Sensitivity to Amphetamine Depends on a lack of Serotonin Transporters

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Abstract

Background: Amphetamine abuse is a worldwide problem. Serotonin and dopamine are important neurotransmitters which are implicated in the acquisition, maintenance and relapse phase of amphetamine addiction. By blocking and reversing the action of dopamine reuptake transporters and reversing the action of serotonin reuptake transporters, amphetamine increases extracellular dopamine and serotonin levels. The present study aims to give insights in the role of the serotonin transporter and accumbal serotonin in amphetamine reward and addiction by combining microdialysis and behavioural testing in serotonin transporter knock out rats (SERT KO) and their wildtype (SERT WT) counterparts.

Method: SERT KO and SERT WT were subjected to an acute amphetamine challenge (2.5 mg/kg) while locomotor activity, ultrasonic vocalizations and extracellular serotonin and dopamine levels were measured in the nucleus accumbens shell. A separate cohort of SERT KO and SERT WT was subjected to amphetamine self-administration (0.03 mg/kg/infusion) followed by a progressive ratio test to measure motivation.

Results: Microdialysis revealed an increase of both serotonin and dopamine levels after amphetamine (2.5 mg/kg) administration, but no significant genotype differences were found. However, the increase in extracellular accumbal serotonin, but not dopamine levels of SERT KO animals was more prolonged. SERT KO rats emitted more 50 kHz ultrasonic vocalizations and had higher locomotor activity compared to SERT WT rats after amphetamine. Self-administration revealed that SERT KO rats self-administered more amphetamine (0.03 mg/kg/infusion) compared to SERT WT rats. SERT KO animals showed a quadratic increase in their intake, whereas SERT WT showed a linear increase in their intake. Furthermore, SERT KO scored higher on the progressive ratio test.

Discussion and conclusion: All our results linked together support the hypothesis that SERT KO rats are more sensitive to amphetamine, which may lead to an increased intake compared to SERT WT rats. Accumbal dopamine levels did not differ between the genotypes, which may imply an important role for accumbal serotonin in individual differences in drug reward and sensitivity. While being careful in generalizing to humans, our findings could have implications for individualized treatments for patients with amphetamine addiction.

Keywords: *accumbal serotonin, amphetamine reward/addiction/sensitivity, dopamine, long short access, microdialysis, nucleus accumbens, self-administration, short long access, SERT knockout (KO) rats, ultrasonic vocalizations (USV).*

Introduction

The psychostimulant amphetamine (AMPH) is used by more than 50 million people worldwide, of whose 30% develop a substance-use disorder (SUD). This disorder is frequently accompanied by alcohol, tobacco and other drug use or abuse (Darke and Hall, 1995, Baker and Dawe, 2005, Statista, 2016), which leads to a preference for animals in studying monodrug abuse.

Serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) are important neurotransmitters which are implicated in regulating SUD acquisition, maintenance, and recovery (Herman and Balogh, 2012). The actions of AMPH on these monoamine systems are diverse and complicated and complex interactions occur (Lott et al., 2006). Its actions include blockade and reversal of dopamine transporters (DAT) and reversal of the 5-HT transporter (SERT), as well as actions on vesicular storage of DA and 5-HT (Gainetdinov et al., 1999, Lott et al., 2006). The rewarding and addictive effects of AMPH depend on the ability to increase extracellular DA and 5-HT levels (Torres et al., 2003). Interestingly, brain DA levels are controlled by 5-HT (Gobert et al., 2000, Di Matteo et al., 2001, Di Matteo et al., 2002). Therefore, genetic variations in these monoaminergic systems, especially the 5-HT system, are among the inherited risk factors for SUD (Lott et al., 2006) and are likely contributing to SUD patient heterogeneity (Herman and Balogh, 2012). Indeed, decreased function of the 5-HT transporter, mediated in humans by the short allelic polymorphism of the 5-HT linked promoter region (5-HTTLPR), is associated with increased effects of the psychostimulants cocaine and ecstasy (Gerra et al., 2004, Lott et al., 2006, Johnson, 2010), however this genetic link with AMPH is unclear and needs to be investigated.

The serotonin transporter (SERT) knock out (KO) rat model can be highly informative to study both the biochemical as well as the behavioural consequences of disturbed 5-HT homeostasis on AMPH addiction, since SERT KO rats lack an important substrate for the 5-HT increasing action of AMPH (Homberg et al., 2007). Sensitivity to AMPH in rats can be measured by for example the frequency of 50 kHz ultrasonic vocalisations (USV) which is believed to reflect drug reward (Knutson et al., 2002, Wohr et al., 2015, Avvisati et al., 2016). Recently, the involvement of both the dopaminergic and serotonergic system in USV has been demonstrated (Wright et al., 2013). Another measure involves locomotor activity, which is drastically increased after AMPH administration (Randrup et al., 1963, Wohr et al., 2015). Self-administration (SA) procedures have high face validity since they provide the most direct correspondence with addictive behaviour that occurs in the natural environment (Panlilio and Goldberg, 2007). Differential drug access influences the intake in humans and rats. Rats with short access (ShA, daily 1h sessions) to the drug show stable and low drug intake, whereas long access (LgA, daily 6h sessions), results in gradual escalation of drug intake. Therefore, this paradigm is used to study the transition from drug use to drug addiction (Ahmed and Koob, 1998).

The present study aims to give insights in the role of SERT and extracellular accumbal 5-HT levels in AMPH reward and addiction by combining microdialysis and behavioural testing in SERT KO and their SERT WT counterparts. DA and 5-HT levels in the nucleus accumbens (NAc) shell will be measured, after acute administration of AMPH. The NAc shell is an important structure within the drug reward and reinforcement circuitry (Di Chiara, 2002). Furthermore, locomotor activity and 50 kHz USV will be measured. Finally, AMPH self-administration will be performed in a separate cohort to investigate characteristics of drug addiction. Based on the repeated finding that both humans and rats with reduced SERT functioning

are more sensitive to psychostimulants like cocaine and ecstasy (Gerra et al., 2004, Lott et al., 2006, Homberg et al., 2008, Johnson, 2010, Martin-Santos et al., 2010, Oakly et al., 2014, Verheij et al., 2014) we hypothesize that SERT KO rats are more sensitive to AMPH compared to SERT WT rats, reflected by higher emission rates of 50 kHz USV, increased locomotor activity and more self-administered AMPH possibly accompanied by genotype differences in AMPH induced changes in extracellular accumbal DA/5-HT levels.

In the future, genetic variants in the monoaminergic system may inform individualized choices of treatment, which ultimately may lead to reduced SUD-related problems for both patients and society, since AMPH addictions cause an enormous public health burden (Herman and Balogh, 2012).

Materials and Methods

Animals and housing

Subjects for experiment 1 (self-administration) were 20 naïve male Wistar SERT KO and 20 Wistar male SERT WT rats, weighing respectively 370 – 525 g and 420 – 560 g at the time of intravenous (i.v.) surgery. Subjects for experiment 2 (microdialysis, US and locomotor activity) were 12 naïve male Wistar SERT KO and 11 Wistar male SERT WT rats, weighing 240 – 390 g and 365 – 405 g respectively at the time of stereotactic surgery. The SERT rat model was generated by N-ethyl-N-nitrosurea (ENU)-driven target-selected mutagenesis (Smits et al., 2006, Homberg et al., 2007). Rats were born and raised at the Central Animal Laboratory of the University of Nijmegen and were housed in pairs or triplets in Macrolon type III cages (L 42 cm, W 26 cm, H 15 cm). Following surgery all animals were individually housed, in a temperature (21±1 °C) and humidity (55±5 %) controlled colony room, where music was broadcasted. Light and dark phases were on a 12:12 h cycle (lights off at 0800 AM), and all behavioural testing was conducted during the dark phase. Before the start of the experiment rats were allowed to acclimate to the reversed day/night cycle and the colony for two weeks. Subjects had *ad libitum* access to food (SSNIFF®, 10mm pellets: V1534-703) and acidified water (pH: 2.5 – 3.0), except during test sessions. Procedures were approved by the Animal Welfare Body of the Radboud University medical centre, the Netherlands, conforming to the guideline of the European Council for Animal Care.

Experiment 1 Amphetamine self-administration

Procedures

Intravenous Catheterization I.v. catheterization (Ahmed and Koob, 1998; Verheij et al., in press) was performed under Isoflurane anaesthesia (1 ml/min O_2, 0.5 ml/min N_2, 5% Isoflurane for induction and 2-3% for maintenance). Animals were subcutaneously (s.c.) treated with the antibiotic Kefzol® (Cefazolin 15 mg/ml/kg) and general analgesia Finadyne® (Flunixin 2.5 mg/ml/kg). Local analgesia (Xylocaine 10%, Lidocaine 100mg/ml) and disinfectant (Betadine, 100 mg/ml povidone-iodine) were applied cutaneously before incising. To prevent dehydration of the eyes ointment (Ophtosan®) was applied. Rats were implanted with chronic i.v. jugular catheters (IVSAp40; manufactured by Camcaths, Saint Thomas Place, Ely, UK). The catheter was inserted in the right jugular vein and passed subcutaneously over the right shoulder to exit on the back of the animal were the stainless steel cannula with a silicone mesh was subcutaneously secured. Subjects were given at least 14 days to

recover from surgery before they entered behavioural testing. During recovery, the condition of the animals was daily monitored (weight, behaviour, condition of the wounds, fur condition). Catheter patency was maintained by flushing (i.v.) daily with 0.2 ml of 0.9% sterile saline containing Heparin (50U/ml) and Kefzol ® (Cefazolin 15 mg/ml/kg).

Self-administration boxes Testing was conducted in 20 standard operant boxes (MED Associates Inc., VT, USA), with inside dimensions of 28 cm x 21cm x 21cm. End walls of the operant chamber were made of aluminium, the front and back walls were made of Plexiglas. A removable tray filled with sawdust was placed underneath the floor which comprised 19 stainless rods. Each box contained on the right side wall a recessed sugar pellet tray, two retractable response levers (5 cm wide and 6 cm above the floor) and a stimulus light located 3 cm above each lever. Behavioural data measured as the number of lever presses on both the drug-paired and the inactive lever and number of infusions were recorded via a computer interface system (MED Associates Inc., VT, USA). Delivery of AMPH occurred via an infusion pump (MED Associates Inc., VT, USA) that was attached to the swivel via silicon tubing. At the other end of the swivel, silicon tubing, encased in a stainless steel tether, connected the animal's catheter to the syringe via the swivel.

Intravenous Amphetamine Self-Administration & Progressive Ratio Following recovery animals entered a training paradigm lasting 13 days in which they had restricted access (1h, maximum number of rewards was set to 20) to an AMPH-paired lever on a fixed ratio one (FR1, every response gives an infusion) schedule. The inactive (no programmed consequences) and active (AMPH paired) levers were counterbalanced for position among subjects. Responding to the active lever resulted in both illumination of the cue light and an i.v. AMPH delivery of 0.03 mg/kg/infusion (Klebaur et al., 2001, Gipson and Bardo, 2009) of on average 0.1 ml over 3 seconds (2.4 – 3.5 seconds, based on the body weight of the animal). This was followed by a 20s time-out period, in which the cue light was on and in which responding to both the active and inactive lever had no programmed consequences. After the time out period, the cue light turned off and responding to the active lever again resulted in previously described consequences. Following the initial 1h training paradigm, rats were assigned to either the short access (ShA, 1h) or the long access (LgA, 6h) group, so that the average drug intake over the last two training days did not differ significantly between groups. Both the ShA and the LgA group were then subjected to 19 daily sessions identical to those in training, with the exception that LgA animals had extended access (6h) to the AMPH self-administration. Hereafter, rats were subjected to a progressive ratio (PR) schedule (every 1,1,2,4,8, etc. lever press gives an infusion) to test the maximum effort that the animals will expend in order to receive an AMPH infusion (Richardson and Roberts, 1996). For a schematic overview of the setup see table 1 (Verheij et al., in press).

Table 1 *Schematic overview of the experimental setup*

Day 1-14	Day 15 – 34	Day 35	Day 36	24h later
Training paradigm 1h	Daily ShA (1h) or LgA (6h) sessions	PR schedule	ShA (1h) and LgA (6h) session	Decapitation

Drugs
Amphetamine sulphate (Sigma, UK) was dissolved in 275 ml bags of 0.9% sterile saline and stored at room temperature for a maximum of five days. The dose used in both the training and the experimental sessions was 0.03 mg/kg per infusion (average of 0.1 ml per infusion) (Klebaur et al., 2001, Gipson and Bardo, 2009).

Data analysis
Number of rewards, incorrect responses, and time-out responses were analysed with a 2 (genotype: KO/WT) x 2 (access type: ShA/LgA) x 19 (days) RMANOVA. All analyses were performed with IBM SPSS Statistics 21 (Chicago, IL). For all analyses, significance was set at $p \leq 0.05$, and RM ANOVA and student t-tests were used as post hoc tests to determine differences between groups.

Histology
After completion of the experiment rats were decapitated and their brains were removed and stored at –80 ºC. Further analysis (e.g. protein analysis) on the brains will be executed in a later stadium.

Experiment 2 Extracellular 5-HT and DA levels after acute AMPH challenge

Procedures
Stereotactic surgery Stereotactic surgery (see for example: Verheij et al., 2008) was performed under Isoflurane anaesthesia (1 ml/min O2, 0.5 ml/min N2, 5% Isoflurane for induction and 2-3% for maintenance). Animals were s.c. injected with the antibiotic Kefzol® (Cefazolin 15 mg/ml/kg) and the analgesia Finadyne® (Flunixin 2.5 mg/ml/kg). Local analgesia Xylocaine® 10% (Lidocaine 100mg/ml) and disinfectant (Betadine, 100 mg/ml povidone-iodine) were applied cutaneously before making the incision. To prevent dehydration of the eyes ointment (Ophtosan®) was applied. Rats were implanted with a unilateral cannula targeted at the right nucleus accumbens (NAc) shell (measured from Bregma: ML 0.80 mm, AP 1.60 mm, DV -5.80 mm). Stainless screws (n=4) were placed around the cannula implant site, followed by application of carboxylate cement (Durelon™) over the open surface of the skull to fixate the cannula. Coordinates were determined according to the brain atlas of Paxinos and Watson (2007). Subjects were given at least seven days to recover from surgery before they entered behavioural testing. During recovery, the condition of the animals was daily monitored (e.g. weight, behaviour, condition of the wound, fur condition).

Microdialysis Following recovery, a microdialysis probe (type AI-8–02/ AZ-8-02, outer diameter: 0.22 mm, 50000 molecular-weight cut-off, Eicom, Tokyo, Japan) was inserted into the guide cannula in a conscious rat at least 16h before the start of the experiment (Verheij and Cools, 2007). At the same moment the cage of the rat was changed to the experimental cage (L 25 cm, W 25 cm, H 35 cm, equipped with bedding material and a wooden stick). These procedures are believed to reduce stress levels and it ensures more stable basal DA and 5-HT levels before the experimental intervention (Tuinstra and Cools, 2000, van der Elst et al., 2005, De Leonibus et al., 2006). Standard solutions of DA and 5-HT were injected in duplicate to calibrate the microdialysis system before experimental sessions (see appendix). Hereafter, the inlet and outlet tubing was attached to a swivel to allow measurement in the freely moving animal. With a rate of 2.0 µL/min the probe was perfused with

modified Ringer solution (See appendix, Verheij and Cools, 2007). Once every 10 minutes the collected outflow was injected into a high performance liquid chromatography (HPLC) system (HTEC-500, software version 2.2.4, Eicom, Tokyo, Japan). Separation of DA and 5-HT was based on reversed phase, ion-paring liquid chromatography using a column (Eicom) with mobile phase containing 1% methanol (flow rate; 500 µl/min, temp 25 °C). An electrochemical detector (ECD) coupled to the HPLC measured extracellular levels of DA and 5-HT at +400 mV. As soon as the extracellular levels of both DA and 5-HT were stabilized (three consecutive samples deviate <10%) (Tuinstra & Cools, 2000), the three subsequent samples were used as baseline. After the last baseline sample was injected into the HPLC the animal was i.p. injected with either 1 ml/kg AMPH (2.5 mg/kg) or 1 ml/kg Saline (0.9% NaClWohr et al., 2015). Locomotor activity was recorded throughout the experiment, starting 60 minutes prior to the AMPH/saline injection till at least for 180 minutes following the injection with an automated Photobeam Activity System (PAS, San Diego Instruments, USA).

Ultrasonic vocalizations. An UltraSoundGate Conderson Microphone (CM16; Avisoft Bioacoustics, Berlin, Germany), which was sensitive to a frequency range of 10-200 kHz with input-referred self- noise level of 18 dB Sound Pressure Level was placed above the centre of the experimental cage. This cage was encased with polystyrene foam for isolation purposes to minimize environmental noise as much as possible. The microphone was connected to an UltraSoundGate 116H audio device (Avisoft Bioacoustics), which was connected to a computer. The acoustic data were recorded with a sampling rate of 250,000 Hz in 16-bit format by (recording range: 0-250 kHz) an Avisoft recorder (version 4.2.22; Avisoft Bioacoustics). Ultrasonic vocalizations were measured throughout the experiment, starting 60 minutes prior to injection and lasting until at least 180 minutes following injection (Wohr et al., 2015).

Histology

Upon completion of the experiment the position of the microdialysis probe was verified (see figure 1). Rats were deeply anesthetised by an i.p. injection pentobarbital (1.0 ml; Apotheek Faculteit Diergeneeskunde Utrecht, 60 mg/ml). After respiration had stopped, the animals were transcardially perfused with 30 ml 10% paraformaldehyde solution (J.T. Baker®) and decapitated. Brains were removed and fixed in 10% paraformaldehyde solution for at least three days and stored at 7ºC. The exact placement of the microdialysis probe was verified by Vibratome sections (Leica® VT1000, slice thickness 100 µm), with reference to the atlas by Paxinos and Watson (2007). Only those animals with a confirmed placement of the probe in the target area (see figure 1) were included in statistical analyses.

Data analysis

Changes in extracellular accumbal DA and 5-HT levels and locomotor activity were quantified as percentage change compared to baseline levels and analysed with a 2 (genotype: SERT KO/WT) x 2 (treatment: saline/AMPH) x 21 (time points) RM ANOVA. Total numbers of 50kHz USV were counted for the baseline session and following injection (Wohr et al., 2015) and were analysed with a 2 (genotype: SERT KO/WT) x 2 (treatment: saline/AMPH) x 21 (time points) RM ANOVA. Statistical testing was done using IBM SPSS Statistics 21 (Chicago, IL). Differences were considered to be statistically significant when $p \leq 0.05$ and RM ANOVA and student t-tests were used as posthoc analyses.

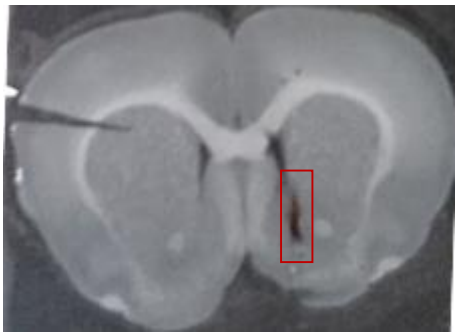

Figure 1. Correct placement of a microdialysis probe in the NAc shell indicated with the red rectangular box

Results experiment 1 Self-administration & progressive ratio

One rat had a leaking i.v. catheter and was excluded from all statistical analyses. Outlier detection on the mean values of rewards, incorrect and timeout responses revealed that one SERT WT rat in the short access group on correct responses and timeout responses, two SERT KO rats in the short access group on incorrect responses and one SERT KO animal in the long access group on timeout responses had extreme values (>3*IQR) and therefore these values were removed from the data set and excluded from statistical analysis. Groups were based on mean intake of the last two training days and did not differ significantly (SERT KO ShA: M=6.9±1.3, SERT KO LgA: M=6.6± 1.9, SET WT ShA: M=7.0± 1.9, SERT WT LgA: M=7.1±2.0, all p>0.05, student t-tests)

*Self-administratio*n Overall, animals in the LgA group (M=58.6 ±6.5) self-administered more AMPH compared to the ShA animals (M= 9.2±6.3, $F_{1,35}$=29.666, p=0.000, eta^2=0.459), indicating that our paradigm was successful (Ahmed and Koob, 1998).

A repeated measures ANOVA revealed a significant three-way interaction of day x genotype x access ($F_{3,111}$=5.975, p=0.001, eta^2=0.146). Follow up RM-ANOVA (split on genotype) showed a significant interaction of day x access for both SERT KO ($F_{3,50}$=16.145, p=0.000, eta^2=0.487) and SERT WT rats ($F_{3,46}$=2.874, p=0.05, eta^2=0.138). There was no day effect on AMPH intake in SERT WT ($F_{3,26}$=1.480, p=0.244, n.s.) nor in SERT KO ($F_{4,33}$=1.695, p=0.179, n.s.) in the ShA group. These animals had a stable and similar intake pattern (SERT KO: M=9.2±1.9, SERT WT: M=9.2±3.3, split on access type, day x genotype: $F_{5,95}$=1.266, p=0.284, n.s., see figure 2A). However, in the LgA group the day effect was significant for both SERT WT ($F_{2,21}$=3.703, p=0.036, eta^2=0.292) and SERT KO ($F_{3,23}$=15.296, p=0.000, eta^2=0.657). There was a significant day x genotype in the LgA group ($F_{3,51}$=6.058, p=0.001, eta^2=0.263) and posthoc analysis revealed a linear increase in intake ($F_{1,9}$=6.220, p=0.034, eta^2=0.409, figure 2B) in the SERT WT LgA group, but a quadratic increase in intake ($F_{1,8}$=8.986, p=0.017, eta^2=0.529, figure 2B) in the SERT KO LgA group. Post hoc t-tests showed that SERT KO self-administered more AMPH on day 17 (t=2.204, p=0.028), day 18 (t=2.291, p=0.035) and day 19 (t=2.786, p=0.013). No interaction or main effects were found on time-out responses (all p>0.05, see supplementary figure 9 in appendix), nor on incorrect responses (all p>0.05, see supplementary figure 10 in appendix)

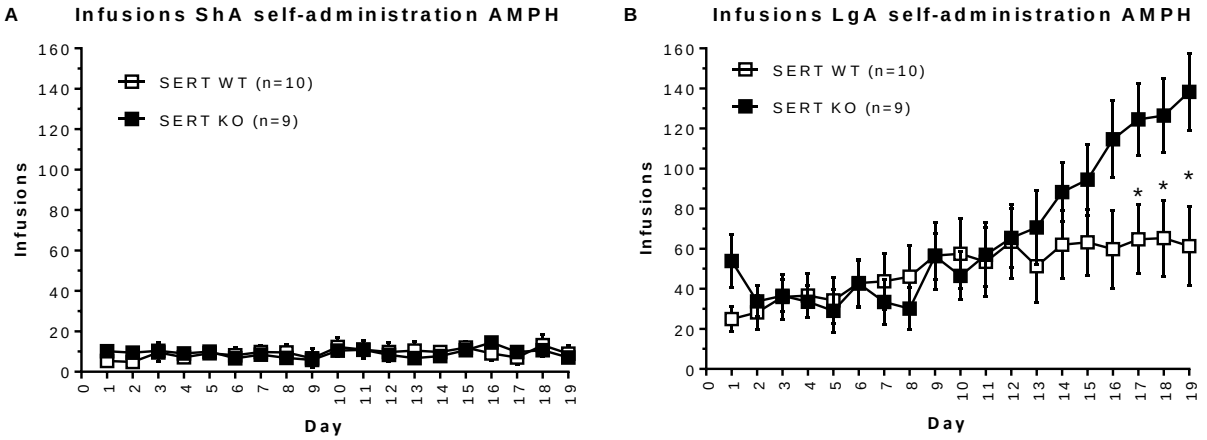

Figure 2. Mean (±SEM) numbers of self-administered amphetamine infusions (0.03 mg/kg/infusion) of LgA and ShA sessions over 19 days are shown. (A) Number of infusions of SERT WT and SERT KO rats in the ShA sessions over nineteen days. (B) Number of infusions of SERT WT and SERT KO rats in the LgA sessions over 19 days. * p<0.05 (student t-tests). Note: values of one SERT WT in the LgA group on day 18 and 19 were substituted by the values of day 17, because of physical and health problems of this rat.

Progressive ratio The main effect of access type was significant, LgA animals (M=7.44±0.78) administered more AMPH under PR conditions compared to ShA animals (M=3.14±0.74; $F_{1,32}$=15.885, p=0.000, eta^2=0.332). Although the interaction effect (genotype x access type) was only marginally significant ($F_{1,32}$=3.706 p=0.068, eta^2=0.104), there was a significant genotype effect in the LgA group ($F_{1,16}$=5.574, p=0.032, eta^2=0.271, see figure 3), whereas this effect was absent in the ShA group ($F_{1,18}$=0.677, p=0.422, n.s.). SERT KO (M=9.88 ± 1.56) animals in the LgA group were more motivated compared to SERT WT (M=5.00 ± 1.36) rats in the LgA group to receive AMPH. There were no interaction or main effects on incorrect and time-out responses, except for an access effect for both variables (incorrect $F_{1,32}$=4.737, p=0.037; timeout $F_{1,32}$=4.193, p= 0.049).

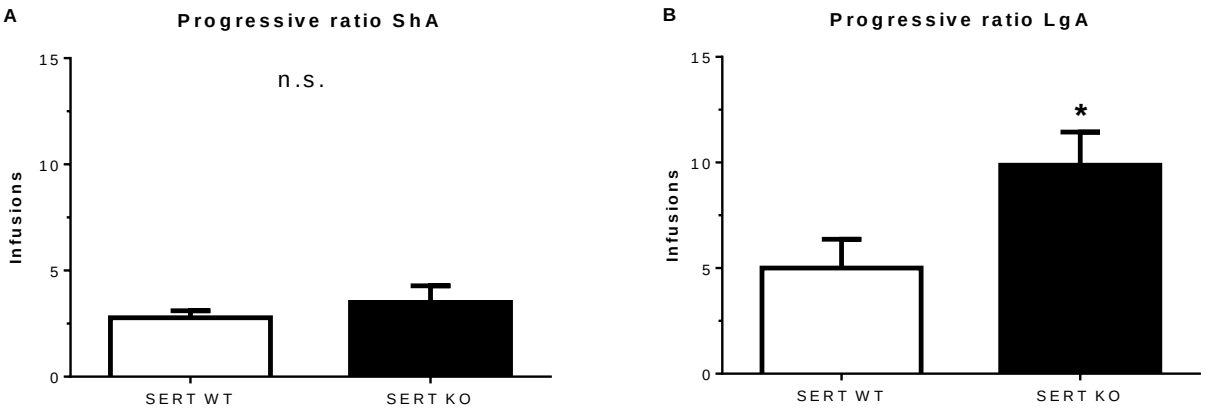

Figure 3. Mean (±SEM) numbers of self-administered amphetamine infusions (0.03 mg/kg/infusion) of SERT WT (LgA, n=9; ShA, n=9) and SERT KO (LgA, n=8; ShA, n=10) on the progressive ratio test are shown of the ShA group (A) and the LgA group (B). *p<0.05 (student t-test).

Results experiment 2: Extracellular levels of 5-HT and DA after acute AMPH

Four SERT WT and two SERT KO rats were excluded from statistical analysis due to erroneous placement of the microdialysis guide cannula or absence of a probe track, detected in post-experimental histology. Additionally, a substantial number of SERT

WT and SERT KO rats had to be excluded from analysis due to leaking probes (approximately 4mm from the distal end, see figure 4). Consequently, data of only two saline-treated SERT WT and six saline-treated SERT KO rats, as well as four AMPH-treated SERT WT and five AMPH-treated SERT KO could be used.

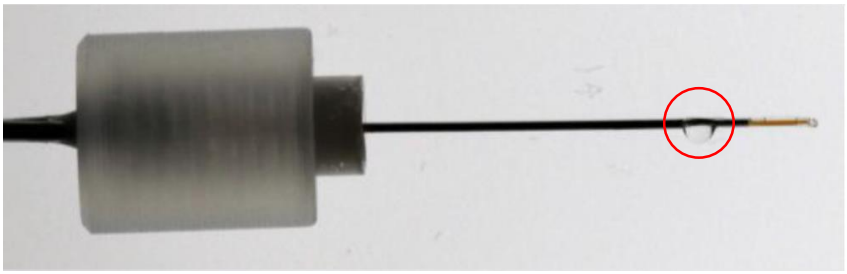

Figure 4. Leaking microdialysis probe at red circle at approximately 4mm from distal end

Baseline extracellular DA and 5-HT levels A one-way ANOVA revealed that baseline extracellular levels of DA in the NAc shell did not differ significantly ($F_{1,16}$=0.486, p=0.496, n.s; Figure 5A) between SERT WT rats (M=0.81 ± 0.46 pg/sample) and SERT KO rats (M=1.24 ± 0.38 pg/sample). In contrast, 5-HT baseline extracellular levels were significantly higher ($F_{1,16}$=10.346, p=0.006, eta^2=0.408; Figure 5C) in SERT KO rats (M=7.18 ± 1.48 pg/sample) compared to SERT WT rats (M=0.63 ± 0.20 pg/sample)

Extracellular DA levels after AMPH There were no significant three or two-way interactions between time, genotype and treatment on extracellular accumbal DA levels (genotype x treatment x time: $F_{2,23}$=0.537, p=0.584, n.s.; time x treatment: $F_{2,23}$=1.961, p=0.165, n.s.; time x genotype: $F_{2,23}$=0.507, p=0.600, n.s., genotype x treatment: $F_{1,12}$=0.345, p=0.568, n.s.). However, there was a significant treatment effect ($F_{1,12}$= 6.438, p=0.026, eta^2=0.349, Figure 5B). AMPH induced an increase in DA levels (M=675 ±150%), whereas DA levels stayed stable after saline (M=103±167%).

Extracellular 5-HT levels after AMPH There was a significant time x treatment effect on 5-HT levels ($F_{5,63}$=2.488, p=0.036, eta^2=0.172, Figure 5D). Post hoc RM ANOVA revealed that there was a significant main effect of time for the AMPH treatment ($F_{4,24}$=3.432, p =0.023, eta^2=0.364) but not for the saline treatment ($F_{2,14}$=1.135, p=0.357, n.s.). Simple contrasts in the RM ANOVA showed that 5-HT levels differed significantly from baseline at 20 – 60, 90 and 100 minutes (all p<0.05) following injection of amphetamine. None of the three-way and two-way interactions were significant (all p>0.05). Although there was no significant effect of genotype in the AMPH treatment group ($F_{1,6}$=3.354, p=0.117, eta^2=0.359) nor in the saline group ($F_{1,6}$=0.022, p=0.889, n.s.), the effect size of genotype on 5-HT levels (eta^2=0.359), but not DA levels (eta^2=0.061) in the AMPH treated group is surprisingly large. Low statistical power, caused by small number of rats per condition due to dropout because of leaking microdialysis probes, probably prevents the effect from being significant (VanVoorhis and Morgan, 2007). Post hoc student t-tests revealed that 5-HT levels of SERT KO rats remained high and stable after AMPH treatment until the end of the measurement (180 minutes), whereas 5-HT levels of SERT WT rats recovered to baseline after 180 minutes (supplementary figure 11 C&D).

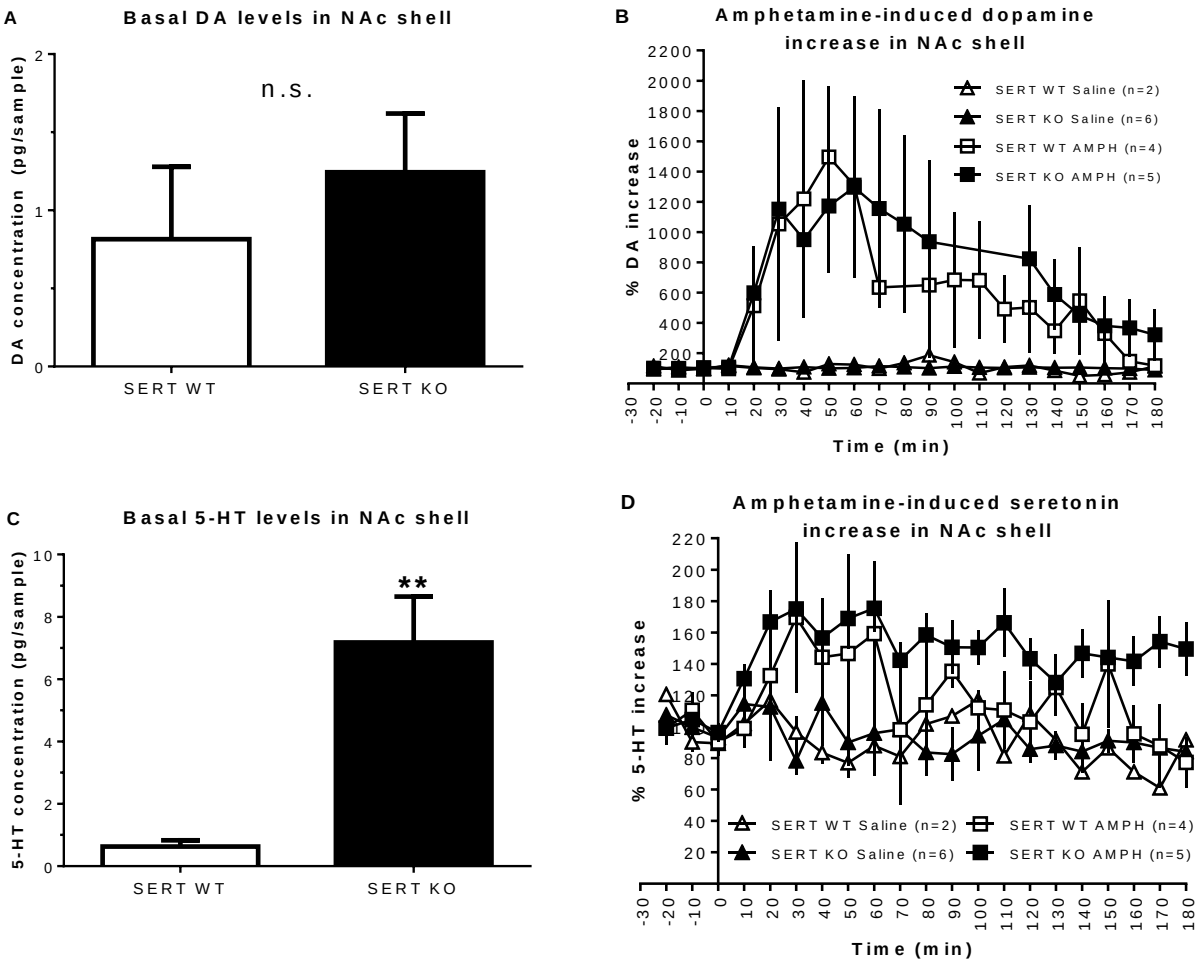

Figure 5. Mean (±SEM) DA and 5-HT levels of in the NAc shell are shown. Basal levels of DA (A) and 5-HT (C) in SERT WT (n=6) and SERT KO rats (n=11). Percentage increase relative to basal levels in DA (B) and 5-HT (D) concentration in the NAc shell. **p<0.01 (student t-test). Note: x-axis values represent time blocks of 10 minutes. Supplementary figures available in appendix.

Ultrasonic vocalizations The analysis of the 50 kHz USV of the present study is not finished at the time of writing this manuscript, since it is very labour intensive. However, we received unpublished data from a separate cohort of AMPH-treated (2.5 mg/kg/injection) SERT WT and SERT KO rats (Wohr et al., unpublished findings). SERT KO rats emitted significant more 50 kHz vocalizations compared to SERT WT rats (Figure 6A: genotype effect p<0.05). The number of USV/min of both SERT KO and SERT WT peaked around 50 USV/min. As for accumbal 5-HT levels, the number gradually decreased to baseline level in SERT WT rats but remained stable and high in SERT KO rats, even 180 minutes after the injection (see Figure 6).

Locomotor activity One SERT WT rat, treated with AMPH, had to be excluded from analysis because of blockage of the signals by sawdust. Therefore, the total number of beam interruptions were obtained from four saline treated SERT WT and seven SERT KO rats, and six AMPH treated SERT WT and six SERT KO rats. Rats with leaking or erroneously placed probes were included.

The three-way interaction of genotype, treatment and time and the interaction of time x genotype were not significant (respectively $F_{3,60}$=2.139, p=0.101, n.s. and $F_{3,60}$=2.105, p=0.106, n.s.). However, the time x treatment interaction was significant ($F_{3,60}$=11.986, p=0.000, eta^2=0.387). Follow-up analysis revealed a time effect after

AMPH treatment ($F_{2,27}$=12.754, p=0.000, eta2=0.537) but not after saline treatment $F_{3,31}$=1.191, p=0.330, n.s.). Furthermore, there was a significant interaction effect of genotype and treatment on the locomotor activity ($F_{1,19}$=5.095, p=0.036, eta^2=0.211). Follow up repeated measures showed that that there was a significant effect of genotype in the amphetamine group, but not in the saline group ($F_{1,10}$=5.347, p=0.043, eta^2=0.348). In other words, SERT KO animals had a higher locomotor activity after the AMPH injection compared to SERT WT rats. The larger activity was mainly visible in the first 90 minutes; however, this was only marginally significant for all those time points. In SERT WT rats, locomotor activity after the amphetamine injection increased up to 1286 ± 254% after 40 minutes, in SERT KO the locomotor activity increased up to 2574 ± 634% after 60 minutes (see Figure 7).

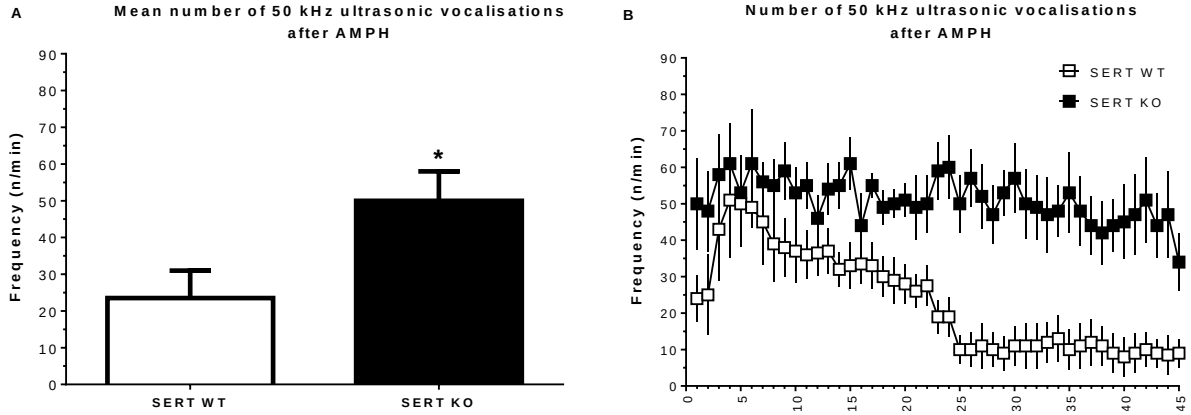

Figure 6. Mean (±SEM) number of 50 kHz ultrasonic vocalisations after 2.5 mg/kg amphetamine injection. * p<0.05 (student t-test).

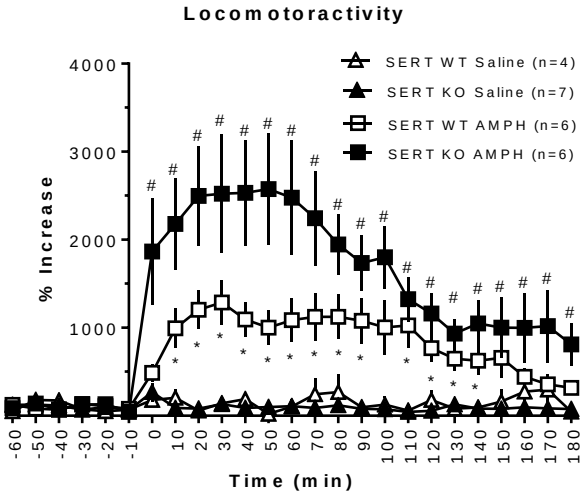

Figure 7. Mean (±SEM) percentage increase in locomotor activity of SERT WT and SERT KO rats relative to baseline after amphetamine (2.5 mg/kg/injection) or saline injection. # p<0.05 (student t-tests) SERT KO saline vs AMPH, * p<0.05 (student t-tests) SERT WT saline vs AMPH.

Discussion

The present study aimed to give insights in the role of SERT and accumbal 5-HT/DA in AMPH reward and addiction by combining self-administration and measuring extracellular 5-HT and DA by microdialysis in SERT KO and their SERT WT counterparts. In experiment 1 SERT KO rats in the LgA group showed a quadratic increase in AMPH intake, whereas SERT WT in the LgA group showed a linear increase, which results in higher drug intake in SERT KO rats at day 17,18 and 19 in

the LgA group. Experiment 2 revealed that SERT KO rats showed more hyperactive behaviour and higher 50 kHz USV emission rates compared to SERT WT rats. USV (and locomotor activity) are believed to be a measure for drug reward (Knutson et al., 2002, Wohr et al., 2015, Avvisati et al., 2016). Furthermore, basal extracellular levels of accumbal 5-HT were significantly higher in SERT KO rats than in SERT WT rats, whereas there was no difference in basal extracellular DA levels. The latter findings are extensions of previous research, where this effect was observed in the ventral hippocampus and the dorsal striatum (Homberg et al., 2007, Verheij et al., 2014). Increased extracellular 5-HT levels are explained by the absence of the transporter in SERT KO animals, which is normally responsible for the reuptake of 5-HT from the synaptic cleft back into the presynaptic cell (Rudnick, 2006). In combination with the lack of genotype differences in basal DA this confirms the absence of 5-HT and DA interactions in SERT KO animals (Verheij et al., 2014).

Increased levels of both extracellular 5-HT and DA were found after acute AMPH treatment (2.5 mg/kg), however, no genotype differences in extracellular accumbal 5-HT or DA levels were found. This finding is based on very low number of observations per condition, due to large dropout which was caused by leaking microdialysis probes. In the future, we can decrease the dropout by using more reliable probes. The small number of rats per conditions led to low statistical power. Therefore it is probably an inaccurate representation of the results (VanVoorhis and Morgan, 2007). This is enforced by the relatively strong effect between SERT KO and SERT WT rats (eta^2=0.359). Furthermore, AMPH induced an increase in accumbal 5-HT levels which more prolonged in SERT KO rats than in SERT WT rats. Furthermore, this prolonged effect is not only visible in 5-HT levels in of SERT KO rats, a similar temporal pattern can be detected in the number of USV and locomotor activity. This may indicate that these processes are interconnected.

Taken together, our data support the hypothesis that SERT KO rats are more sensitive to AMPH compared to SERT WT rats, which may indicate that humans carrying the short allele of the 5-HTTLPR gene are more sensitive to AMPH, as is the case with other psychostimulants like cocaine and ecstasy (Gerra et al., 2004, Lott et al., 2006, Homberg et al., 2008, Johnson, 2010, Martin-Santos et al., 2010, Oakly et al., 2014, Verheij et al., 2014). The resemblance of the temporal patterns of USV, locomotor activity and accumbal 5-HT levels may implicate that AMPH reward is mediated by accumbal 5-HT, and not so much by SERT, DAT or accumbal DA. Taking it one step further, this could imply that the observed escalation of drug self-administration is dependent on the degree of experienced reward and thus on accumbal 5-HT. This is of clinical significance because this could support the hypothesis that humans carrying the short allele of the 5-HTTLPR gene are more sensitive to develop AMPH abuse and dependence (Kitamura et al., 2006). Although we should always be careful in generalizing rat work to humans, our results suggest that genetic variants in the 5-HT system should be considered in patient heterogeneity and in individualized choices of treatment to reduce SUD-related problems for both patients and the society, since AMPH addictions cause an enormous public health burden (Herman and Balogh, 2012).

Unfortunately, we cannot provide extensive insights in the complex working mechanisms of AMPH use and abuse with this study. The precise neurobiological mechanisms responsible for the transition from drug use to abuse remain unclear and needs further investigation (Ahmed and Koob, 1998, Kitamura et al., 2006). Our study provides evidence for a 5-HT dependent, but SERT independent effect of AMPH. Therefore, it might be interesting to study genotype differences in e.g.

vesicular monoamine transport (VMAT) and the storage and release of 5-HT and/or DA in AMPH use and abuse. It could be that SERT KO animals exhibit larger numbers of VMAT, which can result in increased release of monoamines through exocytosis. Furthermore, future studies could give insights in altered gene expression or epigenetic processes in the NAc shell in AMPH use and abuse. Furthermore, the power of our microdialysis experiment should be improved by increasing the number of rats per condition. Thereafter, correlation analysis of AMPH-induced behavioural changes (locomotor activity and USV) and accumbal 5-HT (and DA) could be provided. A positive correlation between behavioural measures, which are thought to reflect drug reward, and accumbal 5-HT levels, would support the hypothesis that accumbal 5-HT mediates AMPH reward.

Conclusion

The present study shows that SERT KO animals are more sensitive to AMPH as reflected by increased AMPH intake, hyper locomotor activity and increased 50 kHz USV. No differences in extracellular levels of DA and 5-HT were seen after AMPH administration. However, post hoc analyses per genotype suggest that there is a strong effect size (eta^2=0.359) for 5-HT levels after AMPH treatment. Post hoc student t-tests revealed that 5-HT levels of SERT KO as well as SERT WT rats increase after AMPH. However, for SERT KO rats this effect is more prolonged. This trend is also visible for USV and locomotor activity. Linking these findings with the self-administration data, it could imply that AMPH reward is mediated by accumbal 5-HT. Future research should increase the number of rats in the microdialysis and correlate AMPH-induced changes in behaviour (USV, locomotor activity) with changes in extracellular 5-HT and DA levels to test this new hypothesis.

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Disclosure

The authors declare no conflict of interest.

Abbreviations

AMPH, amphetamine; SA, self-administration; LgA, long access; ShA, short access; DA, dopamine; 5-HT, serotonin; i.v.; intra venous; ml, millilitre; mg, milligram; ML, medial-lateral; AP, anterior-posterior; DV, dorsal-ventral; NAc, Nucleus accumbens; USV, ultrasonic vocalisations;

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Appendix

Solutions Self-administration

In this study the following solutions were used: 1) Amphetamine: 39.2 mg dexamphetamine sulfas BP (BUFA, Sigma, UK) was dissolved in 275 ml Saline (0.9% NaCl, B. Baxter B.V., NL). 2) Flushing solution: 0.9% sterile saline (Baxter B.V., NL) containing Heparin (50U/ml, LEO pharma, NL) and Kefzol ® (Cefazolin 15 mg/ml/kg, Eurocept pharmaceutics, NL).

Solutions Microdialysis

In this study the following solutions were used: 1) Modified Ringer solution: 8.590 g NaCl (Sigma), 0.298 g KCl (Sigma), 0.176 g CaCl2·2H2O (Sigma) and 0.224 g MgCl2·6H2O (Sigma) was dissolved in ultra-pure water (> 18 MΩ). 2) Mobile phase: 13.45 g NaH2PO4·2H2O (Wako), 4.94 g Na2HPO4·12H2O (Wako), 500 mg sodium 1-decanesulphonate (2 mM, Kasei Kogyo) and 50 mg EDTA (0.1 mM, Dojindo) dissolved in ultra-pure water (> 18 MΩ). The end concentration of 0.1 M phosphate buffer contained 1% methanol. 3) Dopamine standard solution: 0.0125 g DA (Sigma) was dissolved in 10 ml HCL-1M (Boom). After dilution the final concentration was 50 pg in 5 µl of 0.1 M HCL solution. 4) Serotonin standard solution: 0.0121 g 5-HT was dissolved in 10 ml HCL-1M (Boom). Final concentration was 50 pg in 5 µl of 0.1 M HCL solution. 5) Amphetamine: 2.5 mg dexamphetamine sulfas BP (BUFA) was dissolved in 1 ml Saline (0.9% NaCl, B. Braun).

Supplementary figures experiment 1 AMPH self-administration

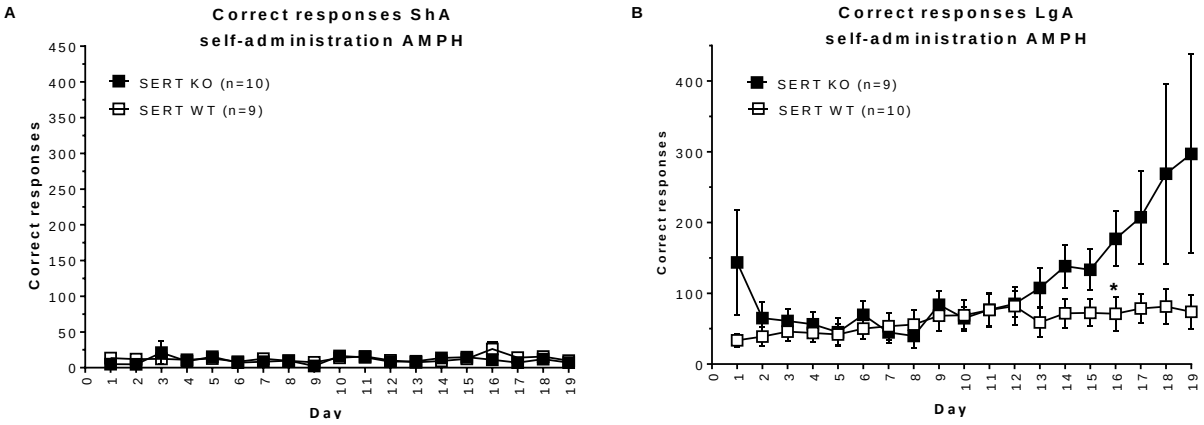

Figure 8. Mean (± SEM) number of correct responses in the (A) ShA group and (B) LgA group. *p<0.05 (student t-test)

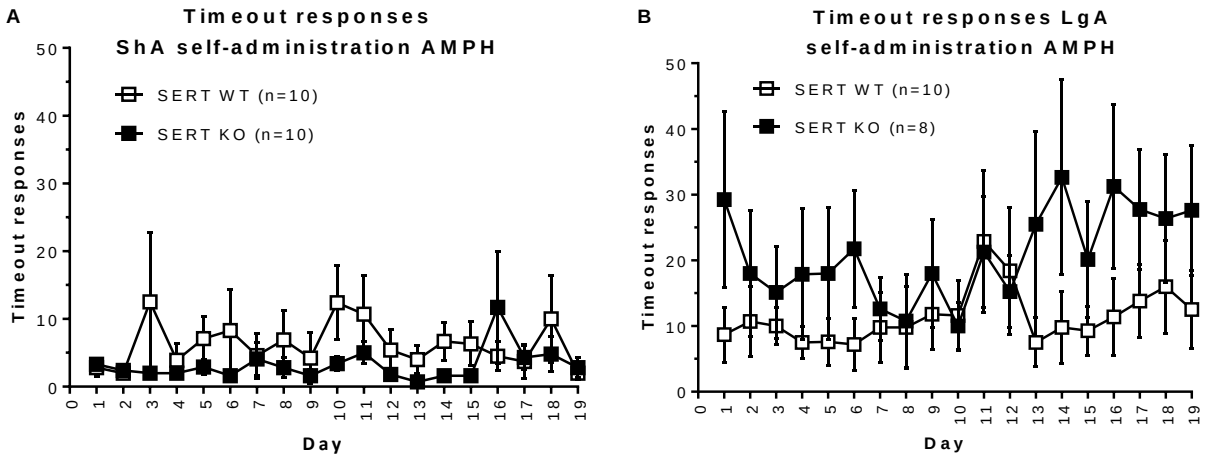

Figure 9. Mean (± SEM) number of timeout responses in the (A) ShA group and (B) LgA group. Note: no significant interactions nor main effects were found on the number of timeout responses.

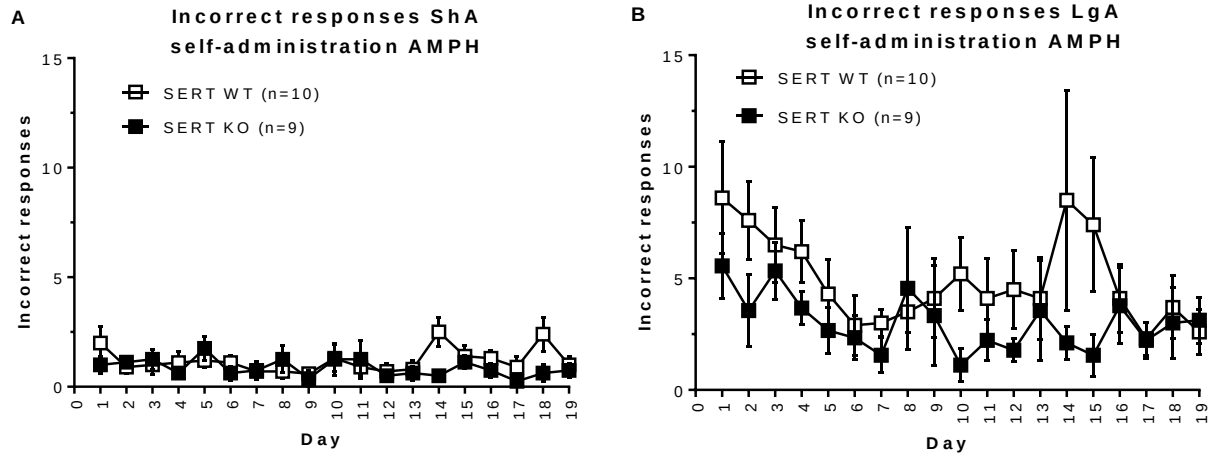

Figure 10. Mean (± SEM) number of incorrect responses in (A) ShA group and (B) LgA group. Note: no significant interactions nor main effects were found on the number of incorrect responses.

Supplementary Figures experiment 2 Microdialysis

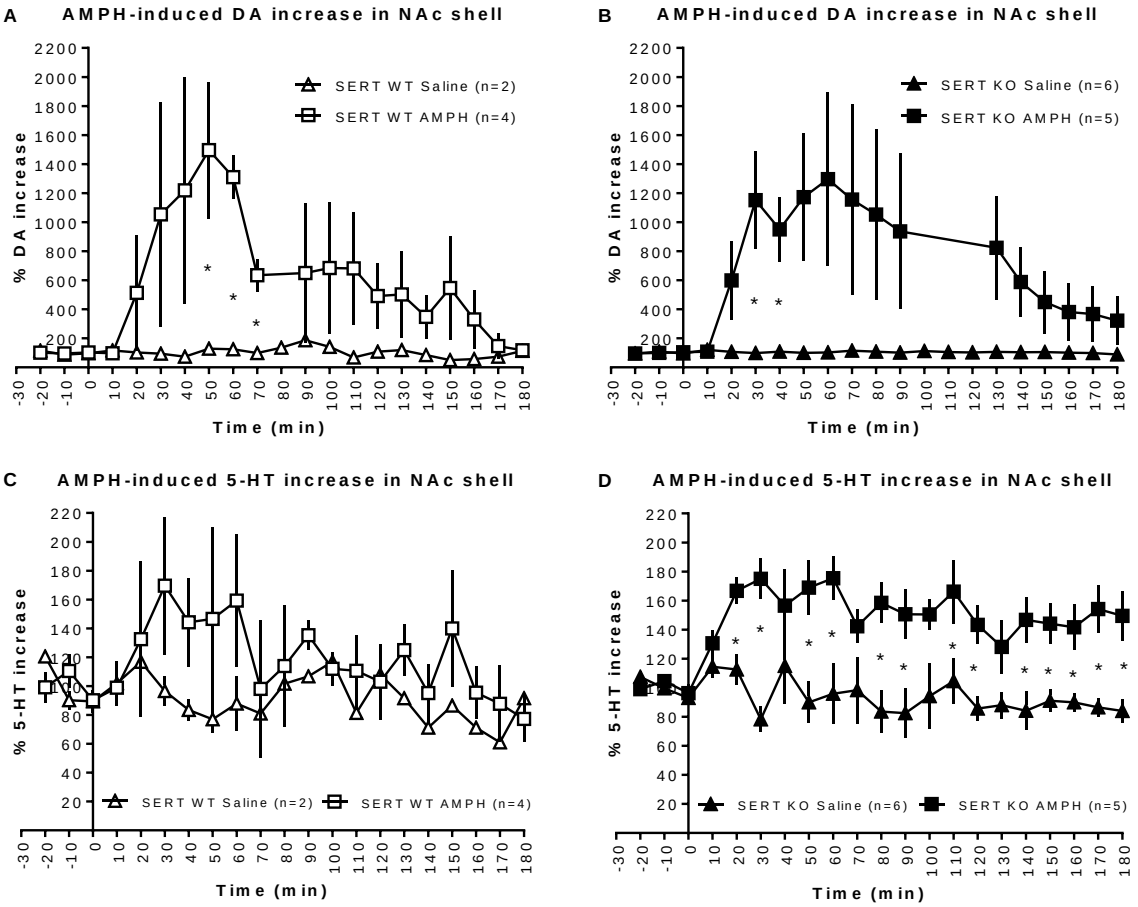

Figure 11. Mean (± SEM) increase (compared to the average of baseline samples) in (A) dopamine levels in SERT WT rats, (B) dopamine levels in SERT KO rats, (C) serotonin levels in SERT WT rats and (D) serotonin levels in SERT KO rats. Note: the absence of SEM bars from 0 – 180 minutes of the SERT WT animals in 5-HT graph is because only one animal had a reliable measure at these points. * $p<0.05$ (student t-tests).