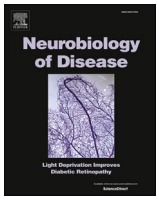
Neurobiology of Disease 206 (2025) 106800

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi



Brain acid sphingomyelinase controls addiction-related behaviours in a sex-specific way

Liubov S. Kalinichenko [a,*], Iulia Zoicas [a], Anne-Marie Bienia [a], Clara Bühner [a], Julia Robinson [a], Joshua Küttermeyer [a], Annika Labonte [a], Thadshajiny Raveendran [a], Lena Warth [a], Irena Smaga [b], Malgorzata Filip [b], Volker Eulenburg [c], Cosima Rhein [a], Anna Fejtova [a], Erich Gulbins [d], Johannes Kornhuber [a], Christian P. Müller [a,e]

[a] Department of Psychiatry and Psychotherapy, University Clinic, Friedrich-Alexander-University of Erlangen-Nürnberg, Schwabachanlage 6, 91054 Erlangen, Germany
[b] Maj Institute of Pharmacology, Polish Academy of Sciences, Department of Drug Addiction Pharmacology, Smętna 12, 31-343 Kraków, Poland
[c] Department for Translational Anaesthesiology and Intensive Care Medicine, Medical Faculty University of Augsburg, Stenglinstr. 2, 86156 Augsburg, Germany
[d] Institute of Molecular Biology, University Hospital Essen, University of Duisburg-Essen, Hufelandstrasse 55, 45122 Essen, Germany
[e] Institute of Psychopharmacology, Central Institute of Mental Health, University of Heidelberg, J 5, 68159 Heidelberg, Germany

ARTICLE INFO

Keywords:
Acid sphingomyelinase
Forebrain
Addiction
Conditioned place preference
Alcohol
Cocaine
Amphetamine
Food
Ketamine
Serotonin

ABSTRACT

Addiction is a chronic and severe mental disorder with high gender- and sex-specificity. However, the pathogenesis of this disorder is not fully elucidated, and no targeted pharmacotherapy is available. A growing body of evidence points out the potential involvement of the ceramide system in the pathophysiology of addiction. A pathogenic pathway for several mental disorders based on the overexpression of an enzyme involved in ceramide formation, acid sphingomyelinase (ASM), was recently proposed. Here we show a crucial role of ASM specifically overexpressing in the forebrain for various types of addiction-related behaviours in a drug- and sex-specific way. In male mice, a forebrain ASM overexpression led to enhanced alcohol consumption in a free-choice paradigm. It also diminished the reinforcing properties of alcohol and cocaine, but not that of amphetamine, ketamine, or a natural reinforcer fat/carbohydrate-rich food in the conditioned place preference (CPP) test in males. In female mice, a forebrain ASM overexpression enhanced alcohol binge-like drinking, while moderate alcohol consumption was preserved. ASM overexpression in females contributed to CPP establishment for amphetamine, but not for other psychoactive substances. Altogether, this study shows a specific involvement of forebrain ASM in the development of conditioned reinforcing effects of different types of substances with addictive properties in a sex-specific way. Our data enlarge the current knowledge on the specific molecular mechanisms of dependence from various drugs of abuse and might serve as a basis for the development of drug- and sex-specific targeted therapy.

1. Introduction

Addiction is defined as a severe chronic relapsing disorder characterized by compulsive drug seeking and use despite adverse consequences (American Psychiatric Association, 2013). It is estimated that 29 % of Europeans aged 15–64 years have ever used an illicit drug, with about 1.5 times higher prevalence in males than in females (European Monitoring Centre for Drugs and Drug Addiction, 2022). Most of drugs are taken by the vast majority of consumers in a controlled way for instrumentalization (Müller et al., 2021, 2023; Spanagel et al., 2024), which might then result in the transition to addiction (Müller, 2020). Considering severe medical and social consequences of drug dependence and addiction as well as absence of targeted therapeutical approaches, investigation of new potential mechanisms of this disorder is of special interest.

Membrane lipids, and particularly ceramides, have long been considered to have mainly a structural function. However, recent studies suggest an active role of ceramides in synaptic plasticity, thus contributing to synaptic transmission and controlling behavioural plasticity (Kalinichenko et al., 2024). Particularly, the ceramide system is shown to play an important role in several mental and psychiatric disorders including drug use and addiction (Schneider et al., 2017; Kalinichenko

* Corresponding author.
E-mail address: Liubov.Kalinichenko@uk-erlangen.de (L.S. Kalinichenko).

https://doi.org/10.1016/j.nbd.2025.106800
Received 30 August 2024; Received in revised form 9 January 2025; Accepted 9 January 2025
Available online 17 January 2025

et al., 2018, 2022). Enzymes of the ceramide formation, such as acid and neutral sphingomyelinases (ASM and NSM), are shown to be involved in the mechanisms of dependence to drugs such as alcohol and cocaine (Nassogne et al., 2004; Müller et al., 2017; Mühle et al., 2018; Frankowska et al., 2021; Kalinichenko et al., 2021a, 2023). In mice, whole body ASM overexpression is associated with enhanced alcohol consumption in a free-choice paradigm, which allows the individuals to control a genetically-induced depression (Gulbins et al., 2013; Müller et al., 2017). Moreover, cocaine self-administration results in a decrease in the expression of the *Smpd1* gene coding for ASM in the prefrontal cortex, but not other brain structures of rats (Frankowska et al., 2021). Taking into account the specific importance of the ceramide system for brain functioning, we propose that the brain, and particularly the forebrain ASM is crucial for modulating the drug use-specific phenotype. Here we analyzed the response to common addictive drugs, such as alcohol, cocaine, amphetamine, and ketamine, as well as food preparation with high addictive potential (Hess et al., 2019) in transgenic mice with forebrain-specific ASM overexpression. Alcohol, cocaine, and amphetamine have been selected as the major drugs relevant for clinical practice (UNODC, 2023). Due to recent establishment of ketamine as an antidepressant treatment, prevalence of ketamine misuse has been substantially growing over last years (Liu et al., 2016). In addition, we used a food preparation with high addictive potential, which composition and fat/carbohydrate ratio corresponds to the "cafeteria diet" (de Macedo et al., 2016; Hess et al., 2019). Drug addiction is mediated by a variety of molecular mechanisms, among which the involvement of brain monoaminergic systems is frequently studied. Among others, the serotonergic system is playing a crucial role in the pathogenesis of addiction for virtually all addictive substances including those we are focusing on in the present study (Yoshimoto et al., 2012; Müller and Homberg, 2015; Tanaka and Watanabe, 2020; Campbell et al., 2021; Gretler and McClain, 2023). Therefore, we analyzed the differences in serotonin (5-HT) receptor expression in the brain of transgenic mice with forebrain-specific ASM overexpression after alcohol administration. As the ceramide system is characterized by high sex-specificity (Mühle et al., 2018; Zoicas et al., 2020b; Kalinichenko et al., 2021a, 2023), we investigated the role of the forebrain ASM in female and male mice separately.

2. Materials and methods

2.1. Animals

Male and female mice (8–18 weeks old) overexpressing ASM in the forebrain ($ASMtg^{fb}$) as well as wild-type (wt) littermates were utilized in the experiments. These mice were generated by crossing female ASMtg mice with male Emx1IREScre homozygous mice as described previously (Zoicas et al., 2020b). As the ASM transgene in this mouse line is located on the X-chromosome, female $ASMtg^{fb}$ mice are heterozygous, while males were hemizygous for the transgene (Zoicas et al., 2020b). Therefore, in this study the data from female and male $ASMtg^{fb}$ were analyzed and discussed separately. The mice were maintained in a standard light-dark cycle (housed between two and five per cage or individually, depending on experimental requirements), with lights off from 07:00 pm to 07:00 am. All animals had ad libitum access to food and water.

Experiments were performed accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Government of Unterfranken, EU Directive 2010/63/EU for animal experiments, and complied with the ARRIVE guidelines. Ethical approval was obtained from the German Animal Protection Law Authority, Regierung von Unterfranken. The experimenter conducting the tests remained blind to the study groups or genotypes of the animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Alcohol-drinking phenotype in the free-choice unlimited access paradigm

Naïve mice of both sexes ($ASMtg^{fb}$ - $n = 10$ males/14 females; wt - $n = 9$ males/12 females) were individually housed for 2 weeks before the initiation of alcohol exposure. Each cage was equipped with two drinking bottles with tap water. After the acclimatization phase, water in one of the bottles was replaced with alcohol at increasing concentrations of 2, 4, 8, 12, and 16 vol% for 4 days each, while another bottle contained tap water. The positions of the bottles were changed and the bottles were weighted every second day. Subsequently, the alcohol concentration was maintained at 16 vol% for 8 days.

To assess the alcohol deprivation effect, alcohol was withdrawn for 3 weeks, during which both bottles contained tap water. Afterwards, alcohol was reintroduced at a concentration of 16 vol% for 4 days. This procedure (3 weeks withdrawal +4 days of reinstatement) was performed twice. The bottles were changed and weighed on a daily basis. The daily amount of alcohol consumed relative to body weight and the preference for alcohol over water were calculated. Control group animals ($ASMtg^{fb}$ - $n = 6$ males/6 females; wt - n = 6 males/6 females), which were utilized for the analysis of the serotonergic receptor expression, had access to two bottles containing only water within the whole experiment (Müller et al., 2017; Kalinichenko et al., 2021b).

2.3. Taste preference test

Alcohol-experienced animals, which had been exposed to the two-bottle free-choice unlimited access, were employed for this test. Sucrose preference (0.5 % and 5) and quinine aversion (2 mg/dl and 20 mg/dl) were assessed in a two-bottle free-choice test against water. The test was conducted three days after the last exposure to alcohol. Each dose of sucrose and quinine was provided for 3 days, with the positions of the bottles being switched and their weights recorded daily. A one-day washout period was provided between the sucrose and quinine testing phases (Müller et al., 2017; Kalinichenko et al., 2021b).

2.4. Alcohol-drinking phenotype in the intermittent alcohol consumption paradigm

Another alcohol consumption phenotype can be observed in the drinking in the dark (DID) schedule, where alcohol is available for 2 h (starting 3 h into the dark cycle) for 5 consecutive days. This paradigm induces high and consistent alcohol consumption in mice (Rhodes et al., 2005; Zoicas et al., 2020a) as it is performed in the active phase of mice when ingestive behaviours are highest, but still leaves mice the choice of drinking or avoiding the alcohol solution. This protocol is proposed as a model for human binge-like alcohol drinking and is intended to reflect the mechanisms underlying alcohol-dependent behaviours in humans (Smutek et al., 2014). Naïve mice of both sexes ($ASMtg^{fb}$ - $n = 9$ males/10 females; wt - $n = 8$ males/8 females) were individually housed for 2 weeks before the commencement of alcohol exposure. Each cage was equipped with one drinking bottle with tap water. The water bottle was replaced by a bottle containing 10 vol% alcohol solution for 2 h, beginning 3 h into the dark cycle, for 5 days. Control (water drinking) mice received a bottle of water for 2 h. All mice received a water bottle for remaining 22 h. The bottles were weighed, and the amount of alcohol consumed relative to body weight was calculated (König et al., 2020).

2.5. Blood alcohol determination

Naïve male mice ($ASMtg^{fb}$ - n = 8 males; wt - $n = 5$ males) were administered an alcohol injection (3.0 g/kg, i.p.). Subsequently, 20 µl blood samples were collected from the submandibular vein at 1, 2, and 3 h after the injection, without the use of anaesthesia. These blood samples were immediately mixed with 80 µl of 6.25 % (*w/v*) trichloroacetic acid. After centrifugation, 15 µl of the supernatant were subjected to

enzymatic alcohol determination using the alcohol dehydrogenase method, as described previously (Zheng et al., 2016).

2.6. Conditioned place preference (CPP)

2.6.1. Alcohol, cocaine, or amphetamine CPP

Drug-seeking behaviour is often triggered by certain contextual cues associated with drugs of abuse. Reinforcing effects of natural and pharmacological stimuli, including drugs of addiction, and associations between cues and drug seeking phenotype are captured in the CPP test (Huston et al., 2013; Pati et al., 2019). The establishment of drug-induced CPP was tested in naïve ASMtg^{fb} and wt mice with a time-course CPP model described previously using the TSE Place Preference test boxes (TSE Systems, Bad Homburg, Germany) (Easton et al., 2014; Kogias et al., 2020). The following substances were administered to naïve animals: alcohol (ASMtg^{fb} - $n = 7$ males/6 females; wt - $n = 9$ males/20 females), cocaine (ASMtg^{fb} - n = 9 males; wt - $n = 10$ males), and D-amphetamine (ASMtg^{fb} - n = 9 males/8 females; wt - n = 9 males/8 females). All animals were handled for 5 days before the experiment. The experiment involved four phases; habituation trial (one session), baseline testing (pre-conditioning, Bl), conditioning trials (14 sessions) and post-conditioning preference tests (3 sessions, T1–T3). For the drug-induced CPP (alcohol, cocaine, amphetamine), one week before the test animals were injected with saline (10 ml/kg) once a day for 3 days (Mon, Wed, Fri) to minimize injection-induced stress during testing.

2.6.1.1. Baseline test. The pretest was designed to establish a baseline level of preference for each individual animal. Mice were injected with saline and introduced into the center compartment with free access to all three compartments for 20 min.

2.6.1.2. Conditioning trials. An unbiased experimental design was used: half of the mice were conditioned to their preferred compartment, and half of them to their non-preferred compartment. Animals were injected i.p. immediately before each of 14 sessions with either saline or a psychostimulant (2 mg/kg alcohol; 20 mg/kg cocaine; 2 mg/kg amphetamine), and introduced into one of two compartments, with restricted access, for 5 min (alcohol) or 20 min (cocaine or amphetamine). All animals received seven pairings with saline and seven pairings with a drug. All drugs were diluted in saline and injected in the volume of 10 ml/kg.

2.6.1.3. Post-conditioning preference tests. To monitor the time course of CPP establishment, preference tests were systematically performed after one, three and seven conditioning/pseudo-conditioning trials. Before each test, mice were injected with saline and introduced into the center compartment with free access to all three compartments for 20 min.

To evaluate conditioned drug effects, time, entries, and distance passed in the compartments during the baseline and test trials were evaluated separately for conditioning and pseudo-conditioning compartments (CC and PC). An increase in behavioural frequency/time between baseline and test provides an indicator for the development of CPP and allow to evaluate reinforcing or aversive properties of a drug. Additional parameters, such as time per entry and distance per entry, which allow more detailed analysis of animal behaviours during the test, were also evaluated. As drug-induced hyperlocomotion may mask possible reinforcing processes, we control drug-induced locomotion during the conditioned trials (Huston et al., 2013).

2.6.2. Ketamine CPP

The ketamine-induced CPP in naïve ASMtg^{fb} and wt mice (ASMtg^{fb} - $n = 14$ males/9 females; wt - $n = 11$ males/12 females) was conducted similar to other psychostimulants. However, in this protocol the baseline test was followed by four conditioning trials (four pairings with saline and four pairings with ketamine) and one preference test. Ketamine was diluted in saline in the dose of 10 mg/kg and injected i.p. at a volume of 10 ml/kg (Guo et al., 2016).

2.6.3. Conditioned place preference induced by high fat/carbohydrate rich food

Preference of high fat/carbohydrate content (FCH) food consisting of powdered standard chow, sunflower oil, and potato flour (53:17.5:29.5 % as described in (Hess et al., 2019) was analyzed in naïve ASMtg^{fb} and wt mice (ASMtg^{fb} - $n = 9$ males/10 females; wt - n = 9 males/13 females) in the CPP test as described above (Hess et al., 2019). During the 20 min conditioning trials, 1 g of FCH was placed in a small petri dish in the middle of the CC, while the petri dish was left empty during pseudo-conditioning trials.

2.7. Loss of righting reflex

Naïve ASMtg^{fb} and wt mice (ASMtg^{fb} - $n = 8$ males/14 females; wt - $n = 16$ males/7 females) were administered with ketamine in the doses of 1, 10, 20, 50, 100 and 200 mg/kg (i.p.) in saline (10 ml/kg) to induce a loss of the righting reflex (LORR), and immediately placed in an empty cage. LORR was observed when the mouse became ataxic and stopped moving for at least 30 s. The animal was then placed on its back. Recovery from alcohol administration was defined as the mouse being able to right itself three times within a minute. A 2 h cut off was used. Time taken for the animal to lose its righting reflex, and time to recovery from alcohol's effect were recorded (Müller et al., 2017).

2.8. RT-qPCR analysis

Taking into account the importance of the serotonergic system for addiction, and particularly alcohol use disorder (Yoshimoto et al., 2012; Müller and Homberg, 2015; (Campbell et al., 2021; Gretler and McClain, 2023; Müller and Homberg, 2015; Tanaka and Watanabe, 2020; Campbell et al., 2021; Gretler and McClain, 2023), we analyzed the effects of forebrain ASM overexpression on 5-HT receptor expression after alcohol drinking in a free-choice model. The brains of alcohol-experienced and control mice of both sexes (ASMtg^{fb} - $n = 6$ males/6 females; wt - n = 6 males/6 females) from the free-access drinking study described above were frozen in dry ice and stored at −80 °C. Subsequently, the nucleus accumbens (Nac) and dorsal hippocampus (DH) were isolated. The expression of 5-HT$_{1a}$, 5-HT$_{2a}$, and 5-HT$_{2c}$ in these brain structures was determined. For this purpose, total RNA was isolated from DH and Nac using the RNA Mini Kit (A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's instructions. The synthesis of the cDNA by reverse transcription from equal amounts of RNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Life Technologies, Waltham, MA, USA). RT-qPCR was performed using the QuantStudio 3 (Applied Biosystems, Foster City, CA, USA) and TaqMan Gene Expression Assays (Applied Biosystems, Waltham, MA, USA; 5-HT$_{1a}$: Assay ID Mm00434106_s1; 5-HT$_{2a}$: Assay ID Mm00555764_m1; 5-HT$_{2c}$: Assay ID Mm00434127_m1). The PCR cycling conditions were as follows: an initial step at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and then 60 °C for 60 s. The relative level of mRNA was assessed using the comparative CT method ($2 - \Delta\Delta Ct$) and normalized to the level of the 18S ribosomal RNA (Mm03928990_g1). All values are expressed as the fold change relative to the group average in the control (WT water group).

2.9. Statistics

All quantitative data were expressed as mean ± SEM. Data were analyzed using two-way ANOVAs for repeated measures where appropriate followed by pre-planned comparisons using Fisher's LSD tests for drinking and receptor expression studies or one-tailed *t*-test for CPP studies. Between-gender comparisons were made using three-way

ANOVA, for repeated measures where appropriate. Correlations were performed using the Pearson test. For single group comparisons of normally distributed data (DID analysis) two-tailed *t*-tests were used. A significance level of $p < 0.05$ was used.

3. Results

3.1. Role of the forebrain ASM overexpression in drug response in male mice

3.1.1. Forebrain ASM overexpression enhances alcohol consumption in a free choice paradigm

In male ASMtg^{fb} mice, no genotype-specific differences were observed in the alcohol consumption (genotype: F(1,16) = 3.404, p = 0.084), while alcohol concentration had a pronounced effect on consumption (F(4,64) = 101.286, $p < 0.001$). No genotype*concentration interaction was observed for this parameter (F(4,64) = 1.355, p = 0.259). Pre-planned comparison revealed significantly enhanced consumption of low concentrations of alcohol by male ASMtg^{fb} mice (2 %, p = 0.043; 4 %, p = 0.012; 8 %, p = 0.012; 12 and 16 %, $p > 0.05$; Fig. 1a). A slight effect of the genotype as well as significant effect of alcohol concentration on alcohol preference were observed (genotype: F(1,16) = 4.327, p = 0.054; concentration: F(4,64) = 12.841, p < 0.001), while no interaction effect was found (F(4,64) = 1.371, p = 0.254). Pre-planned comparison showed enhanced preference of 4 % alcohol only (4 %, p = 0.004; other concentrations, $p > 0.05$; Fig. 1b). The analysis revealed a significant effect of alcohol concentration on water consumption (F(4,64) = 11.247, p < 0.001), but no genotype effect (F(1,16) = 1.145, p = 0.301) or their interaction (F(4,64) = 1.548, p = 0.199). Pre-planned comparisons also did not show genotype-specific differences in water consumption (p > 0.05 for all concentrations; Fig. 1c). Similar, a significant effect of alcohol concentration on total fluid intake (F(4,64) = 2.579, p = 0.046), but no genotype effect (F(1,16) = 2.445, p = 0.137) or their interaction (F(4,64) = 1.270, p = 0.291) were observed. Pre-planned comparisons showed higher fluid consumption at 2 % alcohol (p = 0.028), but not at other concentrations (p > 0.05; Fig. 1d). The alcohol deprivation effect (ADE) was significantly enhanced in male ASMtg^{fb} mice comparing to wt littermates (genotype: F(1,16) = 0.154, p = 0.700), time: F(8,128) = 2.196, p = 0.032; genotype * time interaction: F(8,128) = 2.528, p = 0.020), particularly during the first reinstatement episode. Pre-planned comparisons showed significantly higher alcohol consumption by ASMtg^{fb} mice at the second and third days of the first reinstatement episode (p = 0.020 and p = 0.011; other days p > 0.05; Fig. 1e). Analysis of the taste preference did not reveal genotype differences in the preference of sucrose and avoidance of quinine in all studied doses (p > 0.05; Fig. 1f-g). Altogether, forebrain ASM overexpression in male mice led to an enhanced consumption of alcohol in low and moderate concentrations, as well as an enhanced ADE.

3.1.2. No effects of forebrain ASM overexpression on drinking in the dark (DID) paradigm

In male ASMtg^{fb} mice, no significant differences in the alcohol consumption in the DID paradigm (shown as a mean of the 5 days) were observed ($t = -1.015$, p = 0.326; Fig. 1h).

3.1.3. Forebrain ASM overexpression does not affect alcohol bioavailability

To check whether the genotype-specific differences in alcohol bioavailability might explain the observed differences in alcohol consumption, we have measured changes in blood alcohol concentration (BAC) after a single alcohol injection. An ANOVA revealed a significant effect of time on BAC (F(2,22) = 123.128, $p < 0.001$), but no genotype effect (F(1,11) = 1.014, p = 0.336) or genotype*time interaction (F(2,22) = 1.282, p = 0.297; Fig. 1i). Therefore, the observed enhanced consumption of alcohol by male ASMtg^{fb} mice is not related to the differences in alcohol bioavailability.

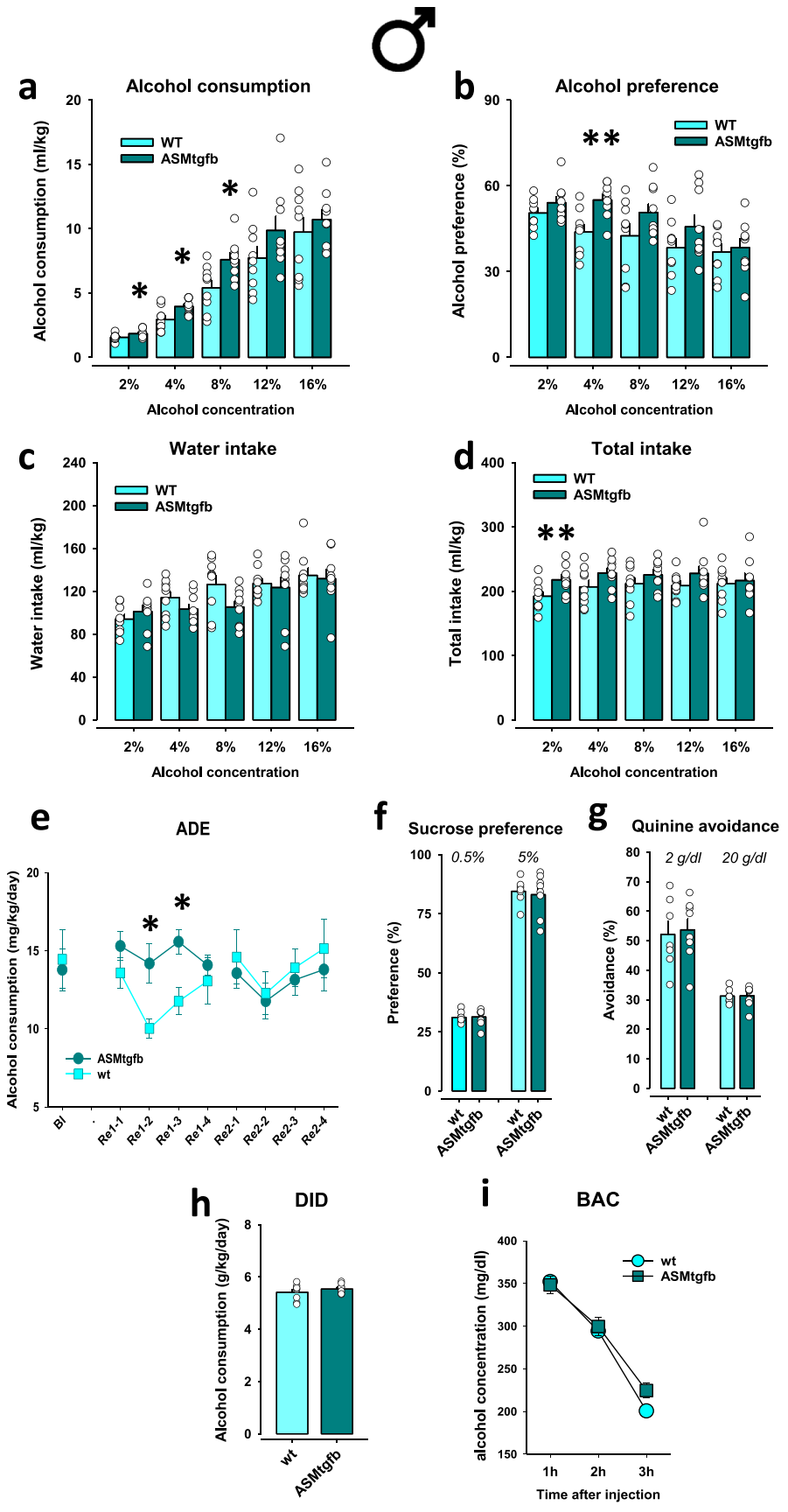
3.1.4. Forebrain ASM overexpression diminishes alcohol-induced place preference

We analyzed the effects of ASM overexpression in the forebrain on the reinforcing effects of alcohol. A CPP was established after a repeated conditioning with alcohol (2 g/kg, i.p.; Factor Test trial F(1,14) = 3.394, p = 0.026) as shown by the time spent in the conditioned compartment (CC) (Fig. 2a). However, two-way ANOVA for repeated measures did not show a genotype effect (F(3,42) = 1.010, p = 0.332) on this parameter or genotype*test trial interaction (F(3,42) = 2.372, p = 0.084; Fig. 2a). Pre-planned comparisons of the time spent in the CC during the test days showed that male ASMtg^{fb} mice spent significantly less time in the CC compared to wt littermates (p = 0.026; Fig. 2a). In the analysis of the time spent in the pseudoconditioning compartment (PC), factors Test trial, Group, and Group*Test trial interaction did not play a significant role ($p > 0.05$; Suppl. Fig. 1a). Male ASMtg^{fb} mice were shown to spent significantly more time in the PC compared to wt mice (p = 0.027). No pronounced genotype-specific differences were found in the alcohol-induced (Trial: F(6,78) = 4.661, $p < 0.001$; Genotype: F(1,13) = 0.035, p = 0.855; Genotype*trial: F(6,78) = 0.402, p = 0.875; Fig. 2f) as well as saline-induced locomotion (Trial: F(6,78) = 25.183, p < 0.001; Genotype: F(1,13) = 0.022, p = 0.884; Genotype*trial: F(6,78) = 1.258, p = 0.287; Suppl. Fig. 1f). Number of entries and distance passed in the CC did not significantly differ between the transgenic and wt animals (two-way ANOVA for repeated measures; Test trial: F(3,39) = 11.333, p < 0.001 and F(3,42) = 7.048, p < 0.001 for entries and distance, respectively; Genotype: F(1,13) = 0.264, p = 0.616 and F(1,14) = 0.451, p = 0.513 for entries and distance, respectively; Genotype*trial: F(3,39) = 0.708, p = 0.553 and F(3,42) = 1.080, p = 0.360 for entries and distance, respectively; pre-planned comparison: $p > 0.05$; Fig. 2b-c). Number of entries and distance in the PC were higher in male ASMtg^{fb} mice compared to wt animals, but reached significant level only for the distance (pre-planned comparison: p = 0.003; Suppl. Fig. 1b-c). Time per entry and distance per entry in the CC were also analyzed by two-way ANOVA for repeated measures, which demonstrated a significant effect of both factors Genotype (F(1,13) = 8.322, p = 0.013 and F(1,13) = 8.849, p = 0.011 for time per entry and distance per entry, respectively) and Test trial (F(3,39) = 6.220, p < 0.001 and F(3,39) = 3.858, p = 0.016 for time per entry and distance per entry, respectively; Fig. 2d-e). Prep-planned comparison showed significantly lower time per entry and distance per entry in the CC during the test trials in male ASMtg^{fb} mice compared to wt littermates (p < 0.001 for both parameters). No genotype-driven differences were observed in the time per entry and distance per entry in the PC (p > 0.05; Suppl. Fig. 1d-e).

These findings suggest that reinforcing effects of alcohol are significantly less pronounced in male ASMtg^{fb} mice compared to wt animals.

3.1.5. Forebrain ASM overexpression determines cocaine-induced place preference

Exposure to cocaine affects peripheral and central ASM activity (Frankowska et al., 2021), but the contribution of the brain ASM in the rewarding effects of cocaine remained unclear. We found that male ASMtg^{fb} mice show diminished development of cocaine CPP. ANOVA did not reveal significant effects of the factor Genotype on the time spent in CC and PC (F(1,17) = 0.012, p = 0.916 and F(1,17) = 0.417, p = 0.527, respectively), while the analysis of the factor Test Trial showed the development of cocaine-induced CPP evaluated by the time spent in the CC (F(3,51) = 4.467, p = 0.007). Moreover, pre-planned comparison showed that male ASMtg^{fb} mice spent significantly less time in the CC (p = 0.016) and more time in the PC (p = 0.002) compared to wt animals (Fig. 3a; Suppl. Fig. 2a). Neither ANOVA, nor pre-planned comparison revealed the genotype-specific differences in the number of entries in the CC and PC as well as distance in the CC ($p > 0.05$; Fig. 3b; Suppl. Fig. 2b-c). Distance passed in the PC was higher in male ASMtg^{fb} mice at the baseline (pre-planned comparison: p = 0.036) as well as during testing (pre-planned comparison: p = 0.003; Suppl. Fig. 2c). In the analysis of the time per entry in the CC and PC, factors Test trial, Genotype, and



(caption on next page)

Fig. 1. Effects of forebrain ASM overexpression on alcohol drinking in male mice. Alcohol consumption (a) and alcohol preference (b) in a free-choice voluntary drinking model were enhanced in male ASMtg^{fb} mice (n = 10 ASMtg^{fb}/9 wt). Water intake (c) did not differ between the genotypes, while total fluid consumption (d) was higher in ASMtg^{fb} mice only at 2 % alcohol. The alcohol deprivation effect (e) was significantly enhanced in male ASMtg^{fb} mice comparing to wt mice. Preference of sucrose (f) and avoidance of quinine (g) did not differ between the genotypes. Forebrain ASM did not affect alcohol consumption in the drinking in the dark (DID, h) model (n = 9 ASMtg^{fb}/8 wt). Blood alcohol availability was similar in male ASMtg^{fb} and wt mice (n = 8 ASMtg^{fb}/5 wt; i). Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using Fisher's LSD tests (a-g, i) or two-tailed t-test (h). (*p < 0.05, **p < 0.01 ASMtg^{fb} vs wt).

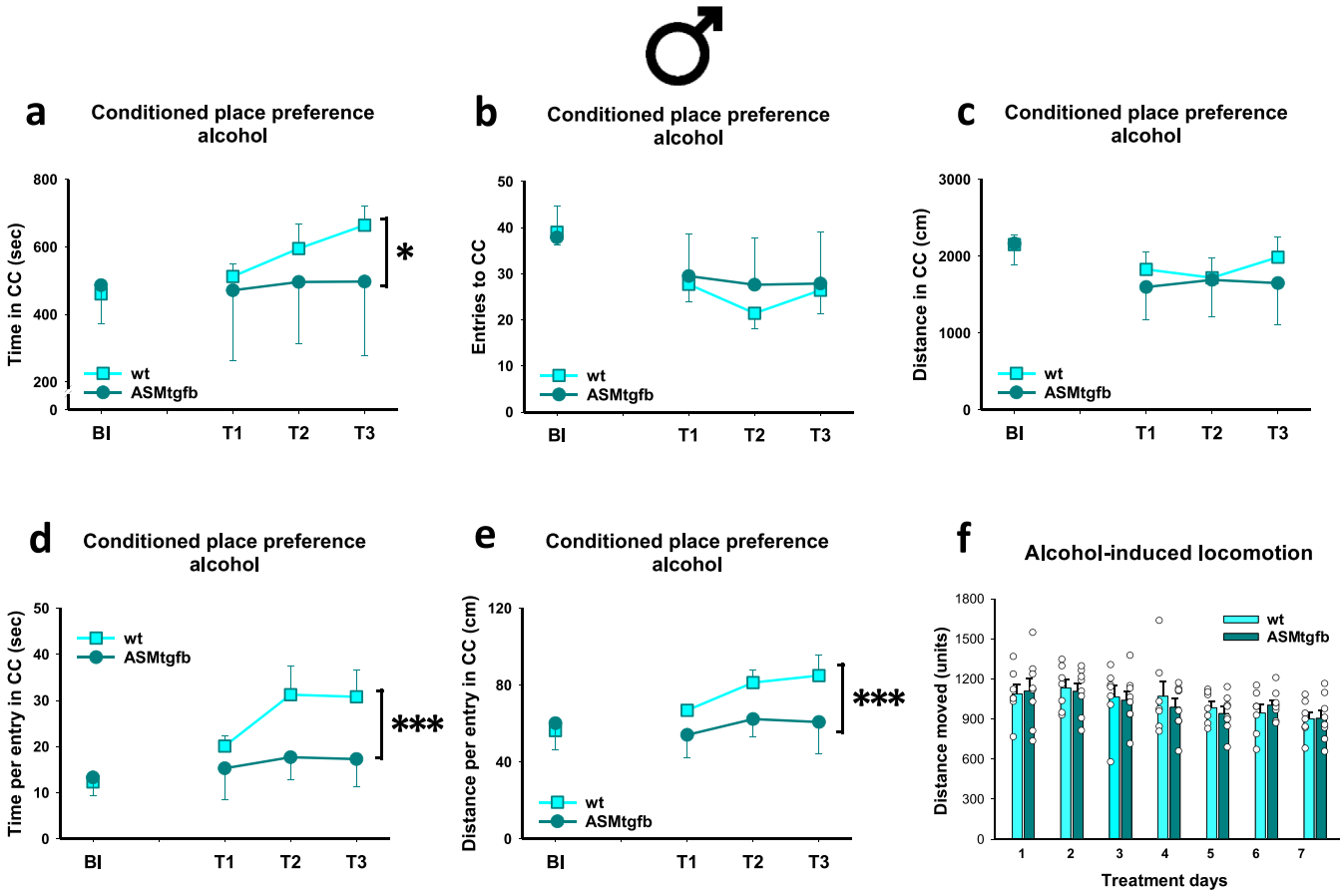


Fig. 2. Forebrain ASM overexpression diminished alcohol-induced place preference in males. Time in the CC (a), time per entry (d) and distance per entry (e) were lower in male ASMtg^{fb} mice compared to wt animals, while number of entries to CC (b) and distance passed in CC (c) did not differ. No genotype-driven differences in alcohol-induced locomotion (f) were found (n = 7 ASMtg^{fb}/9 wt). Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using one-tailed t-test (*p < 0.05, ***p < 0.001 ASMtg^{fb} vs wt).

Genotype*Test trial interaction did not play a significant role (two-way ANOVA for repeated measures), while pre-planned comparison revealed a decrease in this parameter in the CC in ASMtg^{fb} mice at the baseline (p = 0.025) and during testing (p = 0.010; Fig. 3d; Suppl. Fig. 2d). An ANOVA revealed significant effects of the factors Test trial and Genotype on the distance per entry in the CC (F(3,48) = 5.313, p = 0.003 and F(1,16) = 4.848, p = 0.043, respectively), but not in the PC. Pre-planned comparison confirmed that male ASMtg^{fb} mice passed less distance per entry during the baseline (p = 0.008), but not during test trials (p = 0.087; Fig. 3e; Suppl. Fig. 2e). The genotype-driven differences at the baseline were observed only for the parameters time per entry and distance per entry, while parameters time, entries, and distance in the CC did not differ between genotypes. Therefore, it can be proposed that the initial differences did not significantly affects the cocaine preference. Cocaine-induced locomotion significantly increased during the experiment (factor Test trial: F(6,102) = 32.902, p = 0.001), but had a similar pattern in male ASMtg^{fb} and wt mice (factor Genotype: F(1,17) = 0.289, p = 0.598; factor Genotype*Test trial: F(6,102) = 0.306, p = 0.933; pre-planned comparison: p > 0.05; Fig. 3f). ANOVA for repeated measures showed the same phenotype for the locomotion in the PC (Test trial: F(6,96) = 3.189, p = 0.007; factor Genotype: F(1,16) = 1.891, p = 0.188; factor Genotype*Test trial: F(6,96) = 0.971, p = 0.449; pre-planned comparison: p > 0.05; Suppl. Fig. 2f). These data indicate that the forebrain ASM overexpression reduces cocaine's reinforcing effects in males.

3.1.6. *Forebrain ASM overexpression does not affect amphetamine-induced place preference*

Amphetamines are among the five most commonly used illicit drugs. Amphetamine use and dependence were progressively increasing over the last years (Degenhardt et al., 2018). A successful amphetamine CPP was established, but we did not observe a significant contribution of forebrain ASM in the CPP establishment in males. Furthermore, mice made more entries and had higher locomotion in the CC over all three preference tests compared to the baseline test (two-way ANOVA for repeated measures, factor Test trial: F(3,48) = 4.396, p = 0.008 and F(3,48) = 11.391, p = 0.001, for entries and distance in the CC, respectively; Fig. 4b-c). However, ANOVA did not reveal effects of the factor Genotype on all studied parameters of the CPP test (p > 0.05; Fig. 4). Neither pre-planned comparison showed genotype-specific differences

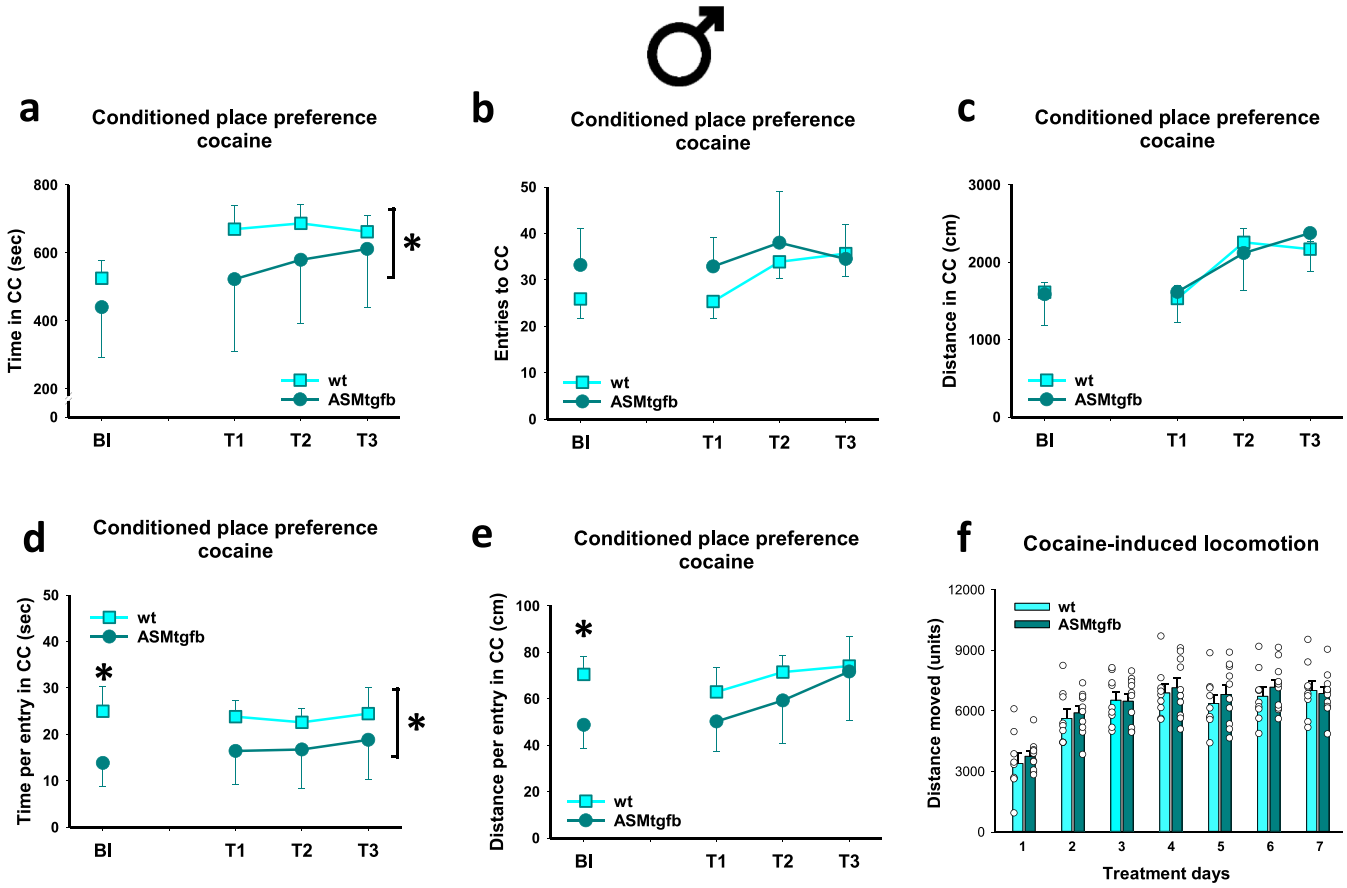


Fig. 3. Forebrain ASM overexpression reduced cocaine-induced place preference in males. Time in the CC (a) and time per entry (d) were lower in male ASMtg^{fb} mice compared to wt animals, while number of entries to CC (b) and distance passed in CC (c), and distance per entry during test trials (e) did not differ (n = 9 ASMtg^{fb}/10 wt). No genotype-driven differences in alcohol-induced locomotion (f) were found. Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using one-tailed t-test (*p < 0.05 ASMtg^{fb} vs wt).

in any of the studied parameters in the CC and PC ($p > 0.05$; Fig. 4, Suppl. Fig. 3). Therefore, ASM can not be considered as an important regulator of the reinforcing effects of amphetamine.

3.1.7. Forebrain ASM overexpression does not mediate ketamine-induced place preference and its sedative properties

The dissociative anaesthetic ketamine is also used as a recreational drug due to its euphoric, dissociative, and hallucinogenic effects. Recent data indicate a rapid growth of the percentage of ketamine users among all drug users (Liu et al., 2016). Here we found that forebrain ASM does not appear to modulate the reinforcing effects of ketamine.

A two-way ANOVA for repeated measures showed the development of ketamine aversion in the CPP, as mice made less entries and had lower locomotion in the CC during the test compared to the baseline (Factor Test trial: $F(1,49) = 26.054$, $p < 0.001$ and $F(1,49) = 14.028$, $p < 0.001$, for entries and distance in the CC, respectively; Fig. 5b-c). However, the factor Genotype did not influence any of the studied parameters in the CC ($p > 0.05$; Fig. 5). In line, pre-planned comparison did not show genotype-specific differences in any of the studied parameters in the CC ($p > 0.05$; Fig. 5). Ketamine-induced locomotion at the treatment day 2 was higher in male ASMtg^{fb} mice compared to wt animals ($p = 0.045$), but this difference was not observed at other days (Fig. 5f). An analysis of the sedative effects of ketamine in different doses (1–200 mg/kg) in the LORR test did not show differences between the genotypes (two-way ANOVA for repeated measures for factor Genotype: $F(1,13) = 1.703$, $p = 0.214$ and $F(1,21) = 0.279$, $p = 0.603$ for LORR duration and latency, respectively; pre-planned comparison: $p > 0.05$ at all time points; Fig. 5g-h). Thus, ASM does not contribute to the reinforcing as well as sedative properties of ketamine.

3.1.8. Forebrain ASM overexpression does not affect reinforcing effects of a natural reinforcer

Food is a natural reinforcer, while FCH rich food has very strong reinforcing properties even in satiated mice (Hess et al., 2019). We have analyzed whether the forebrain ASM overexpression in males modulates the effects of this natural compound with enhanced reinforcing properties. A two-way ANOVA showed the development of food preference in the CPP, as factor Test trial significantly affected the parameters time ($F(3,54) = 5.915$, $p = 0.001$), distance ($F(3,54) = 9.221$, $p < 0.001$), entries $F(3,54) = 8.1326$, $p < 0.001$), and time per entry ($F(3,54) = 8.9195$, $p < 0.0001$), in the CC. An ANOVA did not reveal significant effects of the factor Genotype in any of the studied parameters in the CC (time: $F(1,18) = 0.029$, $p = 0.866$; entries: $F(1,18) = 0.3702$, $p = 0.550$; distance: $F(1,18) = 0.050$, $p = 0.826$; time per entry: $F(1,18) = 0.3466$, $p = 0.563$; distance per entry: $F(1,18) = 1.701$, $p = 0.208$; Fig. 6). The only significant genotype effect on the food-induced locomotion during conditioning ($F(1,18) = 4.411$, $p = 0.05$) was driven by significantly higher locomotor activity of male ASMtg^{fb} mice at the first three days of conditioning ($p < 0.05$; Fig. 6f).Pre-planned comparison also showed higher distance per entry in the CC in male ASMtg^{fb} mice compared to wt animals ($p = 0.036$), but did not reveal other genotype-specific differences in any of the studied parameters in both CC and PC ($p > 0.05$; Fig. 6, Suppl. Fig. 5). Altogether, ASM overexpression in the forebrain does not modulate the reinforcing effects of FCH food in males.

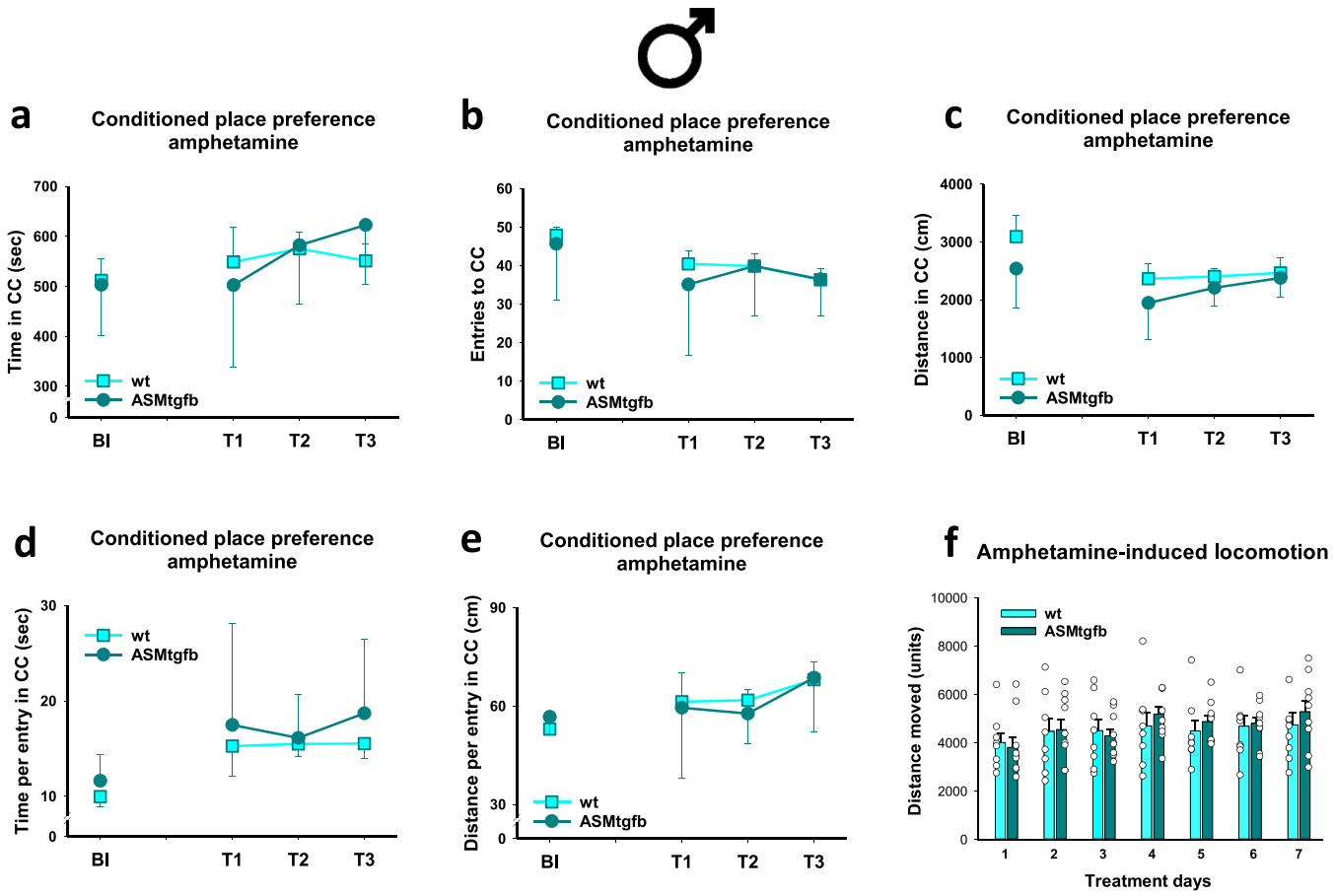


Fig. 4. Forebrain ASM overexpression does not affect amphetamine-induced place preference in males. No genotype-driven differences were observed in the time in the CC (a), entries to the CC (b), distance in the CC (c), time per entry (d), and distance per entry (e), as well as amphetamine-induced locomotion during conditioned trials (f) were registered (n = 9 ASMtg^{fb}/9 wt). Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using one-tailed t-test.

3.1.9. 5-HT$_{2c}$ receptor expression is associated with the drinking phenotype of ASMtg^{fb} mice

A strong interaction between the serotonergic and ceramide systems was shown previously (Müller et al., 2017; Kalinichenko et al., 2024). Here we asked if the changes in the expression of 5-HT receptors in the DH and Nac might mediate the enhanced alcohol preference and consumption observed in the drinking paradigms and CPP test. No genotype-associated differences in the expression of any of the studied receptors were observed in DH or Nac of mice consuming only water ($p > 0.05$; Fig. 7). We observed pronounced effects of alcohol consumption in the free-choice paradigm on the expression of 5-HT$_{2c}$ receptors in both structures. In the Nac, alcohol reduced 5-HT$_{2c}$ receptor expression in male ASMtg^{fb} mice, but did not affect it in wt littermates. An ANOVA revealed a slight treatment effect on the receptor expression ($F(1,24) = 4.109$, $p = 0.056$), but did not show genotype effect ($F(1,24) = 0.711$, $p = 0.409$) or genotype*treatment interaction ($F(1,24) = 1.934$, $p = 0.180$). Pre-planned comparison indicated a significant decrease in 5-HT$_{2c}$ receptor expression in ASMtg^{fb} mice consuming alcohol comparing to water drinking transgenic mice ($p = 0.025$; Fig. 7c). This effect was not observed in wt mice ($p > 0.05$). Similar, alcohol induced a decrease in 5-HT$_{2c}$ receptor in the DH, which was, however, not genotype-specific. An ANOVA showed treatment and genotype effects on the receptor expression ($F(1,24) = 20.683$, $p < 0.001$ and $F(1,24) = 4.673$, $p = 0.043$, respectively), but no genotype*treatment interaction ($F(1,24) = 0.463$, $p = 0.504$). Pre-planned comparison showed an alcohol-induced decrease in the 5-HT$_{2c}$ receptor both in wt and ASMtg^{fb} male mice ($p = 0.001$ and $p = 0.013$, respectively; Fig. 7f). However, self-administration of alcohol did not induce any changes in the expression of 5-HT$_{1a}$ or 5-HT$_{2a}$ receptors in both studied structures of ASMtg^{fb} and wt mice (Fig. 7a, b, d, e). Correlation analysis revealed a significant correlation between the consumption of 16 % alcohol during the initiation of drinking and the expression of 5-HT$_{2a}$ receptors in the DH of wt, but not ASMtg^{fb} male mice ($r = -0.960$, $p = 0.002$; Suppl. Fig. 6a). In turn, 16 % alcohol consumption negatively correlated with the 5-HT$_{2c}$ receptors in the DH of ASMtg^{fb} male mice ($r = -0.894$, $p = 0.016$; Suppl. Fig. 6b). The analysis of interactions between 16 % alcohol during the reinstatement and 5-HT receptor expression showed significant negative correlations for the 5-HT$_{2a}$ receptor expression in the DH and 5-HT$_{2c}$ receptor expression in the Nac of wt animals ($r = -0.881$, $p = 0.020$ and $r = -0.933$, $p = 0.006$, respectively; Suppl. Fig. 6c-d). Only a slight positive correlation between 16 % alcohol during the reinstatement and 5-HT$_{1a}$ receptor expression in the DH was found in ASMtg^{fb} male mice ($r = 0.804$, $p = 0.054$; Suppl. Fig. 6a). Altogether, these data suggest that the serotonergic system contributes to the observed differences in the drinking phenotype induced by the forebrain ASM overexpression.

3.2. Role of the forebrain ASM overexpression in drug response in female mice

3.2.1. Forebrain ASM overexpression does not affects alcohol consumption in a free-choice paradigm

In female ASMtg^{fb} mice, neither ANOVA, nor pre-planned comparison showed genotype-specific differences in the alcohol consumption (genotype: $F(1,11) = 0.004$, $p = 0.954$; concentration: $F(4,44) = 119.372$, $p < 0.001$; genotype*concentration: $F(4,44) = 0.582$, $p = 0.677$; pre-planned comparison: $p > 0.05$; Fig. 8a) or alcohol preference

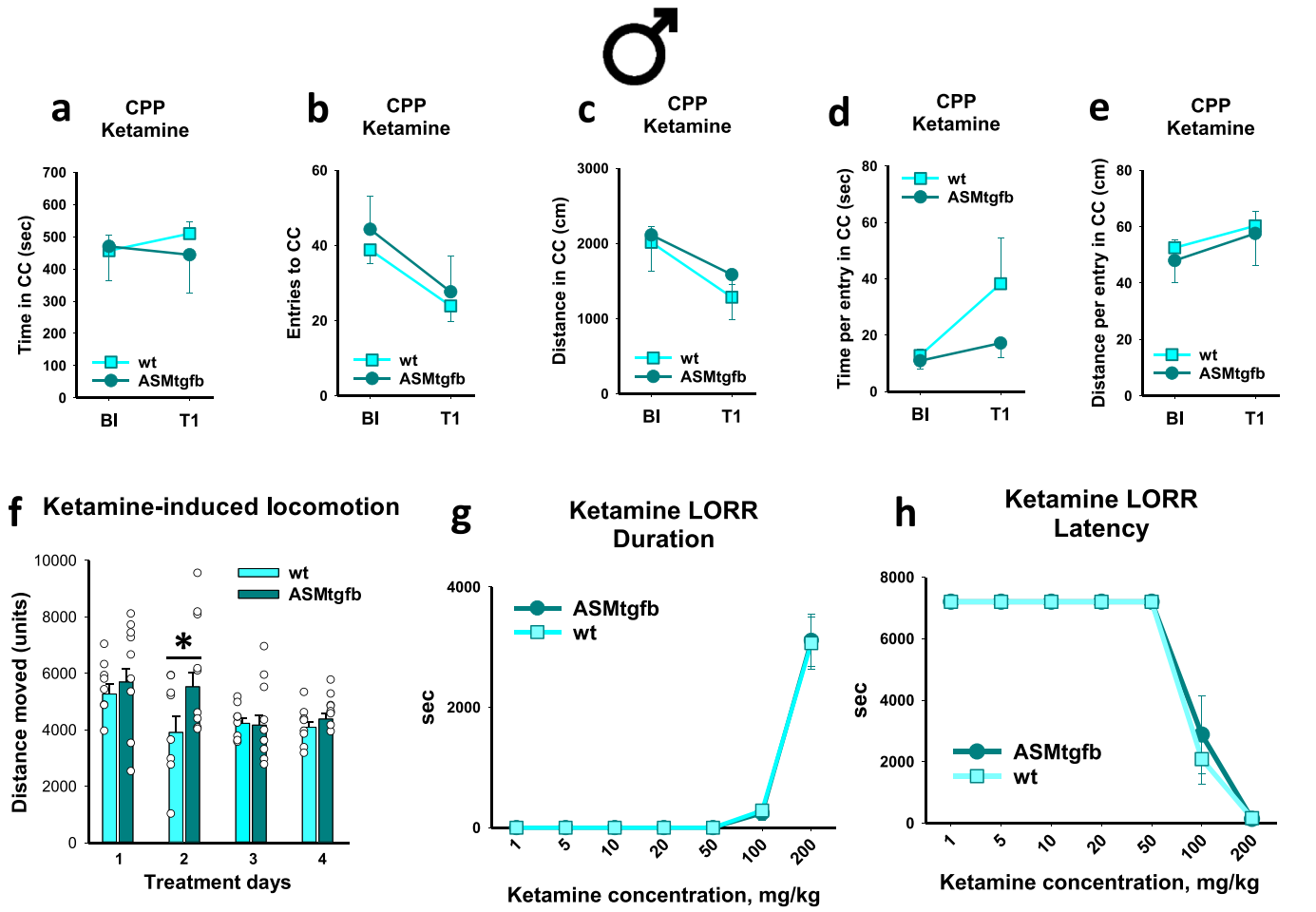


Fig. 5. Forebrain ASM did not mediate ketamine-induced place preference and its sedative properties in males. No genotype-driven differences were observed in the time in the CC (a), entries to the CC (b), distance in the CC (c), time per entry (d), and distance per entry (e) were found (n = 14 ASMtg^{fb}/11 wt). Ketamine-induced locomotion (f) was enhanced in male ASMtg^{fb} mice only at the treatment day 2. Sedative effects of ketamine were not affected by ASM overexpression as shown by duration (g) and latency (h) of loss of righting reflex (LORR) after ketamine administration (n = 8 ASMtg^{fb}/16 wt). Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using one-tailed t-test (*p < 0.05 ASMtg^{fb} vs wt).

(genotype: F(1,11) = 1.317, p = 0.275; concentration: F(4,44) = 2.543, p = 0.053; genotype*concentration: F(4,44) = 0.722, p = 0.582; pre-planned comparison: p > 0.05; Fig. 8b). On the contrary, ANOVA for repeated measurements revealed differences in the water consumption (genotype: F(1,11) = 3.775, p = 0.078; concentration: F(4,44) = 7.508, p = 0.002; genotype*concentration: F(4,44) = 2.882, p = 0.033) as well as total fluid consumption (genotype: F(1,11) = 7.541, p = 0.019; concentration: F(4,44) = 12.340, p < 0.001; genotype*concentration: F (4,44) = 7.372, p < 0.001) in female mice. Female ASMtg^{fb} mice drunk significantly less water when high concentrations of alcohol were presented (12 %, p < 0.001; 16 %, p = 0.002; Fig. 8c). Total intake was also reduced in female ASMtg^{fb} mice compared to wt animals at 12 % alcohol (p = 0.043; Fig. 8d). The alcohol deprivation effect (ADE) was slightly diminished in female ASMtg^{fb} mice comparing to wt littermates, although this difference did not reach the level of statistical significance (genotype effect on alcohol consumption: F(1,14) = 2.125, p = 0.167, time: F(8,112) = 2.499, p = 0.016; genotype*time interaction: F(8,112) = 0.705, p = 0.686; pre-planned comparison: p > 0.05 at all time points; Fig. 8e). Analysis of the taste preference did not show genotype-driven differences in the preference of sucrose and avoidance of quinine at all studied doses (p > 0.05; Fig. 8f-g).

Altogether, forebrain ASM overexpression in female mice was not associated with pronounced changes in alcohol consumption in a free-choice paradigm.

3.2.2. *Forebrain ASM overexpression enhances alcohol consumption in a drinking in the dark (DID) paradigm*

In the DID paradigm, female ASMtg^{fb} mice were characterized by higher alcohol consumption (shown as a mean of the 5 test days) comparing to wt littermates (t = −2.412, p = 0.028; Fig. 8h).

3.2.3. *ASM overexpression does not contribute to reinforcing effects of alcohol*

We analyzed the effects of ASM overexpression in the forebrain on the reinforcing effects of alcohol in females. A CPP was established after a repeated conditioning with alcohol (2 g/kg, i.p.) as confirmed by the time spent and entries in the CC (Factor Test trial: F(3,72) = 3.170, p = 0.029 and F(3,72) = 9.237, p < 0.001, respectively; Fig. 9a). However, two-way ANOVA for repeated measurements did not observe a genotype effects or genotype*test trial interaction in any of the studied parameters (p > 0.05). Pre-planned comparison revealed lower distance per entry in the CC during test trials in female ASMtg^{fb} mice compared to wt animals (p = 0.023; Fig. 9e), but no genotype-driven differences in other studied parameters in the CC and PC. Alcohol- and saline-induced locomotion during the conditioning days also did not differ between ASMtg^{fb} and wt animals (p > 0.05; Fig. 8f; Suppl. Fig. 7f). Therefore, forebrain ASM overexpression in females did not contribute to the development of reinforcing effects of alcohol.

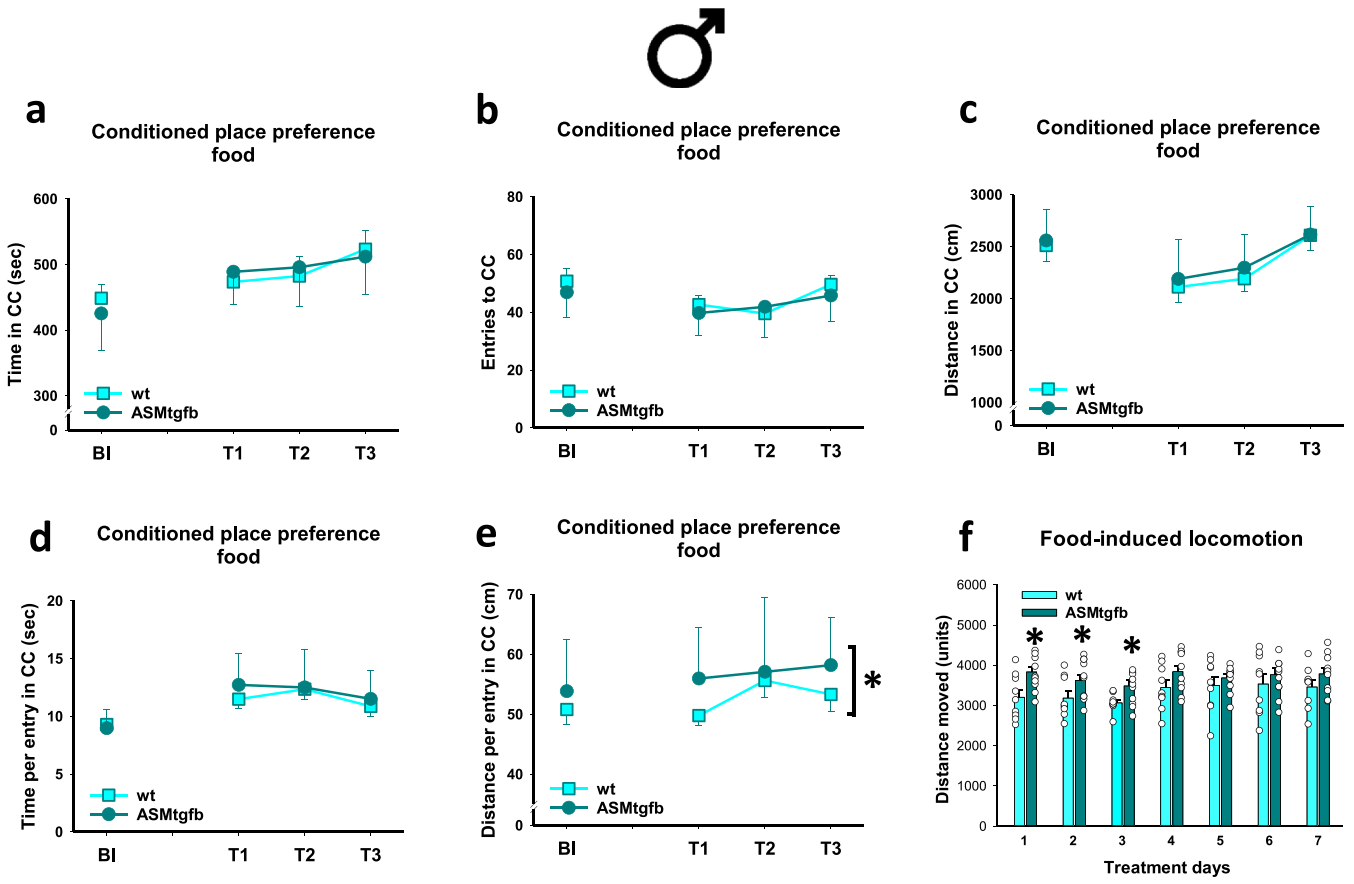


Fig. 6. Forebrain ASM overexpression does not affect reinforcing effects of FCH rich food in males. No genotype-driven differences were observed in the time in the CC (a), entries to the CC (b), distance in the CC (c), time per entry (d; n = 9 ASMtg^{fb}/9 wt). Distance per entry (e) was higher in male ASMtg^{fb} mice compared to wt littermates. Food-induced locomotion (f) was enhanced at conditioning days 1–3 mice with forebrain ASM overexpression. Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using one-tailed t-test (*p < 0.05 ASMtg^{fb} vs wt).

3.2.4. Forebrain ASM overexpression determines amphetamine-induced place preference

Analysis of the amphetamine-induced CPP revealed a significant contribution of forebrain ASM in the CPP establishment in females. A two-way ANOVA for repeated measurements did not show the effect of genotype on the time spent in the CC ($F(1,10) = 3.935$, $p = 0.075$; Fig. 10a), entries to the CC ($F(1,10) = 0.013$, $p = 0.912$; Fig. 10b) or distance passed in the CC ($F(1,10) = 0.025$, $p = 0.878$; Fig. 10c). The factor Test trial had a significant effect on the distance passed in the CC ($F(3,30) = 3.576$, $p = 0.025$), but not other studied parameters. However, the genotype*test trial interaction had a significant effect on the time in the CC ($F(3,30) = 3.029$, $p = 0.045$), entries to the CC ($F(3,30) = 3.214$, $p = 0.037$), and distance passed in the CC ($F(3,30) = 3.891$, $p = 0.018$), but not time per entry or distance per entry ($p > 0.05$; Fig. 10d-e).

Pre-planned comparison showed higher time and entries in the CC during the test trials by female ASMtg^{fb} mice compared to wt animals ($p = 0.004$ and $p = 0.044$; Fig. 10a-b), while wt littermates passed bigger distance per entry in the CC ($p = 0.026$). No genotype-driven differences in all studied parameters in the PC were observed ($p > 0.05$; Suppl. Fig. 8). Therefore, ASM overexpression in the forebrain of females affects the establishment of amphetamine-induced CPP and contributes to its reinforcing effects.

3.2.5. Forebrain ASM overexpression does not determine reinforcing and sedative properties of ketamine

In female mice, the forebrain ASM does not modulate the reinforcing effects of ketamine as shown in the CPP. A two-way ANOVA for repeated measures showed the development of ketamine aversion, as female mice made less entries and had lower locomotion in the CC during the test compared to the baseline (Test trial: $F(1,41) = 41.855$, $p < 0.001$ and $F(1,41) = 42.111$, $p < 0.001$, for entries and distance in the CC, respectively; Fig. 11b-c). The factor Genotype did not influence any of the studied parameters in the CC ($p > 0.05$), and no genotype*test trial interaction was observed ($p > 0.05$). Pre-planned comparison also did not reveal genotype-specific differences in any of the studied parameters in the CC and PC ($p > 0.05$; Fig. 11, Suppl. Fig. 9). Ketamine- and saline-induced locomotion did not differ between female ASMtg^{fb} and wt mice ($p > 0.05$; Fig. 11f, Suppl. Fig. 9f). The analysis of the sedative effects of ketamine in the doses of 1–200 mg/kg in the LORR test also did not reveal any genotype-specific differences (two-way ANOVA for repeated measures for factor Genotype: $F(1,19) = 0.001$, $p = 0.973$ and $F(1,19) = 0.135$, $p = 0.718$ for LORR duration and latency, respectively; pre-planned comparison: $p > 0.05$ at all time points; Fig. 11g-h). Altogether, forebrain ASM in females is not shown to mediate reinforcing and sedative properties of ketamine.

3.2.6. Reinforcing effects of a natural reinforcer are not mediated by forebrain ASM overexpression

Here we found that forebrain ASM overexpression does not play a crucial role in the reinforcing effects FCH enriched food (Hess et al., 2019), in females. An ANOVA did not yield significant effects of the factor Genotype in any of the studied parameters in the CC (time: $F(1,13) = 2.022$, $p = 0.179$; entries: $F(1,13) = 0.223$, $p = 0.645$; distance: $F(1,13) = 1.167$, $p = 0.299$; time per entry: $F(1,13) = 1.711$, $p = 0.213$; distance per entry: $F(1,13) = 2.558$, $p = 0.134$; Fig. 12a-e). Significant

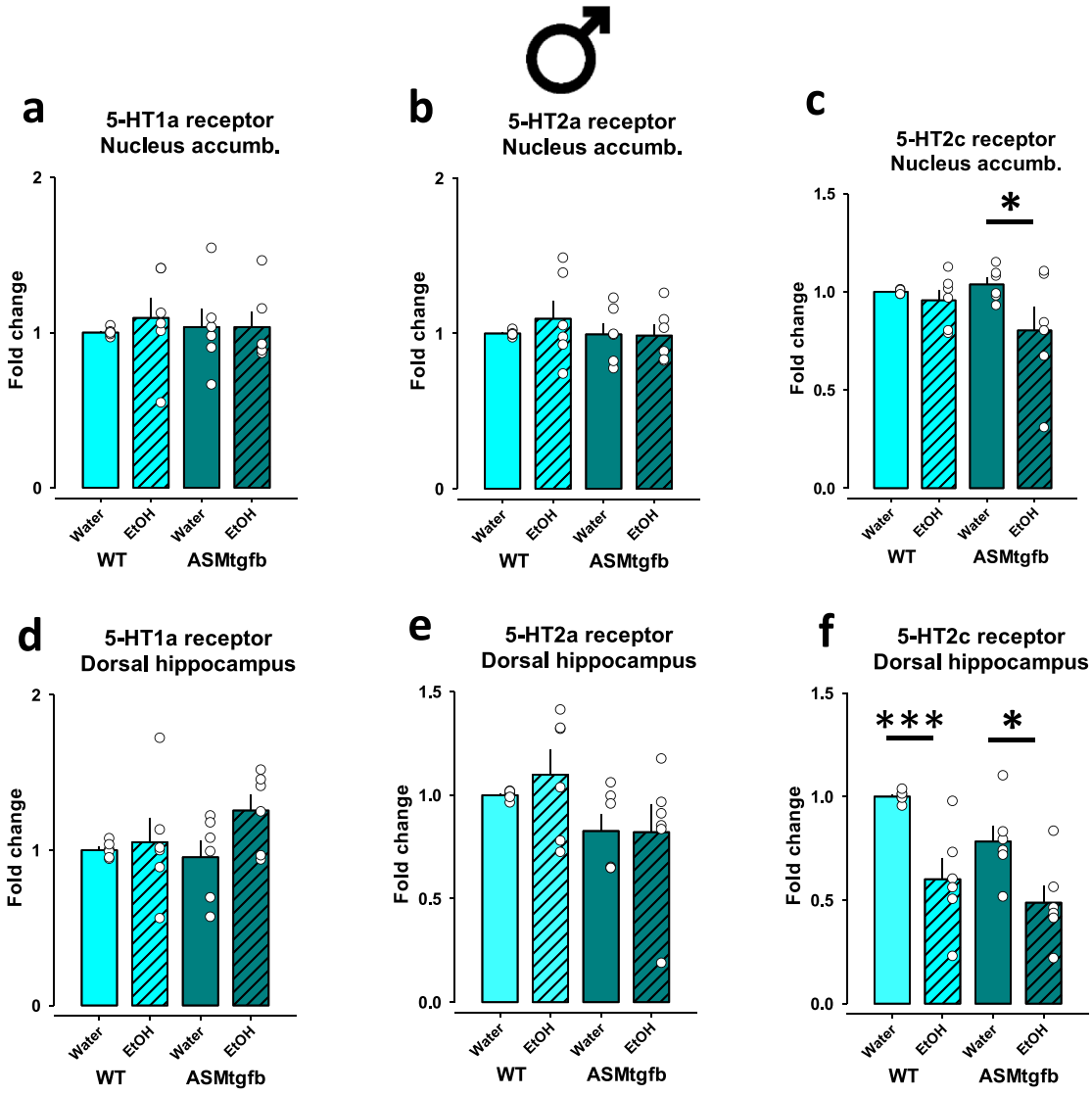


Fig. 7. Forebrain ASM overexpression affects serotonin receptor expression in male mice. No genotype- or treatment-induced changes in the expression of 5-HT_{1a} and 5-HT_{2a} receptors in the nucleus accumbens (a-b) and dorsal hippocampus (d-e) were observed ($n = 6$ in each group). The expression of 5-HT_{2c} receptor was reduced in the nucleus accumbens of male $ASMtg^{fb}$ mice, but not wt mice after alcohol drinking (c). In the dorsal hippocampus, 5-HT_{2c} receptor expression was reduced in alcohol drinking $ASMtg^{fb}$ and wt mice (f). Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using Fisher's LSD tests (*p < 0.05, ***$p < 0.001$ $ASMtg^{fb}$ vs wt).

effects of the factor Test trial on the time spent in the CC ($F(3,39) = 4.168$, $p = 0.012$) and distance moved in the CC ($F(3,39) = 12.960$, $p < 0.0001$) indicated the successful development of FCH rich food-induced CPP (Fig. 12a,c). Pre-planned comparison did not reveal significant genotype-induced differences in any of the studied parameters in the CC and PC during the test, trials as well as during conditioning ($p > 0.05$; Fig. 12, Suppl. Fig. 10). The distance per entry passed in the CC by female $ASMtg^{fb}$ animals during the baseline trial was slightly higher than in wt mice ($p = 0.022$; Fig. 12e). However, no baseline differences in other parameters were found. Altogether, forebrain ASM overexpression in females did not contribute to reinforcing effects of the natural reinforcer.

3.2.7. Forebrain ASM overexpression modulates serotonin receptor expression

Free-choice alcohol consumption was associated with pronounced changes in the serotonergic system of female $ASMtg^{fb}$ and wt animals. In the DH, ANOVA revealed no effects of the factor Genotype on the expression of 5-HT_{1a} and 5-HT_{2a} receptors ($F(1,22) = 4.019$, $p = 0.060$ and $F(1,22) = 0.595$, $p = 0.450$ for 5-HT_{1a} and 5-HT_{2a} receptors), while significant effects of the factor Treatment were observed ($F(1,22) = 8.898$, $p = 0.008$ and $F(1,22) = 12.701$, $p = 0.002$). Pre-planned comparison did not reveal any genotype-driven differences in the expression of these receptors in the DH of $ASMtg^{fb}$ and wt mice receiving only water ($p > 0.05$; Fig. 13a-b). Alcohol induced an increase in the expression of 5-HT_{1a} in the DH only in wt animals (Pre-planned comparison: $p = 0.008$; Fig. 13a), resulting in the significantly higher expression of this receptor in wt animals comparing to $ASMtg^{fb}$ littermates drinking alcohol ($p = 0.044$). Free-choice alcohol consumption was followed by a significant increase in the expression of 5-HT_{2a} receptors in the DH of $ASMtg^{fb}$ ($p = 0.003$), and no increase in wt animals ($p > 0.05$; Fig. 13b). For the 5-HT_{2c} receptors, two-way ANOVA did not show significant effects of the factors Genotype or Treatment ($p > 0.05$), while their interaction was significant ($F(1,23) = 12.109$, $p = 0.003$). Pre-planned comparison revealed significant differences in the expression of these receptors in the DH between $ASMtg^{fb}$ and wt mice receiving only water ($p = 0.023$) and mice having choice between alcohol or water ($p = 0.024$; Fig. 13c). Alcohol consumption induced a decrease in the

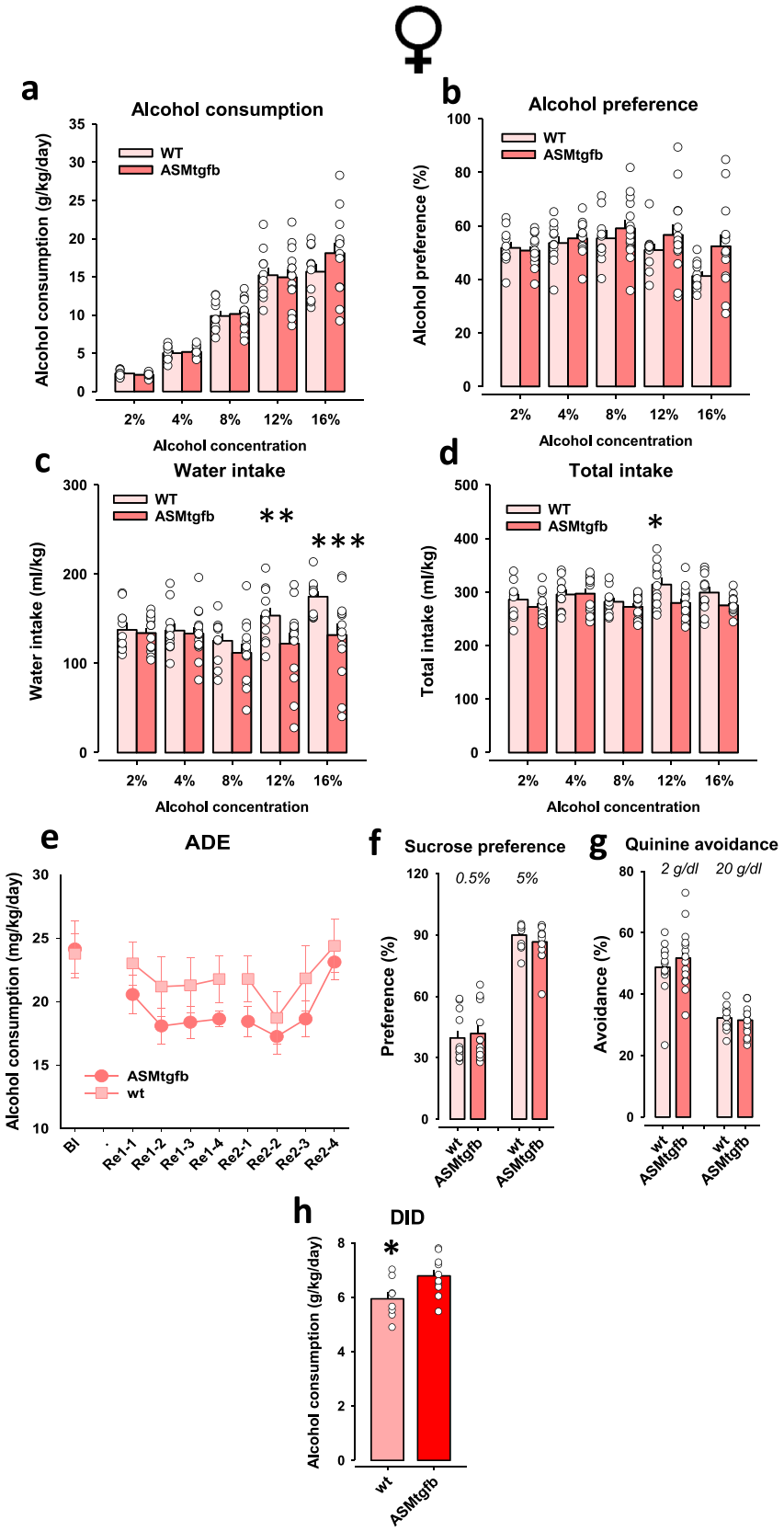


Fig. 8. Effects of forebrain ASM overexpression on alcohol drinking in female mice. Alcohol consumption (a) and alcohol preference (b) in a free-choice voluntary drinking model did not differ in female ASMtg^{fb} and wt mice (n = 14 ASMtg^{fb}/12 wt). Water intake (c) and total fluid consumption (d) was higher in wt animals. Forebrain ASM did not affect the alcohol deprivation effect (e), preference of sucrose (f) and avoidance of quinine (g). Female ASMtg^{fb} drunk more alcohol than wt mice in the DID model ($n = 10$ ASMtg^{fb}/8 wt; h). Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using Fisher's LSD tests (a-g) or two-tailed t-test (h) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ASMtg^{fb} vs wt).

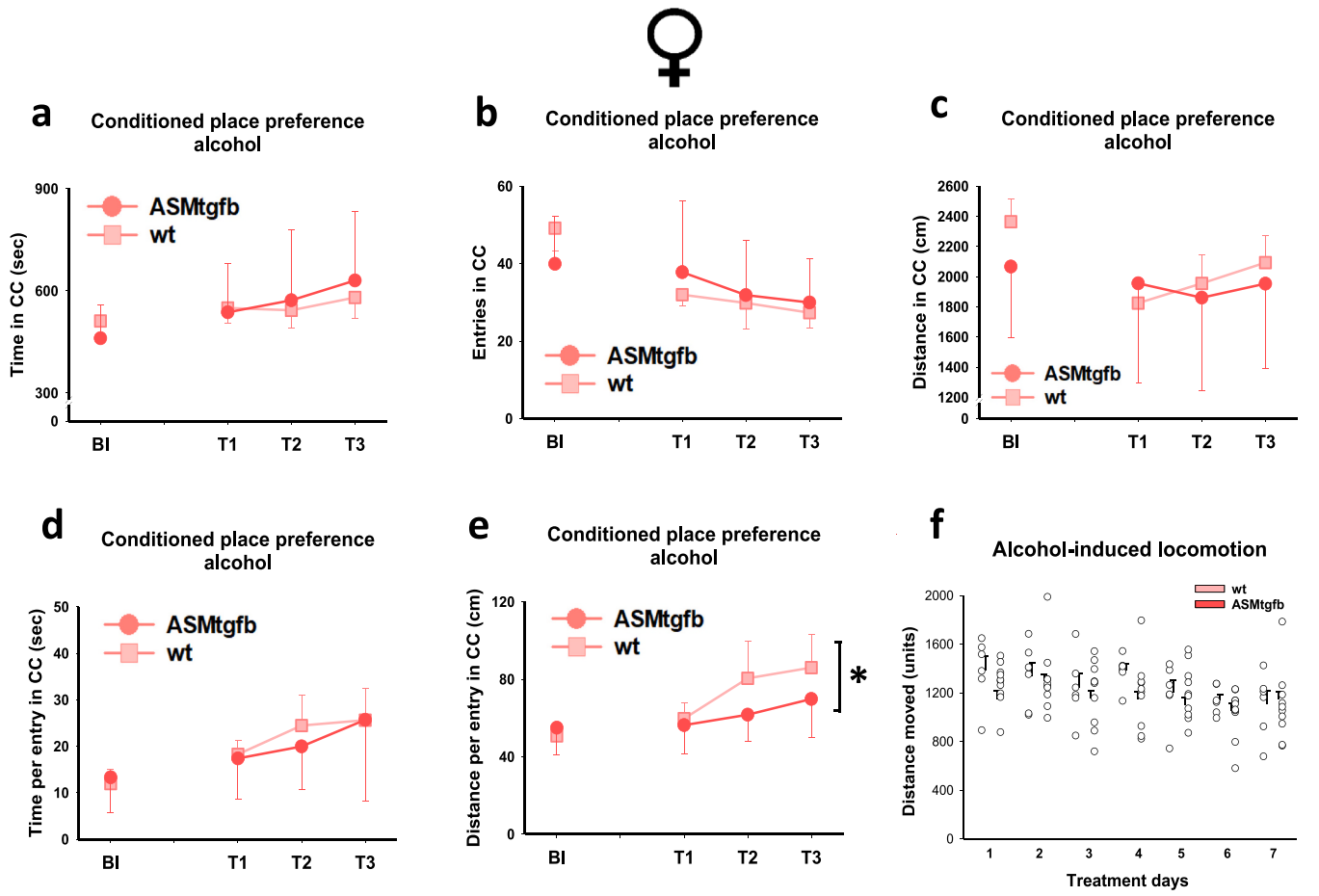


Fig. 9. Forebrain ASM overexpression does not contribute to reinforcing effects of alcohol in females. Time in the CC (a), entries to the CC (b), distance in the CC (c), time per entry (d), and alcohol-induced locomotion (f) did not differ between female ASMtg^{fb} mice and wt animals (n = 6 ASMtg^{fb}/20 wt). Distance per entry (e) was reduced in female ASMtg^{fb} mice compared to wt animals. Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using one-tailed t-test (*p < 0.05 ASMtg^{fb} vs wt).

expression of 5-HT$_{2c}$ receptors in the DH of wt mice ($p = 0.004$), but not ASMtg^{fb} females ($p > 0.05$). In the Nac, no significant changes in the expression of 5-HT$_{1a}$ receptors were found (Genotype: F(1,24) = 0.025, $p = 0.877$; Treatment: F(1,24) = 1.858, $p = 0.188$; Genotype*Treatment: F(1,23) = 1.709, $p = 0.206$; pre-planned comparison: $p > 0.05$; Fig. 13d). Similar patterns of changes in the expression of 5-HT$_{2a}$ and 5-HT$_{2c}$ receptors after the exposure to alcohol were found in the Nac of both ASMtg^{fb} and wt mice. ANOVA showed an effect of the factors Genotype and Treatment on the expression of 5-HT$_{2a}$ receptors (F(1,24) = 4.217, $p = 0.053$ and F(1,23) = 22.051, $p < 0.001$), while their interaction was not significant (F(1,24) = 1.900, $p = 0.183$).

Pre-planned comparison showed a significantly lower expression of these receptors in the Nac of ASMtg^{fb} mice receiving only water ($p = 0.025$; Fig. 13e). The exposure to alcohol reduced the expression of 5-HT$_{2a}$ receptors in the Nac of wt ($p < 0.001$) and ASMtg^{fb} ($p = 0.029$) mice, which eliminated the innate differences between ASMtg^{fb} and wt mice ($p > 0.05$). Two-way ANOVA showed a significant effect of the factor Treatment on the expression of 5-HT$_{2c}$ receptors in the Nac (F(1,24) = 19.717, $p < 0.001$), but the factor Genotype and Genotype*Treatment interaction remained insignificant (F(1,24) = 0.067, $p = 0.798$ and F(1,24) = 0.520, $p = 0.479$). Pre-planned comparison revealed that the exposure to alcohol induced a decrease in the expression of 5-HT$_{2c}$ receptors in the Nac of both wt ($p = 0.016$) and ASMtg^{fb} ($p = 0.002$) mice. However, no genotype-specific differences in this parameter were found ($p > 0.05$; Fig. 13f). Correlation analysis observed only a negative correlation between 16 % alcohol consumption during initiation and 5-HT$_{2c}$ receptor expression in the DH in female ASMtg^{fb} mice ($r = -0.896$, $p = 0.016$; Suppl. Fig. 11), while no other correlations were found. Altogether, pronounced changes in the expression of serotonergic receptors are shown to be induced by free-choice alcohol drinking in female mice. The similarity of patterns of these changes in the majority of the studied receptors in the DH and Nac between wt and ASMtg^{fb} mice is in line with the absence of significant differences in the drinking phenotype.

3.3. Role of the forebrain ASM overexpression in sex-specific differences in alcohol consumption and expression of serotonergic receptors

Addiction is a highly sex-specific psychiatric disorder (Cotto et al., 2010). In our study we analyzed females and males separately, while pronounced sex-specific differences were observed. Particularly, alcohol consumption phenotype differed between males and females. A three-way ANOVA revealed significant sex effects on alcohol consumption (F(1,37) = 41.161, $p < 0.001$), alcohol preference (F(1,37) = 7.688, $p = 0.009$), water consumption (F(1,37) = 7.528, $p = 0.009$), and total fluid consumption (F(1,37) = 101.671, $p < 0.001$) in the model of voluntary drinking. For some of the parameters, such as alcohol preference and water consumption (F(4,148) = 3.030, $p = 0.019$ and F(4,148) = 3.529, $p = 0.009$), the interaction between the factors Sex*Genotype*Alcohol concentration were found to be significant, while for others not ($p > 0.05$; Suppl. Fig. 12). In the model of intermittent drinking (DID), the factor Sex also had strong effects on alcohol consumption (F(1,37) = 22.220, $p < 0.001$), while Sex*Genotype interaction did not reach the level of statistical significance (F(1,35) = 3.606, $p = 0.067$; Suppl.

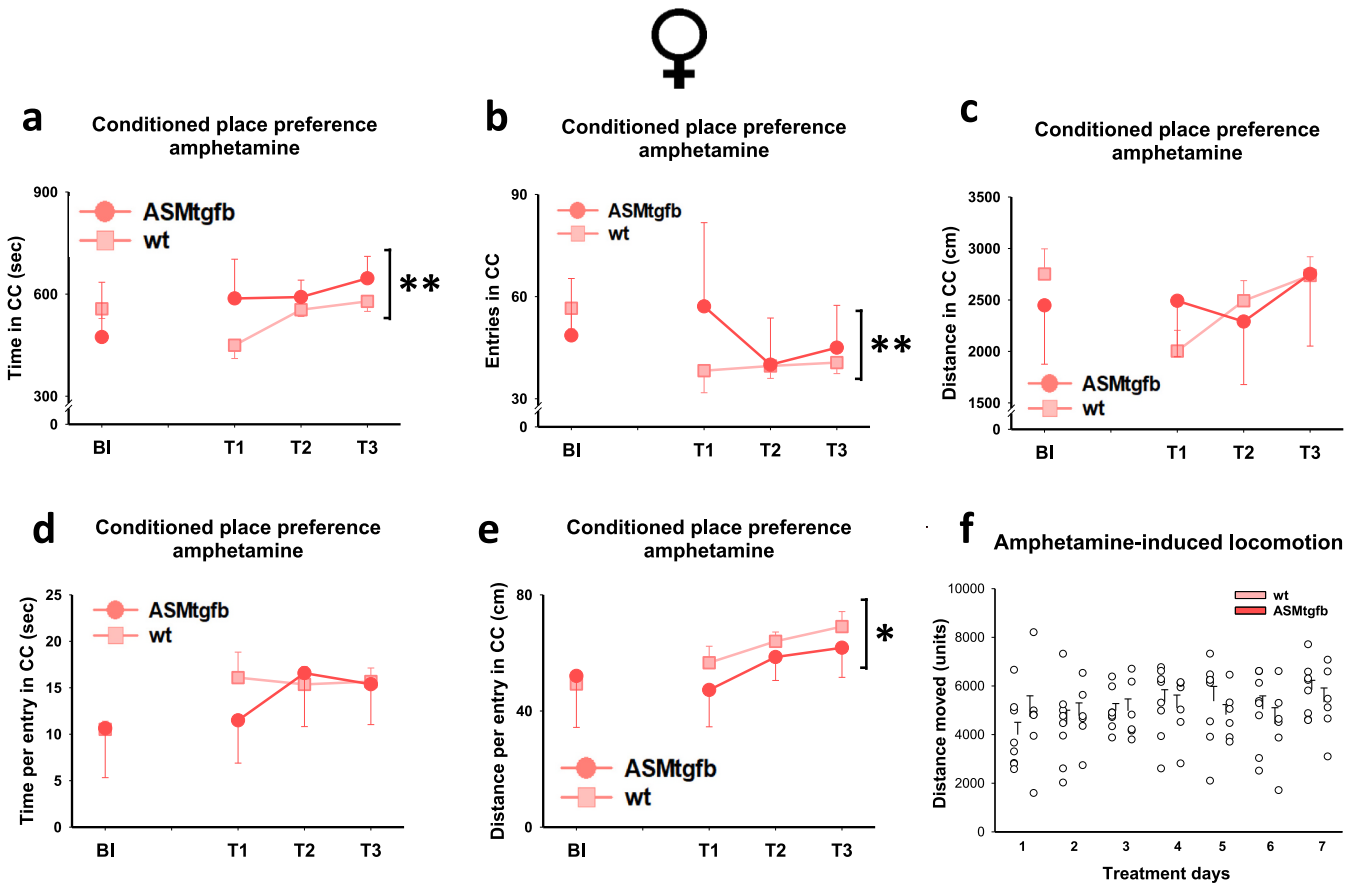


Fig. 10. Forebrain ASM overexpression enhanced amphetamine-induced place preference in males. Time (a) and entries to the CC (b) were enhanced in female ASMtg^{fb} mice compared to wt animals (n = 8 ASMtg^{fb}/8 wt). ASM overexpression did not affect distance passed in the CC (c), time per entry (d) and amphetamine-induced locomotion (f), but reduced distance per entry in the CC (e). Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using one-tailed t-test (*p < 0.05, **p < 0.01 ASMtg^{fb} vs wt).

Fig. 12). An interesting pattern was observed in the serotonergic system: the factor Sex did not have significant influence on the expression of 5-HT$_{1a}$ receptors in the DH or Nac ($F(1,46) = 14.417$, $p = 0.241$ and $F(1,48) = 0.456$, $p = 0.504$). However, it significantly affected the expression of 5-HT$_{2a}$ receptors in the DH and Nac ($F(1,47) = 10.600$, $p = 0.002$ and $F(1,48) = 34.063$, $p < 0.001$). For the 5-HT$_{1c}$ receptors, significant effects of the factor Sex was observed in the DH ($F(1,47) = 8.295$, $p = 0.006$), but not in the Nac ($F(1,48) = 2.002$, $p = 0.165$). The interaction between Sex*Genotype*Treatment was not significant for any of these receptors in any of studied brain structures ($p > 0.05$).

4. Discussion

The ceramide system, and particularly enzymes of the ceramide metabolism, contribute to the development of addiction to certain drugs of abuse, as widely shown in clinical and preclinical studies (Schneider et al., 2017; Kalinichenko et al., 2018, 2021a, 2023). The specific role of the brain sphingolipid system, however, remained elusive. Here we report that enhanced activity of a crucial enzyme of ceramide formation ASM specifically in the murine forebrain mediates the development of drug abuse-related behaviours in a drug- and sex-specific way. In males, the forebrain ASM overexpression is specifically associated with high alcohol consumption in a free-choice paradigm, while the establishment of alcohol and cocaine CPP was diminished. However, this genetic mutation does not affect for the establishment of the conditioned reinforcing effects of amphetamine, ketamine, or a natural reinforcer FCH-enriched food in males (Table 1). On the contrary, a forebrain ASM overexpression in females is related to an enhanced CPP establishment for amphetamine, but not other studied addictive substances. ASM overexpression in females determines alcohol consumption in the DID paradigm, a model of binge-like drinking, but not in a voluntary free-choice paradigm (Table 1). To our knowledge, this study is the first to show the specific involvement of the forebrain ASM to the development of different alcohol drinking phenotypes in a sex-specific way. ASM contributes to the establishment of conditioned reinforcing effects of different types of substances with addictive properties in a sex-specific way.

In this study we observed a specific role of the forebrain ASM overexpression for enhanced alcohol consumption in the free-choice paradigm in males, but not in the in the intermittent access drinking paradigm (DID) mimicking binge drinking of alcohol. These data indicate the importance of ASM for recreational alcohol use, but not for binge alcohol drinking in males. This is in line with the previous studies showing an association between the whole body ASM overexpression and high alcohol consumption in mice with innate depression (Müller et al., 2017; Müller and Kornhuber, 2017). In male ASMtg^{fb} mice, the establishment of CPP for alcohol was diminished as compared to wt animals. It contrasts the previous data showing enhanced alcohol CPP establishment in mice with the whole-body ASM overexpression (Müller et al., 2017). It might be proposed that the exact localisation of ASM is crucial for its contribution to the reinforcing effects of alcohol. However, further research is required to isolate changes of ASM activity in which forebrain structure determines the reinforcing properties of alcohol. Adding on to the drinking phenotype, diminished reinforcing properties of alcohol indicates that the forebrain ASM overexpression in males is not responsible for high-risk alcohol consumption. It might be proposed

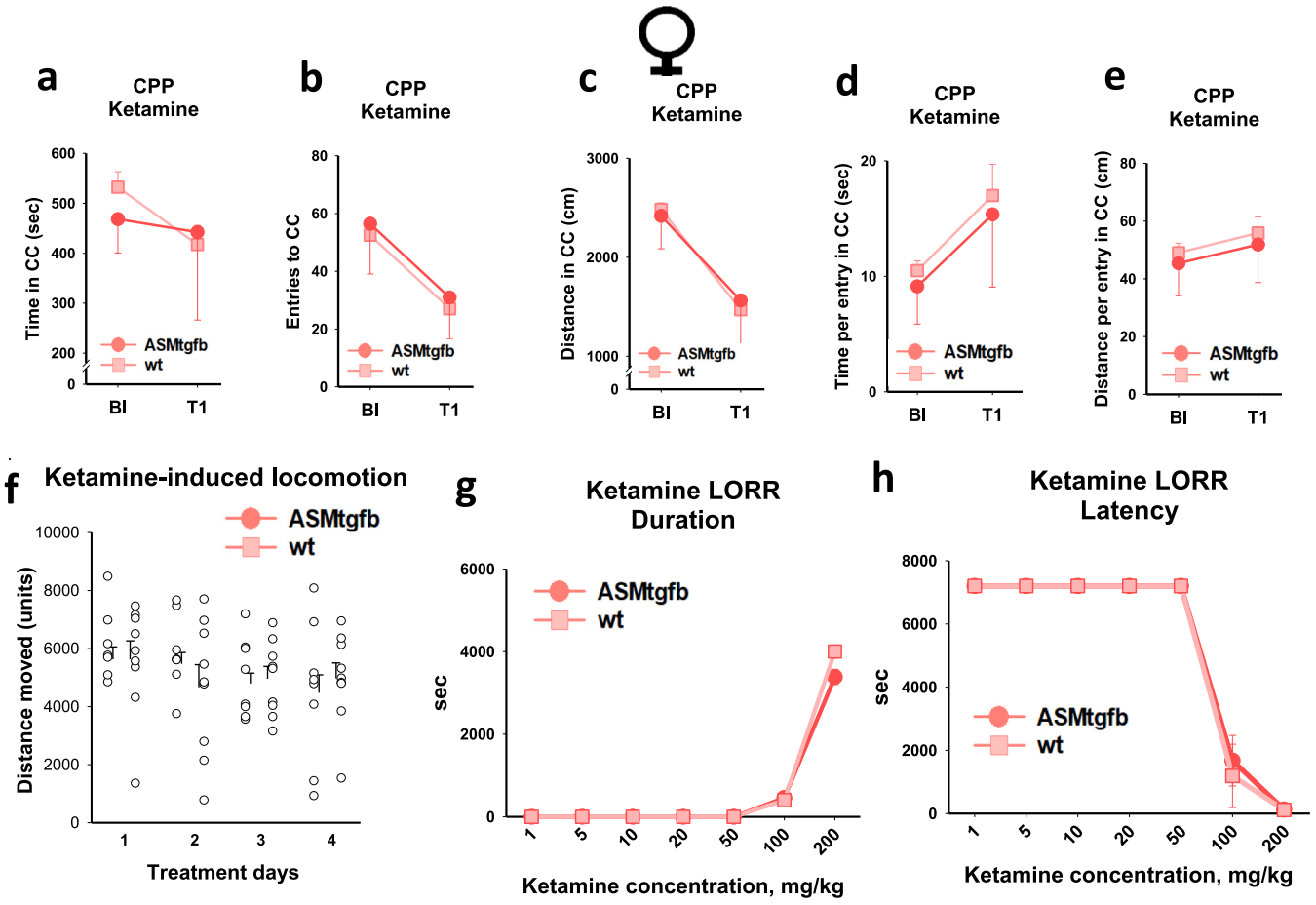


Fig. 11. Forebrain ASM did not mediate ketamine-induced place preference and its sedative properties in females. No genotype-driven differences were observed in the time in the CC (a), entries to the CC (b), distance in the CC (c), time per entry (d), distance per entry (e), and ketamine-induced locomotion (f) were found (n = 9 $ASMtg^{fb}$/12 wt). Sedative effects of ketamine were not affected by ASM overexpression as shown by duration (g) and latency (h) of loss of righting reflex (LORR) after ketamine administration (n = 14 $ASMtg^{fb}$/7 wt). Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using one-tailed t-test.

that male $ASMtg^{fb}$ mice are using alcohol to cope with stress and depression-like behaviour, which were observed previously (Zoicas et al., 2020b), and thus could maintain alcohol drinking at a stable level (Müller, 2020). The observed genotype-driven differences in alcohol consumption might be partially explained by the differences in the functioning of the serotonergic system. Alcohol induced a reduction in the expression of $5\text{-}HT_{2c}$ receptors in the Nac only in males with ASM overexpression. These data are in line with the previous reports showing the specific importance of these serotonergic receptors for alcohol use disorder (Yoshimoto et al., 2012; Tanaka and Watanabe, 2020; Campbell et al., 2021; Gretler and McClain, 2023). Particularly, $5\text{-}HT_{2c}$ receptors in the Nac contribute to the increased alcohol drinking behaviour of C57BL/6 J mice (Yoshimoto et al., 2012). Similar, a $5\text{-}HT_{2c}$ receptor antagonist, SB-242084, increases alcohol consumption (Tomkins et al., 2002; Yoshimoto et al., 2012). It has been recently discussed that the ceramide system directly modulates the functioning of the serotonergic system (Kalinichenko et al., 2024). Therefore, the alcohol consumption pattern observed in $ASMtg^{fb}$ mice might be at least partially determined by the ASM-related changes in the functioning of the serotonergic receptors.

Forebrain ASM overexpression in males is also associated with a reduction of reinforcing properties of cocaine. Previous studies already reported the interaction between cocaine and ASM expression, particularly in the forebrain. The study of Frankowska et al. (2021) revealed that intravenous cocaine self-administration reversibly reduced mRNA expression of *Smpd1*, the gene coding for ASM, specifically in the prefrontal cortex of rats (Frankowska et al., 2021). However, this effect was not limited to the central nervous system. In monkeys, cocaine-induced CPP was associated with a reduction in blood ASM activity (Frankowska et al., 2021). Therefore, it might be proposed that the forebrain ASM is crucial, but not the only mechanism influencing reinforcing properties of cocaine. Altogether, the forebrain ASM might be proposed as a potential target molecule for the development of therapeutic approaches aimed to reduce reinforcing properties of alcohol and cocaine. It should be emphasized that ASM overexpression in the male forebrain did not affect the reinforcing properties of a natural reinforcer, FCH-enriched food, as well as amphetamine and ketamine. Therefore, ASM is probably not a part of a general "addicted brain" (Nestler, 2005; Volkow et al., 2019; Heilig et al., 2021), but is specific for the pathogenesis of addiction to certain drugs of abuse. In males, ASM activity is crucial for susceptibility or resilience to the development of alcohol and cocaine misuse. However, further studies are necessary to isolate how exactly ASM is involved in the pathogenesis of these disorders and how ASM interacts with the currently known molecular mechanisms.

As distinct from males, the forebrain ASM overexpression in female mice did not affect alcohol consumption in the model of free-choice drinking. Considering the enhanced alcohol consumption in mice with the whole-body ASM overexpression (Müller et al., 2017), these data indicate that the forebrain ASM does not contribute to the control of voluntary alcohol consumption. ASM overexpression in other brain structures or peripheral organs probably affects this type of drinking. Interestingly, no pronounced genotype-driven differences in the

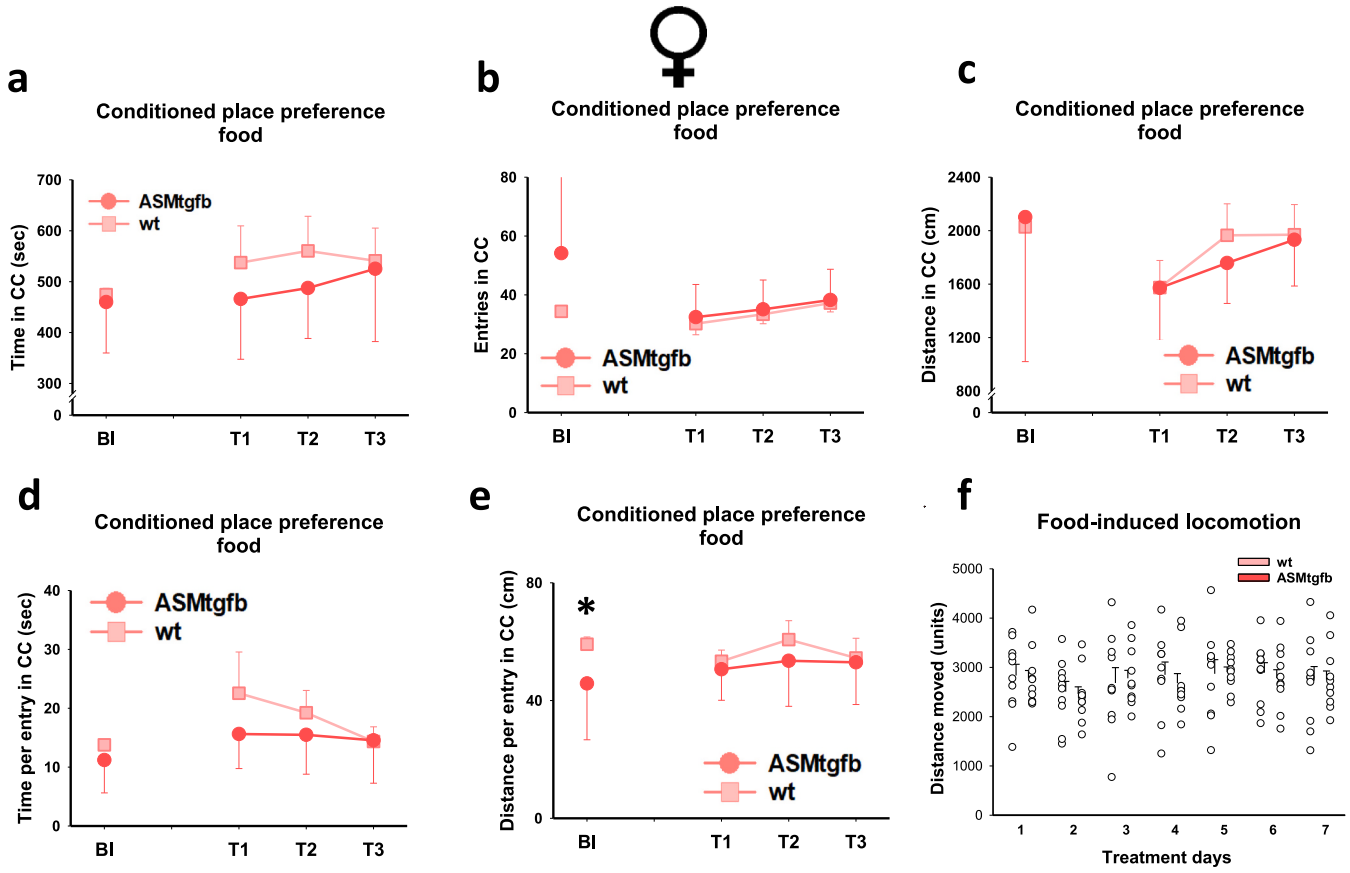


Fig. 12. Forebrain ASM overexpression does not affect reinforcing effects of FCH rich food in females. No genotype-driven differences were observed in the time in the CC (a), entries to the CC (b), distance in the CC (c), time per entry (d) and distance per entry during test trials (n = 10 ASMtg^{fb}/13 wt). Food-induced locomotion (f) was similar in mice with forebrain ASM overexpression and wt littermates. Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using one-tailed t-test.

expression of the serotonergic receptors were observed in alcohol-consuming females. The pattern of alcohol-induced changes in the expression of the majority studied serotonergic receptors was similar in female ASMtg^{fb} and wt mice, which goes in line with absence of strong differences in the drinking phenotype. However, as distinct from males, female ASMtg^{fb} mice were characterized by reduced innate expression of 5-HT$_{2a}$ receptors in the Nac and 5-HT$_{2c}$ receptors in the DH. This is in line with the previous studies showing reduced innate serotonin level in several brain structures of mice with whole-body ASM overexpression (Müller et al., 2017), and probably might determine alterations in other types of behaviour shown previously (Müller et al., 2017; Zoicas et al., 2020a). It should be emphasized that another type of drinking, intermittent access drinking (DID) modelling binge drinking in humans, is shown to be regulated by the forebrain ASM in females. Binge drinking is an important alcohol consumption pattern due to its pronounced social risks, such as high risk injuries, driving accidents, and unwanted pregnancies. Due to fast consumption of high doses of alcohol, binge drinking often results in acute toxicity, particularly neurotoxicity. It is considered as a step to alcohol dependence (Crabbe et al., 2011). Even though there are some molecular mechanisms of binge drinking discovered (Cozzoli et al., 2009, 2016; Gimenez-Gomez et al., 2023), it remains elusive which mechanisms determine the transition from moderate controlled drinking to binge drinking. We propose that ASM expression might be one of these mechanisms, specifically in females. Therefore, deep investigation of ASM involvement in the mechanisms of alcohol consumption are needed as it might be a potential treatment target or can serve as a marker of possible transition from controlled moderate to harmful alcohol consumption pattern.

The analysis of establishment of CPP in female ASMtg^{fb} mice revealed accelerated development of CPP for amphetamine, but not for alcohol, ketamine, or FCH-enriched food. To our knowledge, these are the first data showing the role of ASM for the reinforcing properties of amphetamine. A study of Astarita et al. (2015) revealed that ceramide accumulation is a crucial mechanism determining D-methamphetamine toxicity via cellular senescence and inflammation (Astarita et al., 2015). Self-administration of D-methamphetamine by rats was accompanied by the accumulation of multiple ceramide species in the dorsal striatum and several peripheral organs, probably via the de novo pathway (Astarita et al., 2015). However, this study was performed on male rats and D-methamphetamine, which pharmacological properties differ from those of amphetamine, and thus cannot be compared with our data. Altogether, ASM expression in the forebrain is a female-specific modulator of reinforcing properties of amphetamine, but not of other studied drugs of abuse.

Our study revealed strong sex-specific modulatory effects of the forebrain ASM overexpression on drugs of abuse related behaviour. Comparison of drinking phenotypes of ASMtg^{fb} mice showed pronounced differences between females and males. In particular, wt females consumed significantly more alcohol in a free-choice drinking paradigm than males. However, this was not observed in the DID model. It is well-known that substance use disorders are sex-specific: for example, young males are more likely to be dependent upon marijuana or alcohol, whereas females are more likely to be dependent upon cocaine and psychotherapeutic drugs (Cotto et al., 2010). On another hand, sex-specific differences in the lipid metabolism are also well-known (Zoicas et al., 2020a; Kalinichenko et al., 2021a, 2023). The exact molecular mechanisms of these differences remain unclear, even though several studies indicate the involvement of oestrogens in the

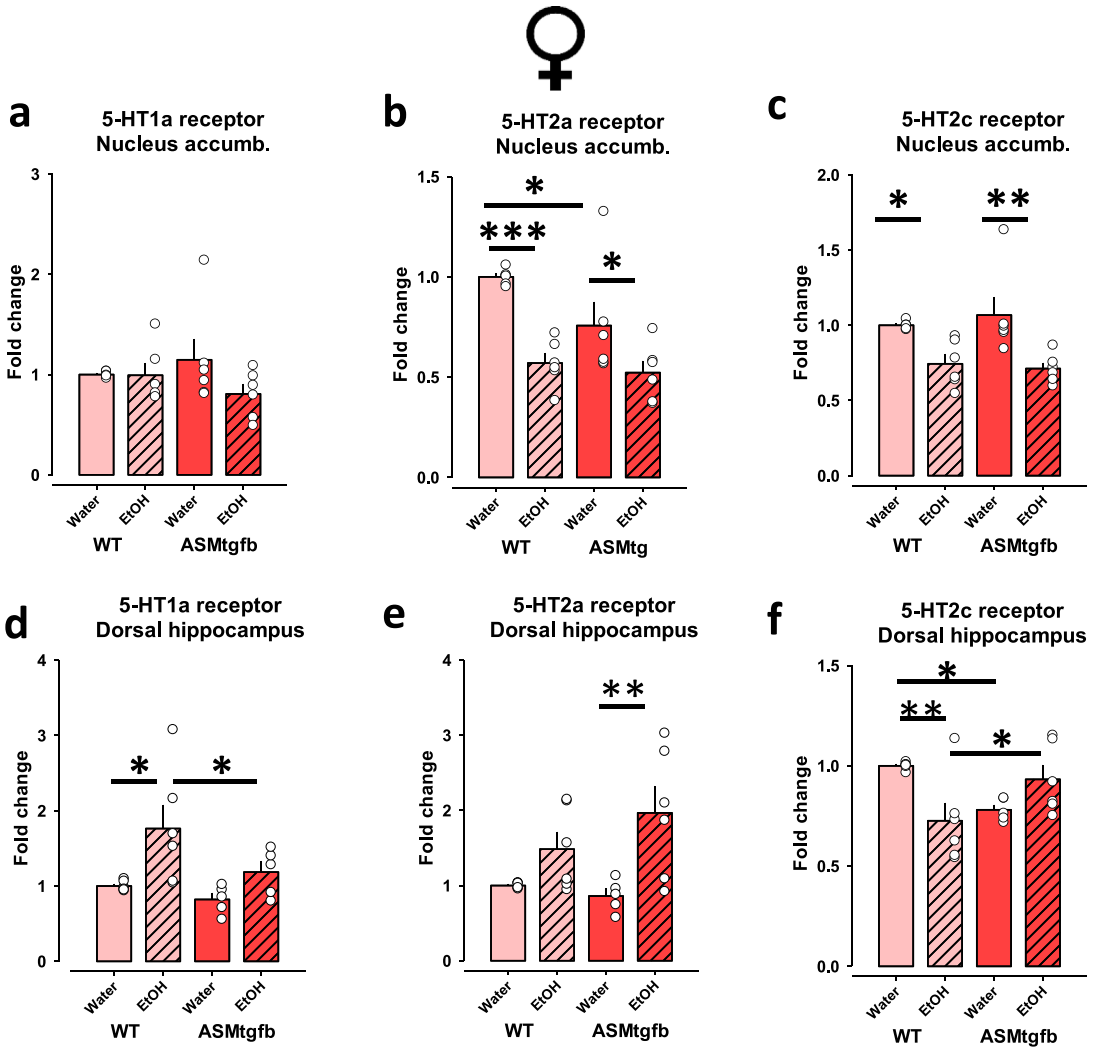


Fig. 13. Forebrain ASM overexpression affects serotonin receptor expression in female mice. No genotype- or treatment-induced changes in the expression of 5-HT_{1a} receptors in the nucleus accumbens (n = 6 in each group; a). Alcohol induced a decrease in the expression of 5-HT_{2a} (b) and 5-HT_{2c} receptors (c) in the nucleus accumbens, as well as 5-HT_{1a} receptors in the dorsal hippocampus (d) both in female $ASMtg^{fb}$ and wt animals. An increase in the expression of 5-HT_{2a} receptors in the dorsal hippocampus (e) reached statistical significance only in female $ASMtg^{fb}$ mice. Alcohol diminished the expression of 5-HT_{2c} receptors in the dorsal hippocampus (b) only in wt, but not $ASMtg^{fb}$ mice. Data were analyzed by two-way ANOVA for repeated measures followed by pre-planned comparisons using Fisher's LSD tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ $ASMtg^{fb}$ vs wt).

Table 1
Contribution of ASM specifically overexpressing in murine forebrain to various types of addiction-related behaviours in a drug- and sex-specific way. ↑ - enhancement, ↓ - diminishment, – - no effect, n.t. – not tested.

		♂	♀
Moderate alcohol drinking		↑	–
Binge alcohol drinking		–	↑
Reinforcing effects	Alcohol	↓	–
	Amphetamine	–	↑
	Cocaine	↓	n.t.
	Ketamine	–	–
	"Cafeteria diet"	–	–

regulation of the ceramide balance (Pan et al., 2014; Yu et al., 2015; Vozella et al., 2019; Kendall et al., 2022; Hoffmann et al., 2024). Our current study does not unravel the exact molecular pathway of the ASM-associated differences in drug response in females and males, but emphasizes the specific role of the ceramide system in the sex- and gender-specific drug use disorders. These differences might to a certain extent be related to the sex-specific changes in 5-HT receptor expression. Interestingly, we observed a lower fold change in the expression of $5HT_{2a}$ in the DH of females of all studied groups, and the opposite pattern in the Nac. Similar, the fold change in the expression of $5HT_{2c}$ was diminished in the Nac of alcohol consuming males of both genotypes, but not in female mice. Our results are supported by literature data widely showing the role of the serotonergic system in the sex-specific differences of alcohol consumption (Castle and Flanigan, 2024; Flanigan et al., 2023; Torres Irizarry et al., 2024). Particularly, Torres Irizarry et al. (2024) showed an oestrogen-dependent mechanism of alcohol-induced activation of 5-HT neurons in the dorsal raphe during DID (Torres Irizarry et al., 2024). Therefore, although our data do not allow to clearly explain the observed differences between female and male wt and $ASMtg^{fb}$ mice, they might add information on the serotonergic mechanisms determining sex-specificity of drug addiction.

A number of factors limits our study. Due to breeding differences, males generally possess higher ASM activity in the forebrain, although not in all brain structures (Zoicas et al., 2020a). Considering forebrain ASM as the causative factor for the observed addictive phenotype, this might to a certain extent influence the observed sex differences. Even though ASM was shown to affect learning and memory performance (Kalinichenko et al., 2021b, 2022; Zoicas et al., 2016), the effects of the

forebrain ASM on these processes, which might influence addiction-like behaviour, have not been investigated yet.

Altogether, our study suggests a specific role of the forebrain ASM for the drug abuse-related behaviours, in a drug- and sex-specific way. In males, forebrain ASM controls moderate low-risk alcohol consumption and prevents against alcohol and cocaine preferences. In turn, in females forebrain ASM overexpression contributes to the development of binge, but not moderate alcohol drinking. It also determines amphetamine use related behaviours in females. The forebrain ASM might serve as a crucial factors for sex- and gender-specificity of substance use disorders. It might also serve as a gender-specific prognostic marker or potential therapeutic target for drug use disorder, in a drug-specific way.

Funding

The authors like to thank Jana Schramm, Laura Emrich and Katrin Ebert for excellent technical assistance. This work was supported by the German National Science Foundation (Deutsche Forschungsgemeinschaft [DFG]), grant MU 2789/18-1, GU 335/29-2, GU 335/29-3, KO 947/13-3, KO 947/15-2, and TRR265 (Project-ID 402170461), the statutory funds of the Maj Institute of Pharmacology Polish Academy of Sciences, Interdisciplinary Center for Clinical Research (IZKF, J93), Johannes und Frieda Marohn-Stiftung, and in part by the Federal Ministry of Education and Research (BMBF) under the e:Med Program (031L0190B and 01KC2004B). This project was in partial fulfilment of the requirements for obtaining the degree “Dr. med.” by AMB, CB, JR, JK, AL, TR, and LW.

CRediT authorship contribution statement

Liubov S. Kalinichenko: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Iulia Zoicas:** Writing – review & editing, Investigation, Formal analysis. **Anne-Marie Bienia:** Writing – review & editing, Investigation, Formal analysis. **Clara Bühner:** Writing – review & editing, Investigation, Formal analysis. **Julia Robinson:** Writing – review & editing, Investigation, Formal analysis. **Joshua Küttermeyer:** Writing – review & editing, Investigation, Formal analysis. **Annika Labonte:** Writing – review & editing, Investigation, Formal analysis. **Thadshajiny Raveendran:** Writing – review & editing, Investigation, Formal analysis. **Lena Warth:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Irena Smaga:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Malgorzata Filip:** Writing – review & editing, Supervision, Investigation, Formal analysis. **Volker Eulenburg:** Writing – review & editing, Investigation, Formal analysis. **Cosima Rhein:** Writing – review & editing, Methodology, Investigation. **Anna Fejtova:** Writing – review & editing, Methodology. **Erich Gulbins:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Johannes Kornhuber:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Christian P. Müller:** Writing – original draft, Supervision, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2025.106800.

References

American Psychiatric Association, 2013. Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Association. https://doi.org/10.1176/appi.books.9780890425596.

Astarita, G., Avanesian, A., Grimaldi, B., Realini, N., Justinova, Z., Panlilio, L.V., Basit, A., Goldberg, S.R., Piomelli, D., 2015. Methamphetamine accelerates cellular senescence through stimulation of De Novo ceramide biosynthesis. PLoS One 10, e0116961. https://doi.org/10.1371/journal.pone.0116961.

Campbell, E.J., Bonomo, Y., Pastor, A., Collins, L., Norman, A., Galettis, P., Johnstone, J., Lawrence, A.J., 2021. The 5-HT 2C receptor as a therapeutic target for alcohol and methamphetamine use disorders: a pilot study in treatment-seeking individuals. Pharmacol. Res. Perspect. 9. https://doi.org/10.1002/prp2.767.

Castle, M.E., Flanigan, M.E., 2024. The role of brain serotonin signaling in excessive alcohol consumption and withdrawal: a call for more research in females. Neurobiol. Stress 30, 100618. https://doi.org/10.1016/j.ynstr.2024.100618.

Cotto, J.H., Davis, E., Dowling, G.J., Elcano, J.C., Staton, A.B., Weiss, S.R.B., 2010. Gender effects on drug use, abuse, and dependence: a special analysis of results from the national survey on drug use and health. Gend. Med. 7, 402–413. https://doi.org/10.1016/j.genm.2010.09.004.

Cozzoli, D.K., Goulding, S.P., Zhang, P.W., Xiao, B., Hu, J.-H., Ary, A.W., Obara, I., Rahn, A., Abou-Ziab, H., Tyrrel, B., Marini, C., Yoneyama, N., Metten, P., Snelling, C., Dehoff, M.H., Crabbe, J.C., Finn, D.A., Klugmann, M., Worley, P.F., Szumlinski, K.K., 2009. Binge drinking upregulates accumbens mGluR5-Homer2-PI3K signaling: functional implications for alcoholism. J. Neurosci. 29, 8655–8668. https://doi.org/10.1523/JNEUROSCI.5900-08.2009.

Cozzoli, D.K., Kaufman, M.N., Nipper, M.A., Hashimoto, J.G., Wiren, K.M., Finn, D.A., 2016. Functional regulation of PI3K-associated signaling in the accumbens by binge alcohol drinking in male but not female mice. Neuropharmacology 105, 164–174. https://doi.org/10.1016/j.neuropharm.2016.01.010.

Crabbe, J.C., Harris, R.A., Koob, G.F., 2011. Preclinical studies of alcohol binge drinking. Ann. N. Y. Acad. Sci. 1216, 24–40. https://doi.org/10.1111/j.1749-6632.2010.05895.x.

de Macedo, I.C., de Freitas, J.S., da Silva Torres, I.L., 2016. The influence of palatable diets in reward system activation: a mini review. Adv. Pharmacol. Sci. 2016, 1–7. https://doi.org/10.1155/2016/7238679.

Degenhardt, L., Charlson, F., Ferrari, A., Santomauro, D., Erskine, H., Mantilla-Herrara, A., Whiteford, H., Leung, J., Naghavi, M., Griswold, M., Rehm, J., Hall, W., Sartorius, B., Scott, J., Vollset, S.E., Knudsen, A.K., Haro, J.M., Patton, G., Kopec, J., Carvalho Malta, D., Topor-Madry, R., McGrath, J., Haagsma, J., Allebeck, P., Phillips, M., Salomon, J., Hay, S., Foreman, K., Lim, S., Mokdad, A., Smith, M., Gakidou, E., Murray, C., Vos, T., 2018. The global burden of disease attributable to alcohol and drug use in 195 countries and territories, 1990–2016: a systematic analysis for the global burden of disease study 2016. Lancet Psychiatry 5, 987–1012. https://doi.org/10.1016/S2215-0366(18)30337-7.

Easton, A.C., Lourdusamy, A., Havranek, M., Mizuno, K., Solati, J., Golub, Y., Clarke, T.-K., Vallada, H., Laranjeira, R., Desrivières, S., Moll, G.H., Mössner, R., Kornhuber, J., Schumann, G., Giese, K.P., Fernandes, C., Quednow, B.B., Müller, C.P., 2014. αCaMKII controls the establishment of cocaine's reinforcing effects in mice and humans. Transl. Psychiatry 4, e457. https://doi.org/10.1038/tp.2014.97.

European Monitoring Centre for Drugs and Drug Addiction, 2022. Publ. Off. Eur. Union, Luxemb. Trends and.

Flanigan, M.E., Hon, O.J., D'Ambrosio, S., Boyt, K.M., Hassanein, L., Castle, M., Haun, H. L., Pina, M.M., Kash, T.L., 2023. Subcortical serotonin 5HT2c receptor-containing neurons sex-specifically regulate binge-like alcohol consumption, social, and arousal behaviors in mice. Nat. Commun. 14 (1), 1800. https://doi.org/10.1038/s41467-023-36808-2.

Frankowska, M., Jesus, F.M., Mühle, C., Pacheco, J.V.N., Maior, R.S., Sadakierska-Chudy, A., Smaga, I., Piechota, M., Kalinichenko, L.S., Gulbins, E., Kornhuber, J., Filip, M., Müller, C.P., Barros, M., 2021. Cocaine attenuates acid sphingomyelinase activity during establishment of addiction-related behavior—a translational study in rats and monkeys. Addict. Biol. 26. https://doi.org/10.1111/adb.12955.

Gimenez-Gomez, P., Le, T., Martin, G.E., 2023. Modulation of neuronal excitability by binge alcohol drinking. Front. Mol. Neurosci. 16. https://doi.org/10.3389/fnmol.2023.1098211.

Gretler, J., McClain, M.L., 2023. Handbook of the behavioral neurobiology of serotonin. Appl. Neuropsychol. Adult 30, 141–142. https://doi.org/10.1080/23279095.2021.1916731.

Gulbins, E., Palmada, M., Reichel, M., Lüth, A., Böhmer, C., Amato, D., Müller, C.P., Tischbirek, C.H., Groemer, T.W., Tabatabai, G., Becker, K.A., Tripal, P., Staedtler, S., Ackermann, T.F., van Brederode, J., Alzheimer, C., Weller, M., Lang, U.E., Kleuser, B., Grassmé, H., Kornhuber, J., 2013. Acid sphingomyelinase–ceramide system mediates effects of antidepressant drugs. Nat. Med. 19, 934–938. https://doi.org/10.1038/nm.3214.

Guo, R., Tang, Q., Ye, Y., Lu, X., Chen, F., Dai, X., Yan, Y., Liao, L., 2016. Effects of gender on ketamine-induced conditioned placed preference and urine metabonomics. Regul. Toxicol. Pharmacol. 77, 263–274. https://doi.org/10.1016/j.yrtph.2016.03.007.

Heilig, M., MacKillop, J., Martinez, D., Rehm, J., Leggio, L., Vanderschuren, L.J.M.J., 2021. Addiction as a brain disease revised: why it still matters, and the need for

consilience. Neuropsychopharmacology 46, 1715–1723. https://doi.org/10.1038/s41386-020-00950-y.
Hess, A., Kress, S., Rakete, S., Muench, G., Kornhuber, J., Pischetsrieder, M., Müller, C.P., 2019. Influence of the fat/carbohydrate component of snack food on energy intake pattern and reinforcing properties in rodents. Behav. Brain Res. 364, 328–333. https://doi.org/10.1016/j.bbr.2019.02.041.
Hoffmann, S., Gerhardt, S., Mühle, C., Reinhard, I., Reichert, D., Bach, P., Boroumand-Jazi, R., Kuehner, C., Aguilera, A., Aslan, A., Bahr, N.S., Belanger, M., Deeken, F., Ebrahimi, C., Fischbach, P.C., Ganz, M., Garbusow, M., Großkopf, C.M., Heigert, M., Hentschel, A., Karl, D., Liu, S., Mazza, M., Pelz, P., Pinger, M., Reichl, M., Riemerschmid, C., Rosenthal, A., Steffen, J., Strehle, J., Wedemeyer, F., Weiss, F., Wenzel, J., Wieder, G., Wieland, A., Zaiser, J., Zech, H.G., Zimmermann, S., Kornhuber, J., Müller, C.P., Sommer, W.H., Spanagel, R., Banaschewski, T., Deserno, L., Ebner-Priemer, U.W., Flor, H., Kirsch, P., Rietschel, M., Vollstädt-Klein, S., Walter, H., Meyer-Lindenberg, A., Rapp, M.A., Witt, S., Smolka, M.N., Heinz, A., Tost, H., Kiefer, F., Reichert, M., Lenz, B., 2024. Associations of menstrual cycle and progesterone-to-estradiol ratio with alcohol consumption in alcohol use disorder: a sex-separated multicenter longitudinal study. Am. J. Psychiatry 181, 445–456. https://doi.org/10.1176/appi.ajp.20230027.
Huston, J.P., de Silva, M.A.S., Topic, B., Müller, C.P., 2013. What's conditioned in conditioned place preference? Trends Pharmacol. Sci. 34, 162–166. https://doi.org/10.1016/j.tips.2013.01.004.
Kalinichenko, L.S., Gulbins, E., Kornhuber, J., Müller, C.P., 2018. The role of sphingolipids in psychoactive drug use and addiction. J. Neural Transm. 125, 651–672. https://doi.org/10.1007/s00702-018-1840-1.
Kalinichenko, Liubov S., Abdel-Hafiz, L., Wang, A.-L., Mühle, C., Rösel, N., Schumacher, F., Kleuser, B., Smaga, I., Frankowska, M., Filip, M., Schaller, G., Richter-Schmidinger, T., Lenz, B., Gulbins, E., Kornhuber, J., Oliveira, A.W.C., Barros, M., Huston, J.P., Müller, C.P., 2021a. Neutral sphingomyelinase is an affective valence-dependent regulator of learning and memory. Cereb. Cortex 31, 1316–1333. https://doi.org/10.1093/cercor/bhaa298.
Kalinichenko, Liubov S., Mühle, C., Jia, T., Anderheiden, F., Datz, M., Eberle, A.-L., Eulenburg, V., Granzow, J., Hofer, M., Hohenschild, J., Huber, S.E., Kämpf, S., Kogias, G., Lacatusu, L., Lugmair, C., Taku, S.M., Meixner, D., Tesch, N., Praetner, M., Rhein, C., Sauer, C., Scholz, J., Ulrich, F., Valenta, F., Weigand, E., Werner, M., Tay, N., Mc Veigh, C.J., Haase, J., Wang, A.-L., Abdel-Hafiz, L., Huston, J.P., Smaga, I., Frankowska, M., Filip, M., Lourdusamy, A., Kirchner, P., Ekici, A.B., Marx, L.M., Suresh, N.P., Frischknecht, R., Fejtova, A., Saied, E.M., Arenz, C., Bozec, A., Wank, I., Kreitz, S., Hess, A., Bäuerle, T., Ledesma, M.D., Mitroi, D.N., Miranda, A.M., Oliveira, T.G., Gulbins, E., Lenz, B., Schumann, G., Kornhuber, J., Müller, C.P., 2021b. Neutral sphingomyelinase mediates the co-morbidity trias of alcohol abuse, major depression and bone defects. Mol. Psychiatry 26, 7403–7416. https://doi.org/10.1038/s41380-021-01304-w.
Kalinichenko, L.S., Gulbins, E., Kornhuber, J., Müller, C.P., 2022. Sphingolipid control of cognitive functions in health and disease. Prog. Lipid Res. 86, 101162. https://doi.org/10.1016/j.plipres.2022.101162.
Kalinichenko, L.S., Mühle, C., Jia, T., Anderheiden, F., Datz, M., Eberle, A.-L., Eulenburg, V., Granzow, J., Hofer, M., Hohenschild, J., Huber, S.E., Kämpf, S., Kogias, G., Lacatusu, L., Lugmair, C., Taku, S.M., Meixner, D., Sembritzki, N.-K., Praetner, M., Rhein, C., Sauer, C., Scholz, J., Ulrich, F., Valenta, F., Weigand, E., Werner, M., Tay, N., Mc Veigh, C.J., Haase, J., Wang, A.-L., Abdel-Hafiz, L., Huston, J.P., Smaga, I., Frankowska, M., Filip, M., Lourdusamy, A., Kirchner, P., Ekici, A.B., Marx, L.M., Suresh, N.P., Frischknecht, R., Fejtova, A., Saied, E.M., Arenz, C., Bozec, A., Wank, I., Kreitz, S., Hess, A., Bäuerle, T., Ledesma, M.D., Mitroi, D.N., Miranda, A.M., Oliveira, T.G., Lenz, B., Schumann, G., Kornhuber, J., Müller, C.P., 2023. Adult alcohol drinking and emotional tone are mediated by neutral sphingomyelinase during development in males. Cereb. Cortex 33, 844–864. https://doi.org/10.1093/cercor/bhac106.
Kalinichenko, L.S., Kornhuber, J., Sinning, S., Haase, J., Müller, C.P., 2024. Serotonin signaling through lipid membranes. ACS Chem. Neurosci. 15, 1298–1320. https://doi.org/10.1021/acschemneuro.3c00823.
Kendall, A.C., Pilkington, S.M., Wray, J.R., Newton, V.L., Griffiths, C.E.M., Bell, M., Watson, R.E.B., Nicolaou, A., 2022. Menopause induces changes to the stratum corneum ceramide profile, which are prevented by hormone replacement therapy. Sci. Rep. 12, 21715. https://doi.org/10.1038/s41598-022-26095-0.
Kogias, G., Zheng, F., Kalinichenko, L.S., Kornhuber, J., Alzheimer, C., Mielenz, D., Müller, C.P., 2020. Swiprosin1/EFhd2 is involved in the monoaminergic and locomotor responses of psychostimulant drugs. J. Neurochem. 154, 424–440. https://doi.org/10.1111/jnc.14959.
König, L., Kalinichenko, L.S., Huber, S.E., Voll, A.M., Bauder, M., Kornhuber, J., Hausch, F., Müller, C.P., 2020. The selective FKBP51 inhibitor SAFit2 reduces alcohol consumption and reinstatement of conditioned alcohol effects in mice. Addict. Biol. 25, e12758. https://doi.org/10.1111/adb.12758.
Liu, Y., Lin, D., Wu, B., Zhou, W., 2016. Ketamine abuse potential and use disorder. Brain Res. Bull. 126, 68–73. https://doi.org/10.1016/j.brainresbull.2016.05.016.
Mühle, C., Weinland, C., Gulbins, E., Lenz, B., Kornhuber, J., 2018. Peripheral acid sphingomyelinase activity is associated with biomarkers and phenotypes of alcohol use and dependence in patients and healthy controls. Int. J. Mol. Sci. 19, 4028. https://doi.org/10.3390/ijms19124028.
Müller, C.P., 2020. Drug instrumentalization. Behav. Brain Res. 390, 112672. https://doi.org/10.1016/j.bbr.2020.112672.
Müller, C.P., Homberg, J.R., 2015. Serotonin revisited. Behav. Brain Res. 277, 1–2. https://doi.org/10.1016/j.bbr.2014.10.008.
Müller, C.P., Kornhuber, J., 2017. Biological evidence for paradoxical improvement of psychiatric disorder symptoms by addictive drugs. Trends Pharmacol. Sci. 38 (6), 501–502.
Müller, C.P., Kalinichenko, L.S., Tiesel, J., Witt, M., Stöckl, T., Sprenger, E., Fuchser, J., Beckmann, J., Praetner, M., Huber, S.E., Amato, D., Mühle, C., Büttner, C., Ekici, A. B., Smaga, I., Pomierny-Chamiolo, L., Pomierny, B., Filip, M., Eulenburg, V., Gulbins, E., Lourdusamy, A., Reichel, M., Kornhuber, J., 2017. Paradoxical antidepressant effects of alcohol are related to acid sphingomyelinase and its control of sphingolipid homeostasis. Acta Neuropathol. 133, 463–483. https://doi.org/10.1007/s00401-016-1658-6.
Müller, C.P., Mühle, C., Kornhuber, J., Lenz, B., 2021. Sex-dependent alcohol instrumentalization goals in non-addicted alcohol consumers versus patients with alcohol use disorder: longitudinal change and outcome prediction. Alcohol. Clin. Exp. Res. 45, 577–586. https://doi.org/10.1111/acer.14550.
Müller, C.P., Schumann, G., Rehm, J., Kornhuber, J., Lenz, B., 2023. Self-management with alcohol over lifespan: psychological mechanisms, neurobiological underpinnings, and risk assessment. Mol. Psychiatry. https://doi.org/10.1038/s41380-023-02074-3.
Nassogne, M.-C., Lizarraga, C., N'Kuli, F., Van Bambeke, F., Van Binst, R., Wallemacq, P., Tulkens, P.M., Mingeot-Leclercq, M.-P., Levade, T., Courtoy, P.J., 2004. Cocaine induces a mixed lysosomal lipidosis in cultured fibroblasts, by inactivation of acid sphingomyelinase and inhibition of phospholipase A1. Toxicol. Appl. Pharmacol. 194, 101–110. https://doi.org/10.1016/j.taap.2003.09.026.
Nestler, E.J., 2005. Is there a common molecular pathway for addiction? Nat. Neurosci. 8, 1445–1449. https://doi.org/10.1038/nn1578.
Pan, W., Yu, J., Shi, R., Yan, L., Yang, T., Li, Y., Zhang, Z., Yu, G., Bai, Y., Schuchman, E. H., He, X., Zhang, G., 2014. Elevation of ceramide and activation of secretory acid sphingomyelinase in patients with acute coronary syndromes. Coron. Artery Dis. 25, 230–235. https://doi.org/10.1097/MCA.0000000000000079.
Pati, D., Pina, M.M., Kash, T.L., 2019. Ethanol-induced conditioned place preference and aversion differentially alter plasticity in the bed nucleus of stria terminalis. Neuropsychopharmacology 44, 1843–1854. https://doi.org/10.1038/s41386-019-0349-0.
Rhodes, J.S., Best, K., Belknap, J.K., Finn, D.A., Crabbe, J.C., 2005. Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. Physiol. Behav. 84, 53–63. https://doi.org/10.1016/j.physbeh.2004.10.007.
Schneider, M., Levant, B., Reichel, M., Gulbins, E., Kornhuber, J., Müller, C.P., 2017. Lipids in psychiatric disorders and preventive medicine. Neurosci. Biobehav. Rev. 76, 336–362. https://doi.org/10.1016/j.neubiorev.2016.06.002.
Smutek, M., Turbasa, M., Sikora, M., Piechota, M., Zajdel, J., Przewlocki, R., Parkitna, J. R., 2014. A model of alcohol drinking under an intermittent access schedule using group-housed mice. PloS one 9 (5), e96787. https://doi.org/10.1371/journal.pone.0096787.
Spanagel, R., Bach, P., Banaschewski, T., Beck, A., Bermpohl, F., Bernardi, R.E., Beste, C., Deserno, L., Durstewitz, D., Ebner-Priemer, U., Endrass, T., Ersche, K.D., Feld, G., Gerchen, M.F., Gerlach, B., Goschke, T., Hansson, A.C., Heim, C., Kiebel, S., Kiefer, F., Kirsch, P., Kirschbaum, C., Koppe, G., Lenz, B., Liu, S., Marxen, M., Meinhardt, M.W., Meyer-Lindenberg, A., Montag, C., Müller, C.P., Nagel, W.E., Oliveria, A.M.M., Owald, D., Pilhatsch, M., Priller, J., Rapp, M.A., Reichert, M., Ripke, S., Ritter, K., Romanczuk-Seiferth, N., Schlagenhauf, F., Schwarz, E., Schwöbel, S., Smolka, M.N., Soekadar, S.R., Sommer, W.H., Stock, A., Ströhle, A., Tost, H., Vollstädt-Klein, S., Walter, H., Waschke, T., Witt, S.H., Heinz, A., 2024. The ReCoDe addiction research consortium: losing and regaining control over drug intake—findings and future perspectives. Addict. Biol. 29. https://doi.org/10.1111/adb.13419.
Tanaka, M., Watanabe, Y., 2020. RNA editing of serotonin 2C receptor and alcohol intake. Front. Neurosci. 13. https://doi.org/10.3389/fnins.2019.01390.
Tomkins, D.M., Joharchi, N., Tampakeras, M., Martin, J., Wichmann, J., Higgins, G., 2002. An investigation of the role of 5-HT2C receptors in modifying ethanol self-administration behaviour. Pharmacol. Biochem. Behav. 71, 735–744. https://doi.org/10.1016/S0091-3057(01)00710-9.
Torres Irizarry, V.C., Feng, B., Yang, X., et al., 2024. Estrogen signaling in the dorsal raphe regulates binge-like drinking in mice. Transl. Psychiatry 14, 122. https://doi.org/10.1038/s41398-024-02821-2.
UNODC, 2023. World Drug Report 2023 doi: 9789210028233.
Volkow, N.D., Michaelides, M., Baler, R., 2019. The neuroscience of drug reward and addiction. Physiol. Rev. 99, 2115–2140. https://doi.org/10.1152/physrev.00014.2018.
Vozella, V., Basit, A., Piras, F., Realini, N., Armirotti, A., Bossù, P., Assogna, F., Sensi, S. L., Spalletta, G., Piomelli, D., 2019. Elevated plasma ceramide levels in post-menopausal women: a cross-sectional study. Aging (Albany NY) 11, 73–88. https://doi.org/10.18632/aging.101719.
Yoshimoto, K., Watanabe, Y., Tanaka, M., Kimura, M., 2012. Serotonin 2C receptors in the nucleus accumbens are involved in enhanced alcohol-drinking behavior. Eur. J. Neurosci. 35, 1368–1380. https://doi.org/10.1111/j.1460-9568.2012.08037.x.
Yu, J., Pan, W., Shi, R., Yang, T., Li, Y., Yu, G., Bai, Y., Schuchman, E.H., He, X., Zhang, G., 2015. Ceramide is upregulated and associated with mortality in patients with chronic heart failure. Can. J. Cardiol. 31, 357–363. https://doi.org/10.1016/j.cjca.2014.12.007.
Zheng, F., Puppel, A., Huber, S.E., Link, A.S., Eulenburg, V., van Brederode, J.F., Müller, C.P., Alzheimer, C., 2016. Activin controls ethanol potentiation of inhibitory synaptic transmission through GABAA receptors and concomitant behavioral sedation. Neuropsychopharmacology 41, 2024–2033. https://doi.org/10.1038/npp.2015.372.

Zoicas, I., Reichel, M., Gulbins, E., Kornhuber, J., 2016. Role of acid sphingomyelinase in the regulation of social behavior and memory. PLoS One 11 (9), e0162498. https://doi.org/10.1371/journal.pone.0162498.

Zoicas, I., Huber, S.E., Kalinichenko, L.S., Gulbins, E., Müller, C.P., Kornhuber, J., 2020a. Ceramides affect alcohol consumption and depressive-like and anxiety-like behavior in a brain region- and ceramide species-specific way in male mice. Addict. Biol. 25. https://doi.org/10.1111/adb.12847.

Zoicas, I., Schumacher, F., Kleuser, B., Reichel, M., Gulbins, E., Fejtova, A., Kornhuber, J., Rhein, C., 2020b. The forebrain-specific overexpression of acid sphingomyelinase induces depressive-like symptoms in mice. Cells 9, 1244. https://doi.org/10.3390/cells9051244.