingly, in the cNAc of SERT^{+/+} rats, we noted that both ShA and LgA to AMPH reduced the expression of GluN2 and GluA2 receptor subunits, an effect observed also

for their respective scaffolding proteins, i.e. SAP102, SAP97 and GRIP. Conversely, in SERT^{-/-} rats, LgA, but not ShA, significantly up-regulated the three GluN and the two GluA subunits (with the exception of GluA2 in ShA-exposed rats), an effect that was not accompanied, though, by a parallel enhancement of the expression of their respective scaffolding proteins, pointing towards an AMPH-induced reduction in the stability of glutamatergic synapse of the cNAc of SERT^{-/-} rats.

Conclusion

Our data show that SERT removal and AMPH self-administration converge on a common set of glutamate abnormalities that may contribute to shape both the behavioral and molecular effects herein observed. [Changes in glutamate signaling fit the glutamate hypothesis for drug addiction \(Kalivas, 2009\)](#). The data show that the combination of SERT deletion and AMPH exposure leads to effects that are unique and distinguishable from those of the individual experimental conditions. Accordingly, we can make several considerations: 1) the glutamate system is differently regulated by AMPH in SERT^{-/-} rats when compared to SERT^{+/+} rats, 2) the molecular determinants of the glutamate synapse are differently regulated in cNAc and sNAc confirming that these two subregions have their own function in regulating drug dependence and, 3) the LgA paradigm shapes the glutamatergic synapse differently from the ShA condition. [Notably, because the nucleus accumbens is tightly connected to various other brain regions, including the prefrontal cortex, thalamus, amygdala, and hippocampus also playing important roles in drug addiction \(Noori et al. 2012\)](#), it is very likely that [beyond the nucleus accumbens molecular changes in connected areas are also implicated in the observed individual differences in responsivity to amphetamine](#). [Because we only tested male rats, further research is required to determine whether](#)

these findings extend to female rats. Taken together, the perturbation of serotonin activity may, via dysregulation of glutamate homeostasis in these NAc regions, contribute to drive the transition from goal-directed drug intake toward addictive states. Hence, we propose that manipulation of serotonergic and/or glutamatergic systems may affect not only cocaine, but also AMPH, dependence.

Figure legends

Figure 1. Schematic representation of the experimental paradigm performed in SERT^{-/-} and SERT^{+/+} rats. (A) The psychomotor effects of AMPH were assessed immediately after AMPH injection and evaluated for 120 min. (B) AMPH self-administration training was performed during 10 days, after which the animals were subdivided into Short (ShA) or Long (LgA) access groups. During the next 18 days the rats were allowed to self-administer AMPH under either of these conditions. (C) 24 hrs after the final ShA or LgA session rats were decapitated, and punches from the nucleus accumbens core (cNAc) and shell (sNAc) sub regions (localized using the Paxinos and Watson 2007 edition of the rat brain atlas) were collected for gene expression analyses of the glutamatergic synapse.

Figure 2. Psychomotor effects of AMPH in SERT^{+/+} and SERT^{-/-} animals. The AMPH-induced and dose-dependent increase in locomotor activity was stronger in SERT^{-/-} vs SERT^{+/+} rats. Data represent mean ± SEM distance moved. *p<0.05 versus respective vehicle; #p<0.05 in AMPH SERT^{-/-} rats versus AMPH SERT^{+/+} rats; §p<0.05 versus respective baseline (time 0).

Figure 3. AMPH self-administration in SERT^{+/+} and SERT^{-/-} animals. The escalation of AMPH intake over the self-administration sessions was larger in rats under LgA than under ShA conditions (A vs B). The daily AMPH intake was larger in SERT^{-/-} versus SERT^{+/+} rats under LgA (B), but not ShA (A) conditions. Data are represented as mean ± SEM. n.s.: not significant. * p≤0.05 (one-sided t-test) versus SERT^{+/+}.

Figure 4. Interaction between SERT deletion and AMPH self-administration on the vesicular glutamate transporter 1 (vGluT1) in the cNAc and sNAc. Protein levels of vGluT1 in (A) cNAc and (B) sNAc are expressed as percentages of SERT^{+/+}-naïve rats. Below the graphs, representative immunoblots are shown for vGluT1 (60 kDa) and β -actin (43 kDa) proteins in the homogenate of cNAc and sNAc. Histograms represent the mean \pm SEM of the following number of rats: naïve (SERT^{+/+} n=6; SERT^{-/-} n=6) ShA (SERT^{+/+} n=14; SERT^{-/-} n=13) and LgA (SERT^{+/+} n=12; SERT^{-/-} n=12). *p<0.05, ***p<0.001 versus SERT^{+/+}-naïve; #p<0.05, ###p<0.001 versus SERT^{-/-}-naïve (Tukey's multiple comparisons test).

N=naïve, S=Amphetamine Short-Access; L= Amphetamine Long-Access

Figure 5. Interaction between SERT deletion and AMPH self-administration on the glial glutamate transporter 1 (GLT-1) in the cNAc and sNAc. Protein levels of GLT-1 in (A) cNAc and (B) sNAc are expressed as percentages of SERT^{+/+}-naïve rats. Below the graphs, representative immunoblots are shown for GLT-1 (62 kDa) and β -actin (43 kDa) proteins in the homogenate of cNAc and sNAc. Histograms represent the mean \pm SEM of the following number of rats: naïve (SERT^{+/+} n=6; SERT^{-/-} n=6) ShA (SERT^{+/+} n=14; SERT^{-/-} n=13) and LgA (SERT^{+/+} n=12; SERT^{-/-} n=12). #p<0.05 versus SERT^{-/-}-naïve; \$\$p<0.01 versus SERT^{-/-}-ShA (Tukey's multiple comparisons test).

N=naïve, S=Amphetamine Short-Access; L= Amphetamine Long-Access

Figure 6. Interaction between SERT deletion and AMPH self-administration on the NMDA receptor subunits in the cNAc and sNAc. Data show GluN1 levels in (A) cNAc and (B) sNAc, GluN2A levels in (C) cNAc and (D) sNAc, and GluN2B levels in (E) cNAc and (F) sNAc. In the lower panel (G) representative immunoblots are shown for GluN2A (180 kDa), GluN2B (180 kDa), GluN1 (120 kDa) and β -actin (43 kDa)

proteins in the cNAc and sNAc homogenates of SERT^{+/+} and SERT^{-/-} rats exposed to AMPH. Results are expressed as percentages of SERT^{+/+}-naïve rats. Histograms represent the mean ± SEM of the following number of rats: naïve (SERT^{+/+} n=6; SERT^{-/-} n=6) ShA (SERT^{+/+} n=14; SERT^{-/-} n=13) and LgA (SERT^{+/+} n=12; SERT^{-/-} n=12). *p<0.05, **p<0.01, ***p<0.001 versus SERT^{+/+}-naïve; #p<0.05, ##p<0.01 versus SERT^{-/-}-naïve; \$p<0.05, \$\$p<0.01 versus SERT^{-/-}-ShA (Tukey's multiple comparisons test). N=naïve, S=Amphetamine Short-Access; L= Amphetamine Long-Access

Figure 7. Interaction between SERT deletion and AMPH self-administration on the scaffolding protein SAP102 in the cNAc and sNAc. Data show protein levels of SAP102 in the (A) cNAc and (B) sNAc expressed as percentage of SERT^{+/+}-naïve rats. Below the graphs, representative immunoblots are shown for SAP102 (102 kDa) and β-actin (43 kDa) proteins in the homogenate of cNAc and sNAc. Histograms represent the mean ± SEM of the following number of rats: naïve (SERT^{+/+} n=6; SERT^{-/-} n=6) ShA (SERT^{+/+} n=14; SERT^{-/-} n=13) and LgA (SERT^{+/+} n=12; SERT^{-/-} n=12). **p<0.01, ***p<0.001 versus SERT^{+/+}-naïve (Tukey's multiple comparisons test). N=naïve, S=Amphetamine Short-Access; L= Amphetamine Long-Access

Figure 8. Interaction between SERT deletion and AMPH self-administration on the AMPA receptor subunits in the cNAc and sNAc. Data show GluA1 levels in the (A) cNAc and (B) sNAc, and GluA2 levels in the (C) cNAc and (D) sNAc. In the lower panel (E) representative immunoblots are shown for GluA1 (108 kDa), GluA2 (108 kDa) and β-actin (43 kDa) proteins in the cNAc and sNAc homogenates of SERT^{+/+} and SERT^{-/-} rats exposed to AMPH. Results are expressed as percentages of SERT^{+/+}-naïve rats. Histograms represent the mean ± SEM of the following number of rats: naïve (SERT^{+/+} n=6; SERT^{-/-} n=6) ShA (SERT^{+/+} n=14; SERT^{-/-} n=13) and LgA (SERT^{+/+}

n=12; SERT^{-/-} n=12). *p<0.05, **p<0.01 versus SERT^{+/+}-naïve; #p<0.05, ##p<0.01, ###p<0.001 versus SERT^{-/-}-naïve; \$\$p<0.01 versus SERT^{-/-}-ShA (Tukey's multiple comparisons test).

N=naïve, S=Amphetamine Short-Access; L= Amphetamine Long-Access

Figure 9. Interaction between SERT deletion and AMPH self-administration on the scaffolding proteins SAP97 and GRIP in the cNAC and sNAC. Data show SAP97 levels in the (A) cNAC and (B) sNAC, and GRIP levels in the (C) cNAC and (D) sNAC. In the lower panel (E) representative immunoblots are shown for GRIP (120 kDa), SAP97 (97 kDa) and β -actin (43 kDa) proteins in the cNAC and sNAC homogenates of SERT^{+/+} and SERT^{-/-} rats exposed to AMPH. Results are expressed as percentage of SERT^{+/+}-naïve rats. Histograms represent the mean \pm SEM of the following number of rats: naïve (SERT^{+/+} n=6; SERT^{-/-} n=6) ShA (SERT^{+/+} n=14; SERT^{-/-} n=13) and LgA (SERT^{+/+} n=12; SERT^{-/-} n=12). *p<0.05, **p<0.01, ***p<0.001 versus SERT^{+/+}-naïve; ##p<0.01 versus SERT^{-/-}-naïve; \$\$\$p<0.001 versus SERT^{-/-}-ShA (Tukey's multiple comparisons test).

N=naïve, S=Amphetamine Short-Access; L= Amphetamine Long-Access

Figure 10. Summary of the protein expression findings in the nucleus accumbens shell and core of naïve SERT^{+/+} and SERT^{-/-} rats versus SERT^{+/+} and SERT^{-/-} rats exposed to ShA and LgA cocaine self-administration.

cNAC=nucleus accumbens core, sNAC=nucleus accumbens shell, ShA=Amphetamine Short-Access; LgA=amphetamine Long-Access, vGlut1=vesicular glutamate transporter 1, GLT-1=glial glutamate transporter 1, NMDA=NMDA receptor unit (e.g. GluN1), Scaffolding=scaffolding protein (e.g. SAP102), AMPA=AMPA receptor subunit

Supplementary figure 1. Number of incorrect lever presses (A, B) and timeout responses (C, D) during AMPH self-administration in SERT^{-/-} and SERT^{+/+} rats under ShA and LgA conditions. No genotype differences were observed. Data are represented as mean ± SEM. n.s.: not significant.

Supplementary figure 2. Uncropped immunoblot related to the expression levels of GluN2A (180 kDa), GluN2B (180 kDa), GluN1 (120 kDa), GRIP (135 kDa), SAP97 (97 kDa), SAP102 (102 kDa), GluA1 (108 kDa), GluA2 (108 kDa), vGlut1 (67 kDa), GLT-1 (62 kDa) and β-Actin (43 kDa) measured in the homogenate of the cNAc of SERT^{+/+} and SERT^{-/-} rats exposed to AMPH ShA and LgA protocol presented in Fig 4, Fig 5, Fig 6, Fig 7, Fig 8, Fig 9. Samples were loaded in three different gels as shown in panel A, B, C.

1= SERT^{+/+}-naïve; 2= SERT^{+/+}-ShA; 3= SERT^{+/+}-LgA; 4= SERT^{-/-}-naïve; 5= SERT^{-/-}-ShA; 6= SERT^{-/-}-LgA.

Supplementary figure 3. Uncropped immunoblot related to the expression levels of GluN2A (180 kDa), GluN2B (180 kDa), GluN1 (120 kDa), GRIP (135 kDa), SAP97 (97 kDa), SAP102 (102 kDa), GluA1 (108 kDa), GluA2 (108 kDa), vGlut1 (67 kDa), GLT-1 (62 kDa) and β-Actin (43 kDa) measured in the homogenate of the sNAc of SERT^{+/+} and SERT^{-/-} rats exposed to AMPH ShA and LgA protocol presented in Fig 4, Fig 5, Fig 6, Fig 7, Fig 8, Fig 9. Samples were loaded in three different gels as shown in panel A, B, C.

[Supplementary table 1. F and p values of both behavioral and molecular data presented in the results section.](#)

ha formattato: Tipo di carattere: Non Grassetto

Author contributions

L.C. conducted the Western blot experiments, analysed the data, and contributed to the writing of the manuscript. M.V. conducted the self-administration experiment, made figures for the self-administration data, and contributed to the writing of the manuscript. K.R. initiated the writing of the manuscript and generated figures. G.T. contributed to sample preparation and conducted the Western blot experiments. F.M. conducted the Western blot analyses and made the graphic abstract and overview figure. P.P. supervised the psychomotor experiment and the project. A.N. supervised the psychomotor experiment and contributed to the writing of the manuscript. J.G. conducted the psychomotor experiment. F.F. conceived and planned the experiments, supervised the molecular analyses, contributed to the interpretation of the results, and contributed to the writing of the manuscript. J.R. conceived and planned the experiments, contributed to the writing of the manuscript, and supervised the project. All authors discussed the results and contributed to the final manuscript.

References

- Ahmed, S. H., & Koob, G. F. (1998). Transition from moderate to excessive drug intake: change in hedonic set point. *Science*, 282(5387), 298-300. doi:10.1126/science.282.5387.298
- Alexander, S.P.H., Roberts, R.E., Broughton B.R.S., Sobey, C.G. George C.H., Stanford, S.C., Cirino, G., Docherty, J.R., Giembycz, M.A., Hoyer, D., Insel, P.A., Izzo, A.A., Ji, Y., MacEwan, D.J., Mangum, J., Wonnacott, S., Ahluwalia, A. (2018) Goals and Practicalities of Immunoblotting and Immunohistochemistry: A Guide for Submission to the British Journal of Pharmacology. *Br J Pharmacol*, 175(3), 407-411. doi: 10.1111/bph.14112.
- Anthony, J.C., Warner, L.A., Warner, Kessler, R.C. (1994). Comparative Epidemiology of Dependence on Tobacco, Alcohol, Controlled Substances, and Inhalants: Basic Findings From the National Comorbidity Survey. *Experimental and Clinical Psychopharmacology* 2(3),244-268. doi:10.1037/1064-1297.2.3.244
- Brivio, P., Homberg, J.R., Riva, M.A., Calabrese, F. (2019) Alterations of Glutamatergic Markers in the Prefrontal Cortex of Serotonin Transporter Knockout Rats: A Developmental Timeline. *Cell Mol Neurobiol*. 39(5), 715-720. doi: 10.1007/s10571-019-00673-9.
- Browne, C.J., Fletcher, P.J. (2016) Decreased Incentive Motivation Following Knockout or Acute Blockade of the Serotonin Transporter: Role of the 5-HT_{2C} Receptor. *Neuropsychopharmacology*. 2016 41(10), 2566-76. doi: 10.1038/npp.2016.63.
- Caffino, L., Mottarlini, F., Van Reijmersdal, B., Telese, F., Verheij, M. M. M., Fumagalli, F., & Homberg, J. R. (2020). The role of the serotonin transporter in prefrontal cortex glutamatergic signaling following short- and long-access cocaine self-administration. *Addict Biol*, e12896. doi:10.1111/adb.12896
- Caffino, L., Verheij, M. M. M., Que, L., Guo, C., Homberg, J. R., & Fumagalli, F. (2019). Increased cocaine self-administration in rats lacking the serotonin transporter: a role for glutamatergic signaling in the habenula. *Addict Biol*, 24(6), 1167-1178. doi:10.1111/adb.12673.
- Calabrese, F., Guidotti, G., Middelman, A., Racagni, G., Homberg, J., Riva, M.A. (2013) Lack of serotonin transporter alters BDNF expression in the rat brain during early postnatal development. *Mol Neurobiol*. 2013 48(1), 244-56. doi: 10.1007/s12035-013-8449-z.
- Cao, J., Hudziak, J.J., Li, D. (2013) Multi-cultural Association of the Serotonin Transporter Gene (SLC6A4) With Substance Use Disorder. *Neuropsychopharmacology*, 38(9), 1737-47 doi: 10.1038/npp.2013.73.
- Caspi, A., Hariri, A. R., Holmes, A., Uher, R., & Moffitt, T. E. (2010). Genetic sensitivity to the environment: the case of the serotonin transporter gene and its implications for studying complex diseases and traits. *Am J Psychiatry*, 167(5), 509-527. doi:10.1176/appi.ajp.2010.09101452

- Curtis, M. J., Alexander, S., Cirino, G., Docherty, J. R., George, C. H., Giembycz, M. A., . . . Ahluwalia, A. (2018). Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. *Br J Pharmacol*, *175*(7), 987-993. doi:10.1111/bph.14153
- Degenhardt, L., Baxter, A. J., Lee, Y. Y., Hall, W., Sara, G. E., Johns, N., . . . Vos, T. (2014). The global epidemiology and burden of psychostimulant dependence: findings from the Global Burden of Disease Study 2010. *Drug Alcohol Depend*, *137*, 36-47. doi:10.1016/j.drugalcdep.2013.12.025
- El Mestikawy, S., Wallen-Mackenzie, A., Fortin, G. M., Descarries, L., & Trudeau, L. E. (2011). From glutamate co-release to vesicular synergy: vesicular glutamate transporters. *Nat Rev Neurosci*, *12*(4), 204-216. doi:10.1038/nrn2969
- Enoch, M. A., Gorodetsky, E., Hodgkinson, C., Roy, A., & Goldman, D. (2011). Functional genetic variants that increase synaptic serotonin and 5-HT₃ receptor sensitivity predict alcohol and drug dependence. *Mol Psychiatry*, *16*(11), 1139-1146. doi:10.1038/mp.2010.94
- Everitt, B. J., & Robbins, T. W. (2016). Drug Addiction: Updating Actions to Habits to Compulsions Ten Years On. *Annu Rev Psychol*, *67*, 23-50. doi:10.1146/annurev-psych-122414-033457
- Faraone, S. V. (2018). The pharmacology of amphetamine and methylphenidate: Relevance to the neurobiology of attention-deficit/hyperactivity disorder and other psychiatric comorbidities. *Neurosci Biobehav Rev*, *87*, 255-270. doi:10.1016/j.neubiorev.2018.02.001
- Perturbed Developmental Serotonin Signaling Affects Prefrontal Catecholaminergic Innervation and Cortical Integrity.
- Garcia, L.P., Witteveen, J.S., Middelman, A., van Hulten, J.A., Martens, G.J.M., Homberg, J.R., Kolk, S.M. (2019) Perturbed Developmental Serotonin Signaling Affects Prefrontal Catecholaminergic Innervation and Cortical Integrity. *Mol Neurobiol*. *56*(2), 1405-1420. doi: 10.1007/s12035-018-1105-x.
- Gerra, G., Zaimovic, A., Garofano, L., Ciusa, F., Moi, G., Avanzini, P., . . . Donnini, C. (2007). Perceived parenting behavior in the childhood of cocaine users: relationship with genotype and personality traits. *Am J Med Genet B Neuropsychiatr Genet*, *144B*(1), 52-57. doi:10.1002/ajmg.b.30388
- Giannotti, G., Caffino, L., Mottarlini, F., Racagni, G., & Fumagalli, F. (2016). Region-specific effects of developmental exposure to cocaine on fibroblast growth factor-2 expression in the rat brain. *Psychopharmacology (Berl)*, *233*(14), 2699-2704. doi:10.1007/s00213-016-4315-9
- Gipson, C. D., & Bardo, M. T. (2009). Extended access to amphetamine self-administration increases impulsive choice in a delay discounting task in rats. *Psychopharmacology (Berl)*, *207*(3), 391-400. doi:10.1007/s00213-009-1667-4

- Guillem, K., Ahmed, S. H., & Peoples, L. L. (2014). Escalation of cocaine intake and incubation of cocaine seeking are correlated with dissociable neuronal processes in different accumbens subregions. *Biol Psychiatry*, *76*(1), 31-39. doi:10.1016/j.biopsych.2013.08.032
- Guidotti, G., Calabrese, F., Auletta, F., Olivier, J., Racagni, G., Homberg, J., Riva, M.A. (2012) Developmental influence of the serotonin transporter on the expression of npas4 and GABAergic markers: modulation by antidepressant treatment. *Neuropsychopharmacology*. *37*(3), 746-58. doi: 10.1038/npp.2011.252.
- Hall, F.S., Sora, I., Hen, R., Uhl, G.R. (2014) Serotonin/dopamine interactions in a hyperactive mouse: reduced serotonin receptor 1B activity reverses effects of dopamine transporter knockout. *PLoS One*. *9*(12), e115009. doi: 10.1371/journal.pone.0115009.
- Holmes, A. J., Bogdan, R., & Pizzagalli, D. A. (2010). Serotonin transporter genotype and action monitoring dysfunction: a possible substrate underlying increased vulnerability to depression. *Neuropsychopharmacology*, *35*(5), 1186-1197. doi:10.1038/npp.2009.223
- Homberg, J. R., De Boer, S. F., Raaso, H. S., Olivier, J. D., Verheul, M., Ronken, E., . . . Cuppen, E. (2008). Adaptations in pre- and postsynaptic 5-HT1A receptor function and cocaine supersensitivity in serotonin transporter knockout rats. *Psychopharmacology (Berl)*, *200*(3), 367-380. doi:10.1007/s00213-008-1212-x
- Homberg, J.R., Schubert, D., Gaspar, P. (2010) New perspectives on the neurodevelopmental effects of SSRIs. *Trends Pharmacol Sci*. *31*(2), 60-5. doi: 10.1016/j.tips.2009.11.003.
- Homberg, J. R., Olivier, J. D., Smits, B. M., Mul, J. D., Mudde, J., Verheul, M., . . . Cuppen, E. (2007). Characterization of the serotonin transporter knockout rat: a selective change in the functioning of the serotonergic system. *Neuroscience*, *146*(4), 1662-1676. doi:10.1016/j.neuroscience.2007.03.030
- Homberg, J. R., Wöhr, M., & Alenina, N. (2017). Comeback of the Rat in Biomedical Research. *ACS Chem Neurosci*, *8*(5), 900-903. doi:10.1021/acscchemneuro.6b00415
- Howell, L. L., & Kimmel, H. L. (2008). Monoamine transporters and psychostimulant addiction. *Biochem Pharmacol*, *75*(1), 196-217. doi:10.1016/j.bcp.2007.08.003
- Igari, M., Shen, H.W., Hagino, Y., Fukushima, S., Kasahara, Y., Lesch, K.P., Murphy, D.L., Hall, F.S., Uhl, G.R., Ikeda, K., Yaegashi, N., Sora, I. (2015) Attenuated Methamphetamine-Induced Locomotor Sensitization in Serotonin Transporter Knockout Mice Is Restored by Serotonin 1B Receptor Antagonist Treatment. Comparative Study *Behav Pharmacol*, *26*(1-2), 167-79. doi: 10.1097/FBP.0000000000000120.
- Kalivas, P. W., & Volkow, N. D. (2005). The neural basis of addiction: a pathology of motivation and choice. *Am J Psychiatry*, *162*(8), 1403-1413. doi:10.1176/appi.ajp.162.8.1403

- Kalivas, P.W. (2009) The glutamate homeostasis hypothesis of addiction. *Nat Rev Neurosci*. 10(8):561-72. doi: 10.1038/nrn2515.
- Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *J Pharmacol Pharmacother*, 1(2), 94-99. doi:10.4103/0976-500X.72351
- Koob, G. F. (1992). Neural mechanisms of drug reinforcement. *Ann N Y Acad Sci*, 654, 171-191. doi:10.1111/j.1749-6632.1992.tb25966.x
- Lile J, Nader M. (2003) The abuse liability and therapeutic potential of drugs evaluated for cocaine addiction as predicted by animal models. *Curr Neuropsychopharmacol*. 1(1), 21-46(26) doi:10.2174/1570159033360566
- Mannelli, P., Patkar, A. A., Murray, H. W., Certa, K., Peindl, K., Mattila-Evenden, M., & Berrettini, W. H. (2005). Polymorphism in the serotonin transporter gene and response to treatment in African American cocaine and alcohol-abusing individuals. *Addict Biol*, 10(3), 261-268. doi:10.1080/13556210500235540
- Martin-Santos, R., Torrens, M., Poudevida, S., Langohr, K., Cuyas, E., Pacifici, R., de la Torre, R. (2010). 5-HTTLPR polymorphism, mood disorders and MDMA use in a 3-year follow-up study. *Addict Biol*, 15(1), 15-22. doi:10.1111/j.1369-1600.2009.00180.x
- Natarajan, R., & Yamamoto, B. K. (2011). The Basal Ganglia as a Substrate for the Multiple Actions of Amphetamines. *Basal Ganglia*, 1(2), 49-57. doi:10.1016/j.baga.2011.05.003
- Noori, H.R., Spanagel, R., Hansson, A.C. (2012) Neurocircuitry for Modeling Drug Effects. *Addict Biol*, 17(5):827-64. doi: 10.1111/j.1369-1600.2012.00485.x.
- Oakly, A. C., Brox, B. W., Schenk, S., & Ellenbroek, B. A. (2014). A genetic deletion of the serotonin transporter greatly enhances the reinforcing properties of MDMA in rats. *Mol Psychiatry*, 19(5), 534-535. doi:10.1038/mp.2013.75
- Oliva, C., Escobedo, P., Astorga, C., Molina, C., & Sierralta, J. (2012). Role of the MAGUK protein family in synapse formation and function. *Dev Neurobiol*, 72(1), 57-72. doi:10.1002/dneu.20949
- Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates, 6th edn. Academic Press/Elsevier, Amsterdam
- Pettie, M., Oakly, A., Harper, D. N., & Ellenbroek, B. A. (2019). A genetic deletion of the serotonin transporter differentially influences the behavioural effects of MDMA. *J Psychopharmacol*, 33(3), 355-363. doi:10.1177/0269881118822156

- Roberts-Wolfe, D. J., & Kalivas, P. W. (2015). Glutamate Transporter GLT-1 as a Therapeutic Target for Substance Use Disorders. *CNS Neurol Disord Drug Targets*, *14*(6), 745-756. doi:10.2174/1871527314666150529144655
- Rocha, B. A., Fumagalli, F., Gainetdinov, R. R., Jones, S. R., Ator, R., Giros, B., . . . Caron, M. G. (1998). Cocaine self-administration in dopamine-transporter knockout mice. *Nat Neurosci*, *1*(2), 132-137. doi:10.1038/381
- Rothman, R. B., & Glowa, J. R. (1995). A review of the effects of dopaminergic agents on humans, animals, and drug-seeking behavior, and its implications for medication development. Focus on GBR 12909. *Mol Neurobiol*, *11*(1-3), 1-19. doi:10.1007/BF02740680
- Schipper, P., Hiemstra, M., Bosch, K., Nieuwenhuis, D., Adinolfi, A., Glotzbach, S., . . . Homberg, J. R. (2019). The association between serotonin transporter availability and the neural correlates of fear bradycardia. *Proc Natl Acad Sci U S A*, *116*(51), 25941-25947. doi:10.1073/pnas.1904843116
- Shaffer, C., Guo, M. L., Fibuch, E. E., Mao, L. M., & Wang, J. Q. (2010). Regulation of group I metabotropic glutamate receptor expression in the rat striatum and prefrontal cortex in response to amphetamine in vivo. *Brain Res*, *1326*, 184-192. doi:10.1016/j.brainres.2010.02.062
- Smits, B. M., Mudde, J. B., van de Belt, J., Verheul, M., Olivier, J., Homberg, J., . . . Cuppen, E. (2006). Generation of gene knockouts and mutant models in the laboratory rat by ENU-driven target-selected mutagenesis. *Pharmacogenet Genomics*, *16*(3), 159-169. doi:10.1097/01.fpc.0000184960.82903.8f
- Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., . . . Dingledine, R. (2010). Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev*, *62*(3), 405-496. doi:10.1124/pr.109.002451
- UNODOC World Drug Report (2018) <https://www.unodc.org/wdr2018/>
- Verheij, M. M., Karel, P., Cools, A. R., & Homberg, J. R. (2014). Reduced cocaine-induced serotonin, but not dopamine and noradrenaline, release in rats with a genetic deletion of serotonin transporters. *Eur Neuropsychopharmacol*, *24*(11), 1850-1854. doi:10.1016/j.euroneuro.2014.09.004
- Verheij, M. M. M., Contet, C., Karel, P., Latour, J., van der Doelen, R. H. A., Geenen, B., . . . Homberg, J. R. (2018). Median and Dorsal Raphe Serotonergic Neurons Control Moderate Versus Compulsive Cocaine Intake. *Biol Psychiatry*, *83*(12), 1024-1035. doi:10.1016/j.biopsych.2017.10.031
- Wee, S., Specio, S. E., & Koob, G. F. (2007). Effects of dose and session duration on cocaine self-administration in rats. *J Pharmacol Exp Ther*, *320*(3), 1134-1143. doi:10.1124/jpet.106.113340

Witteveen, J.S., Middelman, A., van Hulst, J.A., Martens, G.J., Homberg, J.R., Kolk, S.M. (2013) Lack of serotonin reuptake during brain development alters rostral raphe-prefrontal network formation. *Front Cell Neurosci.* 7, 143. doi: 10.3389/fncel.2013.00143.

Wolf, M. E. (2016). Synaptic mechanisms underlying persistent cocaine craving. *Nat Rev Neurosci*, 17(6), 351-365. doi:10.1038/nrn.2016.39

Won, S., Levy, J. M., Nicoll, R. A., & Roche, K. W. (2017). MAGUKs: multifaceted synaptic organizers. *Curr Opin Neurobiol*, 43, 94-101. doi:10.1016/j.conb.2017.01.006

Woolverton, W. L., & Johnson, K. M. (1992). Neurobiology of cocaine abuse. *Trends Pharmacol Sci*, 13(5), 193-200. doi:10.1016/0165-6147(92)90063-c