Ceramides affect alcohol consumption and depressive-like and anxiety-like behavior in a brain region- and ceramide species-specific way in male mice

Iulia Zoicas1 | Sabine E. Huber1,4 | Liubov S. Kalinichenko1 | Erich Gulbins2,3 | Christian P. Müller1 | Johannes Kornhuber1

1 Department of Psychiatry and Psychotherapy, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany
2 Department of Molecular Biology, University of Duisburg-Essen, Essen, Germany
3 Department of Surgery, University of Cincinnati, Cincinnati, Ohio
4 Institute of Physiology I, Westfälische Wilhelms-University Münster, Münster, Germany

Abstract
Depression and alcohol dependence are associated with increased plasma ceramide concentrations in humans. Pharmacological increase in C16 ceramide concentrations in the dorsal hippocampus (DH) induced a depressive-like phenotype in naïve mice. However, the effects of C16 ceramide on alcohol consumption and anxiety-like behavior as well as the behavioral effects of other ceramide species are yet unknown. Therefore, we investigated whether repeated infusion of ceramides with different fatty acid chain lengths (C8, C16, and C20) into the DH and the basolateral amygdala (BLA) alter alcohol consumption, emotional behavior, and tissue monoamine levels. Our results revealed that C16, but not C8 and C20, ceramide altered alcohol drinking and emotional behavior in a brain region-specific way without altering tissue noradrenaline, dopamine, and serotonin levels in the prefrontal cortex, ventral striatum, and dorsal mesencephalon. In more detail, C16 ceramide increased alcohol consumption when infused into the BLA, but not when infused into the DH. Furthermore, C16 ceramide induced a depressive-like phenotype when infused into the DH, but a predominantly anxiogenic-like phenotype (in a non-social, but not a social context) when infused into the BLA. In turn, alcohol drinking normalized C16 ceramide-induced depressive-like and anxiogenic-like phenotypes. This study demonstrates a complex ceramide species-specific and brain region-specific modulation of alcohol consumption and emotional behavior in mice and provides the framework for future studies investigating the involvement of distinct ceramide species in the regulation of emotional behavior.

KEYWORDS
alcohol consumption, basolateral amygdala, C16 ceramide, dorsal hippocampus, emotional behavior

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2019 The Authors. Addiction Biology published by John Wiley & Sons Ltd on behalf of Society for the Study of Addiction

Addiction Biology. 2020;25:e12847.
https://doi.org/10.1111/adb.12847
1 | INTRODUCTION

Ceramides are sphingolipids composed of sphingosine and a fatty acid. They are found in high concentrations within the lipid bilayer of cell membranes where they not only play important roles in membrane structure and function, but also regulate many cellular processes including apoptosis, cell differentiation, and proliferation. Ceramide can be generated by de novo synthesis starting from palmitoyl-CoA and serine or through the salvage pathway involving reacylation of its degradation product sphingosine. Ceramide can also be generated by the hydrolysis of sphingomyelin through the action of sphingomyelinases. Depending on the pH, there are three main forms of sphingomyelinases: neutral sphingomyelinase (NSM), acid sphingomyelinase (ASM), and alkaline sphingomyelinase (alkSM). ASM is a lysosomal glycoprotein catalyzing the degradation of sphingomyelin to ceramide and phosphorylcholine. The ASM/ceramide system was implicated in the pathogenesis of psychiatric disorders such as depression and alcohol use disorder, among others. As such, increased ASM activity was found in peripheral blood mononuclear cells of patients experiencing a major depressive episode and in acutely intoxicated alcoholic-dependent patients. ASM activity was also increased in plasma and serum of alcohol-dependent patients, where it correlated positively with blood alcohol concentrations. Patients experiencing a major depressive episode during the past 2 years showed increased plasma levels of several ceramide species including C16:0, C18:0, C20:0, C24:1, and C26:1, but not C22:0 or C24:0, compared with both healthy controls and patients experiencing a depressive episode more than 2 years ago. Transgenic mice overexpressing ASM (tgASM) showed increased ASM activity and ceramide concentrations in the hippocampus, which were associated with depressive-like and anxiogenic-like phenotypes in both social and non-social contexts. Functional inhibitors of ASM, such as amitriptyline and fluoxetine, reduced ceramide concentrations and ASM protein levels in cultured neurons and in the hippocampus of wild-type and tgASM mice and normalized the depressive-like and anxiogenic-like phenotypes in tgASM mice. tgASM mice also showed increased alcohol consumption, alcohol-deprivation effect, and alcohol-conditioned place preference, which were associated with altered tissue monoamine levels. As such, tgASM mice showed decreased serotonin concentration in the ventral striatum, dorsal hippocampus (DH), and prefrontal cortex and decreased dopamine concentration in the DH, but enhanced dopamine responses to alcohol in the DH and ventral striatum. Voluntary alcohol consumption normalized the depressive-like phenotype in tgASM mice, normalized ASM hyperactivity in the DH, and restored tissue monoamine homeostasis. A direct involvement of ceramide in the pathogenesis of depression was demonstrated in wild-type C57BL/6J mice, which developed a depressive-like phenotype after infusion of C16 ceramide in the DH. However, it is unclear whether the pharmacological increase in ceramide concentrations might also alter alcohol consumption and anxiety-like behavior and whether these effects are specifically mediated by C16 ceramide in the DH. Although depression is highly comorbid with social deficits and tgASM mice showed impaired social preference, as an indicator of increased social anxiety, it is not clear whether ceramides directly influence anxiety-like behavior in a social context.

In the present study, we investigated the effects of two ceramide species with long-chain fatty acid, which have been shown to be increased in the plasma of depressive patients, i.e., C16:0 and C20:0 ceramide, and one ceramide species (C8:0) with medium-chain fatty acid as control, on alcohol consumption, depressive-like behavior, and social and non-social anxiety-like behavior. These ceramide species were infused bilaterally into the DH and basolateral amygdala (BLA), as these brain regions were involved in addiction, depressive-like behavior, anxiety-like behavior, and social behavior.

2 | MATERIALS AND METHODS

2.1 | Animals

Male C57BL/6J mice (Charles River, Germany, 9 weeks of age) were individually housed and remained single housed throughout the experiments. Mice were kept under standard laboratory conditions (12:12 reversed light-dark cycle, lights on at 20:00, 22°C, 60% humidity, food and water ad libitum). Experiments were performed during the dark phase, between 09:00 and 14:00, in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of...
Unterfranken and the guidelines of the National Institutes of Health (NIH).

### 2.2 Experimental procedures

After 2 weeks of single housing (arrival on day −5), mice were implanted bilaterally with guide canulas for intracerebral infusions and were handled for 6 days before experiments started (Figure 1). Starting 1 week after surgery, mice were infused with vehicle, C8 ceramide, C16 ceramide, or C20 ceramide into the DH or BLA every 48 or 72 hours. To investigate the effects of ceramide infusions on alcohol consumption, mice were exposed to the drinking in the dark (DID) paradigm between days 28 and 32 and were given access to 10% alcohol for 2 hours, beginning 3 hours into the dark cycle. Starting from day 32, mice had continuous access to tap water and 10% alcohol in a two-bottle free-choice paradigm, to investigate the effects of alcohol consumption on behavior and to prevent withdrawal symptoms. To investigate the effects of ceramide infusions and alcohol consumption on depressive-like behavior, and social and non-social anxiety-like behavior, mice were tested in the novelty-suppressed feeding (NSF), the elevated plus maze (EPM), the forced swim test (FST), and the social preference response test. In this test, mice were infused bilaterally into the DH with 2 μM, 20 μM, or 200 μM of C8 ceramide, C16 ceramide, or C20 ceramide (5 mice/dose/ceramide species) and were observed in their home cage for 120 minutes. While 20 μM and 200 μM ceramide induced immobility and seizure-like effects (20 μM C8 ceramide: immobility observed in one out of five mice, seizures in 0/5; 200 μM C8 ceramide: immobility in 2/5, seizures in 0/5; 20 μM C16 ceramide: immobility in 2/5, seizures in 0/5; 200 μM C16 ceramide: immobility in 4/5, seizures in 2/5; 20 μM C20 ceramide: immobility in 3/5, seizures in 1/5; 200 μM C20 ceramide: immobility in 4/5, seizures in 1/5), 2 μM ceramide had no acute adverse effects, independent of ceramide species.

#### 2.2.1 Stereotaxic cannula implantation

Implantation of the guide canulas (21G, 8 mm length; Injecta GmbH, Germany) for bilateral infusions was performed under ketamine-xylazine anesthesia (intraperitoneal injection of 120 mg/kg of Ketavet and 16 mg/kg of Rompun) as previously described.11 1 mm above the DH (from bregma: AP − 2.0 mm, ML ± 1.5 mm, DV + 1.4 mm) or BLA (AP − 1.3 mm, ML ± 2.8 mm, DV + 4.0 mm). After surgery, mice were handled for 6 days before experiments started.

#### 2.2.2 Intracerebral infusions

Mice received repeated DH or BLA infusions of vehicle (2% octyl β-D-glucopyranoside [OGP]; 0.2 μL/side; Sigma Aldrich, Germany), C8 ceramide ([d18:1/8:0]; 2 μM/0.2 μL/side; Cayman Chemical, Germany), C16 ceramide ([d18:1/16:0]; 2 μM/0.2 μL/side; Cayman Chemical, Germany), or C20 ceramide ([d18:1/20:0]; 2 μM/0.2 μL/side; Cayman Chemical, Germany). Ceramide solutions were prepared in 2% OGP and sonicated for 10 minutes before use. Infusions were spaced 48 or 72 hours apart and were performed via an infusion cannula (23G, 9 mm length) inserted into the guide cannula and connected via polyethylene tubing to a Hamilton syringe. The infusion system was left in place for 30 seconds following the infusion to allow diffusion of the solution. The correct infusion site was verified (Figure S1) and mice with incorrect cannula placement were removed from the statistical analysis. The infusion protocol and ceramide doses were selected based on a previous study11 and a dose-response test. In this test, mice were infused bilaterally into the DH with 2 μM, 20 μM, or 200 μM of C8 ceramide, C16 ceramide, or C20 ceramide (5 mice/dose/ceramide species) and were observed in their home cage for 120 minutes. While 20 μM and 200 μM ceramide induced immobility and seizure-like effects (20 μM C8 ceramide: immobility observed in one out of five mice, seizures in 0/5; 200 μM C8 ceramide: immobility in 2/5, seizures in 0/5; 20 μM C16 ceramide: immobility in 2/5, seizures in 0/5; 200 μM C16 ceramide: immobility in 4/5, seizures in 2/5; 20 μM C20 ceramide: immobility in 3/5, seizures in 1/5; 200 μM C20 ceramide: immobility in 4/5, seizures in 1/5), 2 μM ceramide had no acute adverse effects, independent of ceramide species.

#### 2.2.3 DID paradigm

To investigate the effects of ceramide infusions on alcohol consumption, the DID paradigm was performed between days 28 and 32. Alcohol-drinking mice were given access to a bottle of 10% alcohol (v/v) prepared from ethanol absolute (Merck Chemicals GmbH, Germany) for 2 hours, beginning 3 hours into the dark cycle. This paradigm was shown to induce high and consistent alcohol consumption in C57BL/6J mice as it is performed in the active phase of mice when ingestive behaviors are highest, but still leaves mice the choice of drinking or avoiding the alcohol solution. The consumed amount of alcohol relative to body weight was calculated.23,24 Control (water-drinking) mice received a bottle of tap water for 2 hours. All mice received a bottle of tap water for the remaining 22 hours.

#### 2.2.4 Two-bottle free-choice paradigm

To investigate the effects of alcohol consumption on behavior and to prevent withdrawal symptoms, the two-bottle free-choice paradigm was performed starting from day 32. Mice were given continuous access to two bottles containing tap water and 10% alcohol. The bottles were weighed daily, and their position was changed daily to rule out any influence of side preference on alcohol drinking. The consumed amount of alcohol relative to body weight was calculated.23,24 Control mice received two bottles of tap water.

#### 2.2.5 NSF paradigm

The depressive-like and non-social anxiety-like behavior of mice was tested in the NSF paradigm as previously described.12 Mice were food-deprived for 24 hours prior to testing with unlimited fluid access. Mice were placed in a novel arena (50 × 50 × 50 cm) with the head facing one of the corners. Immediately afterwards, a single food pellet (ssniff Spezialdiäten GmbH, Germany) was placed in the center of the arena. The latency to feed, defined as biting the food pellet for longer than 3 seconds, was manually analyzed from videos by an observer blind to the treatment condition. An increased feeding latency indicated depressive-like and non-social anxiogenic-like phenotypes.
2.2.6 | EPM test

The non-social anxiety-like behavior of mice was tested in the EPM as previously described and analyzed from videos using JWatcher (V 1.0, Macquarie University and UCLA). A decreased percentage of time spent on the open arms indicated a non-social anxiogenic-like phenotype. The number of entries into the closed arms during the 5-minute testing period indicated locomotor activity.

2.2.7 | Forced swim test

The depressive-like behavior of mice was tested in the FST as previously described. Mice were individually placed into a Plexiglas cylinder (19 cm diameter, 19 cm height) filled with 25°C water to a depth of 13 cm for 6 minutes. The test was recorded and analyzed using JWatcher. An increased percentage of immobility time during the last 4 minutes of the test indicated a depressive-like phenotype.

2.2.8 | Social preference-avoidance test

The social anxiety-like behavior of mice was tested in the SPAT as previously described. Mice were placed in a novel arena (42 × 24 × 35 cm), and after a 30-second habituation period, an empty wire mesh cage (7 × 7 × 6 cm) was placed near one of the short walls. After 2.5 minutes, the empty cage was replaced by an identical cage containing an unfamiliar age- and weight-matched male mouse for additional 2.5 minutes. The test was recorded and analyzed using JWWatcher. A higher investigation time directed towards the mouse versus the empty cage indicated social preference and thus a lack of social anxiety. A decreased investigation time directed towards the mouse versus the empty cage indicated social avoidance and, thus, a social anxiogenic-like phenotype.

2.2.9 | Postmortem neurochemistry

To determine whether ceramide infusions and alcohol consumption alter brain tissue monoamine levels, mice were decapitated and brains were collected and snap frozen with dry ice. After verification of infusion site, the prefrontal cortex, ventral striatum, and dorsal mesencephalon were dissected out of coronal brain slices based on previous studies. Half of the tissue (left or right side, contraballoled within groups) was homogenized in 0.5 M of perchloric acid, centrifuged, filtered, and stored at −80°C until analysis. Samples containing 1377 pg of dihydroxybenzylamine as an internal standard were analyzed by high-performance liquid chromatography (HPLC) with electrochemical detection. The column was an ET 125/2, Nucleosil 120-5, C-18 reversed-phase column (Macherey & Nagel, Germany). The mobile phase consisted of 75 mM of NaH2PO4, 4 mM of KCl, 20 μM of EDTA, 1.5 mM of sodium dodecyl sulfate, 100 μL/I of diethylamine, 12% methanol, and 12% acetonitrile adjusted to pH 6.0 using phosphoric acid. The electrochemical detector (Antec, The Netherlands) was set at 500 mV versus an ISAAC reference electrode (Antec, The Netherlands) at 30°C. Tissue monoamine concentration was expressed as picograms per milligram wet tissue.

2.2.10 | Statistical analysis

For statistical analysis, PASW/SPSS (version 21) was used. Data were analyzed by one-way or two-way analysis of variance (ANOVA) for repeated measures, followed by a Bonferroni post hoc analysis whenever appropriate. Statistical significance was set at P < .05.

3 | RESULTS

3.1 | Effects of DH infusions of C16 ceramide on alcohol drinking, behavior, and brain tissue monoamine levels

C16 ceramide infusions into the DH did not alter alcohol consumption (Figure 2A; treatment effect F1,13 = 1.15; P = .30), but induced a depressive-like phenotype as indicated by an increased feeding latency in the NSF paradigm (Figure 2B; treatment × drinking effect F1,25 = 11.3; P = .002) and an increased immobility time during the FST (Figure 2C; treatment × drinking effect F1,25 = 11.1; P = .003) between water-drinking vehicle- and water-drinking C16-treated mice. Interestingly, alcohol consumption also induced a depressive-like phenotype in vehicle-treated mice (P < .05 versus water-drinking vehicle-treated mice), but normalized C16 ceramide-induced depressive-like phenotype (P < .05 alcohol-drinking C16-treated mice versus water-drinking C16-treated mice). Non-social anxiety-like behavior (Figure 2D; treatment effect F1,25 = 0.21; P = .65), social anxiety-like behavior (Figure 2F; treatment effect F3,50 = 0.55; P = .65), and locomotor activity (Figure 2E; treatment effect F1,25 = 2.34; P = .14) were not altered by C16 ceramide infusions into the DH.

Neither C16 infusions into the DH nor alcohol drinking altered tissue monoamine levels, i.e., noradrenaline, dopamine, and serotonin, in the prefrontal cortex, ventral striatum, and dorsal mesencephalon (Figure S2).

3.2 | Effects of BLA infusions of C16 ceramide on alcohol drinking, behavior, and brain tissue monoamine levels

C16 ceramide infusions into the BLA induced a short-term increase in alcohol consumption during day 1, but not during the following days (Figure 3A; treatment × drinking day effect F4,56 = 3.02; P = .03), and induced depressive-like and non-social anxiogenic-like phenotypes as indicated by an increased feeding latency in the NSF paradigm (Figure 3B; treatment × drinking effect F1,25 = 4.9; P = .04) and a decreased time spent on the open arms of the EPM (Figure 3D; treatment × drinking effect F1,25 = 5.53; P = .03) between water-drinking vehicle- and water-drinking C16-treated mice. However, C16 ceramide infusions into the BLA did not induce a depressive-like phenotype in the FST as indicated by the similar immobility time between water-drinking vehicle- and water-drinking C16-treated mice (Figure 3C; treatment effect F1,25 = 2.65; P = .12), suggesting that C16 ceramide infusions into the BLA induce a
predominantly non-social anxiogenic-like phenotype. Interestingly, alcohol consumption induced a depressive-like phenotype as indicated by an increased feeding latency in the NSF paradigm (Figure 3B) and an increased immobility time during the FST (Figure 3C; drinking effect $F_{1,25} = 5.51; P = .03$; treatment × drinking effect $F_{7,25} = 6.35; P = .02$) between alcohol-drinking vehicle- and water-drinking vehicle-treated mice. Locomotor activity (Figure 3E; treatment effect $F_{1,25} = 0.02; P = .88$) and social anxiety-like behavior (Figure 3F; treatment effect $F_{3,50} = 0.98; P = .41$) were not altered by C16 ceramide infusions into the BLA.

Neither C16 infusions into the BLA nor alcohol drinking altered tissue monoamine levels, i.e., noradrenaline, dopamine, and serotonin, in the prefrontal cortex, ventral striatum, and dorsal mesencephalon (Figure S3).

3.3 Effects of DH infusions of C8 and C20 ceramides on alcohol drinking and behavior

C8 and C20 ceramide infusions into the DH did not alter alcohol consumption (Figure 4A; treatment effect $F_{2,25} = 0.18; P = .84$), depressive-like behavior (Figure 4B; treatment effect $F_{2,70} = 0.26; P = .77$; Figure 4C; treatment effect $F_{2,70} = 0.67; P = .51$), non-social anxiety-like behavior (Figure 4D; treatment effect $F_{2,71} = 0.08; P = .92$), social anxiety-like behavior (Figure 4F; treatment effect $F_{5,142} = 0.90; P = .48$), or locomotor activity (Figure 4E; treatment effect $F_{2,71} = 0.76; P = .47$). However, alcohol consumption consistently induced a depressive-like phenotype as indicated by an increased feeding latency in the NSF paradigm (Figure 4B; drinking effect $F_{1,70} = 17.4; P < .001$) and an increased immobility time during the FST (Figure 4C; drinking effect $F_{1,70} = 12.4; P = .001$) between alcohol-drinking and water-drinking mice, independent of treatment.

4 Discussion

The present study demonstrates for the first time that ceramides affect alcohol consumption and depressive-like and anxiety-like behavior in a brain region- and ceramide species-specific way. In more detail, we show that C16 ceramide induces a depressive-like phenotype when infused into the DH, but a predominantly non-social anxiogenic-like phenotype when infused into the BLA. We also show that C16 ceramide induced a short-term increase in alcohol consumption when infused into the BLA, but not into the DH. Alcohol drinking, on the other hand, normalized C16 ceramide-induced depressive-like and anxiogenic-like phenotypes. These effects were specifically mediated by C16 ceramide in DH and/or BLA, as C8 and C20 ceramide infusions into the DH did not induce any behavioral alterations.
We have previously shown that C16 ceramide infusion into the DH induced a depressive-like phenotype in C57BL/6J mice as assessed in the NSF paradigm and sucrose preference test.\textsuperscript{11} The present study confirmed these effects in the NSF paradigm and FST. Feeding latency, as an indicator of depressive-like and non-social anxiety-like behavior, was assessed on day 36 in the novelty-suppressed feeding paradigm (B). Percentage immobility, as an indicator of depressive-like behavior, was assessed on day 43 in the forced swim test (C). Percentage time spent on the open arms (D) and number of closed arm entries (E), as indicators of non-social anxiety-like behavior and locomotor activity, respectively, were assessed on day 38 in the elevated plus maze test. Percentage investigation towards an unknown mouse compared with an empty cage, as an indicator of social anxiety-like behavior, was assessed on day 45 in the social preference-avoidance test (F). Data represent means ± SEM, and numbers in parentheses indicate group size. *\textit{P} < .05; two-way analysis of variance (ANOVA) for repeated measures. A, Factors treatment × drinking days. B–E, Factors treatment × drinking. F, Factors group × stimulus.

An indirect involvement of BLA ceramide in non-social anxiety-like behavior has been shown by Ono et al.\textsuperscript{32} who demonstrated increased galactosylceramide concentrations in the BLA, but not in the frontal cortex or hippocampus in early weaned mice, which were associated with increased anxiety-like behavior as assessed on the EPM. As galactosylceramide is generated from ceramide by the enzyme galactosylceramide synthase, the ceramide concentrations in the BLA might also be altered in these highly anxious mice.\textsuperscript{32} We could also show that neither C16 nor C8 or C20 ceramide altered social anxiety-like behavior when infused into the DH. Given that female, but not male tgASM mice showed impaired social preference\textsuperscript{12}, as an indicator of increased social anxiety, it might be possible that ceramides influence social anxiety-like behavior in females only. Alternatively, other ceramide species or other brain regions might mediate the effects of ceramides on social anxiety-like behavior.

Our results indicate that DH mediates particularly the effects of C16 ceramide on depressive-like behavior, as neither C8 ceramide nor C20 ceramide induced a similar phenotype. It remains, however, to be investigated whether C8 and C20 ceramides influence behavior when infused in other brain regions. Furthermore, it is not excluded that other ceramide species might also induce a depressive-like phenotype when infused into the DH. In accordance with our study, tgASM mice, which show increased ASM activity and ceramide
Figure 4  Effects of DH infusions of C8 and C20 ceramides on alcohol drinking, depressive-like behavior, and anxiety-like behavior. Mice were infused with C8 ceramide, C20 ceramide, or vehicle bilaterally into the dorsal hippocampus (DH) on days 21, 23, 25, 28, 30, 32, 35, 37, 39, 42, and 44. Alcohol consumption was assessed between days 28 and 32 in the drinking in the dark paradigm (A), defined as days 1-5 in the figure. Feeding latency, as an indicator of depressive-like and non-social anxiety-like behavior, was assessed on day 36 in the novelty-suppressed feeding paradigm (B). Percentage immobility, as an indicator of depressive-like behavior, was assessed on day 43 in the forced swim test (C). Percentage time spent on the open arms (D) and number of closed arm entries (E), as indicators of non-social anxiety-like behavior and locomotor activity, respectively, were assessed on day 38 in the elevated plus maze test. Percentage investigation towards an unknown mouse compared with an empty cage, as an indicator of social anxiety-like behavior, was assessed on day 45 in the social preference-avoidance test (F). Data represent means ± SEM, and numbers in parentheses indicate group size. *P < .05; two-way analysis of variance (ANOVA) for repeated measures. A, Factors treatment × drinking days. B-E, Factors treatment × drinking. F, Factors group × stimulus

concentrations in the hippocampus, also showed depressive-like and anxiogenic-like phenotypes and increased alcohol consumption in a two-bottle free-choice paradigm.11-13 Similarly, chronic unpredictable stress, which was shown to induce depressive-like and anxiogenic-like phenotypes,33,34 also increased the concentration of several ceramide species including C16:0, C16:1, C18:1, C22:1, and C26:1 in the hippocampus and prefrontal cortex, but not in the amygdala and the cerebellum.35 Although previous studies demonstrated that many of the structural and functional hippocampal alterations induced by chronic unpredictable stress were mediated by increased levels of endogenous corticosterone (CORT),36-38 the increase in hippocampal ceramide concentration may lead to a depressive-like phenotype also via a CORT-independent mechanism. As such, chronic CORT treatment (i.e., exogenous CORT) for 28 days did not affect ASM activity and ceramide concentrations in the hippocampus in C57BL/6J mice, although it induced depressive-like and anxiogenic-like phenotypes in these mice.11 Another mechanism might be via the activation of Janus kinase 3 (Jak-3), as chronic stress induced a higher phosphorylation of Jak-3 in wild-type mice compared with ASM-deficient mice.39 Additionally, administration of amitriptyline, which was previously shown to reduce ceramide concentrations and ASM activity in the hippocampus and to normalize the depressive-like and anxiogenic-like phenotypes in tgASM mice,11 also reduced stress-induced Jak-3 phosphorylation in wild-type mice, but not in ASM-deficient mice.39 The stress-induced depressive-like and anxiogenic-like phenotypes in wild-type mice were reversed by administration of a Jak-3 inhibitor, suggesting that Jak-3 is involved in the mediation of stress-induced depression and anxiety, which is at least partly dependent on ASM and its product ceramide.39

The reason for the lack of behavioral effects of C8 and C20 ceramides is unclear to date. Other brain regions might mediate their effects on alcohol consumption and depressive-like and/or anxiety-like behavior, or C8 and C20 ceramides might influence completely different behaviors not tested in this study. Although higher doses of C8, C16, and C20 ceramides had similar negative effects on behavior, it is not excluded that different doses of C8 and C20 might be needed to modify behavior.

Like in our study, voluntary alcohol drinking in a two-bottle free-choice paradigm normalized the depressive-like phenotype in tgASM mice.13 Although the underlying mechanisms are yet unclear, they might include alcohol effects on ASM activity and sphingomyelin levels. As such, alcohol drinking normalized ASM hyperactivity in the DH and restored sphingomyelin levels in the nucleus accumbens in tgASM mice, which showed reduced sphingomyelin levels in this brain region most likely resulting from enhanced ASM-mediated turnover to ceramide.13 Furthermore, alcohol drinking restored tissue monoamine homeostasis in tgASM mice, which showed decreased serotonin concentration in the ventral striatum, DH, and prefrontal cortex and...
decreased dopamine concentration in the DH.\textsuperscript{13} In our study, however, C16 ceramide infusions into the DH and into the BLA were not sufficient to alter tissue monoamine levels, suggesting that either a longer time of ASM/ceramide system imbalance is necessary to alter monoamine homeostasis or other ceramide species and/or brain regions are mediating these effects.

Interestingly, alcohol consumption induced a depressive-like phenotype in vehicle-treated control mice in all our experiments. Although several studies have shown depressive-like effects during alcohol withdrawal in mice, i.e., several days after the last alcohol presentation (for a review, see Holleran and Winder\textsuperscript{40}), a recent study demonstrated depressive-like effects already 1 day after the last alcohol presentation in the DID paradigm,\textsuperscript{41} suggesting that affective symptoms emerge very early after cessation of alcohol drinking and persist into withdrawal. This depressive-like phenotype correlated with a higher expression of the immediate early gene ErG1 in the CeA and bed nucleus of the stria terminalis, both 1 and 21 days into withdrawal, suggesting that a history of binge drinking produces long-lasting neuroadaptations within brain circuits mediating emotional behavior.\textsuperscript{41} Studies using a two-bottle free-choice paradigm, however, have not reported alcohol-induced depressive-like effects before. It might be therefore possible that the alcohol-induced depressive-like effects of phenotype observed in our study is due to the drinking protocol used, i.e., binge drinking in the DID paradigm followed by voluntary alcohol drinking in the two-bottle free-choice paradigm and the long-lasting neuroadaptations that occur after binge drinking.

Slightly different outputs were observed in the behavioral tests used in this study in control water-drinking mice from different experiments, and these differences might relate to several factors including animal batch, experimenter (handling style and scoring style), and season.

Taken together, our study demonstrates a complex brain region-specific modulation of alcohol consumption and emotional behavior by C16, but not by C8 and C20 ceramides, and provides the framework for future studies investigating the involvement of distinct ceramide species in the regulation of emotional behavior. This in turn might promote the development of selective ceramide-targeted tools for the treatment of alcohol use disorders, major depressive disorder, and/or anxiety disorder.

**ACKNOWLEDGEMENTS**

This work was supported by funding from the Forschungsstiftung Medizin at the University Hospital Erlangen (Friedrich-Alexander-Universität Erlangen-Nürnberg to J.K.) and the German Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung, grant number 01EE1401C to J.K.) and by the Deutsche Forschungsgemeinschaft (DFG) grants MU 2789/8-2 (to C.P.M.), KO 947/15-2 (to J.K.), and GU 335/32-2 (to E.G.).

**CONFLICT OF INTEREST**

The authors declare no potential conflict of interests.

**AUTHORS CONTRIBUTION**

IZ, CPM, and JK designed the study. IZ, SEH, and LSK performed experiments and analyzed the data. IZ wrote the manuscript. All authors revised and approved the final version of the manuscript.

**ORCID**

Julia Zocais \(\text{https://orcid.org/0000-0001-7187-3181}\)

Christian P. Müller \(\text{https://orcid.org/0000-0002-5325-9900}\)

Johannes Kornhuber \(\text{https://orcid.org/0000-0002-8096-3987}\)

**REFERENCES**

1. Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol. 2008;9:139-150.

2. Kornhuber J, Müller CP, Becker KA, Reichel M, Gulbins E. The ceramide system as a novel antidepressant target. Trends Pharmacol Sci. 2014;35:293-304.

3. Goñi FM, Alonso A. Sphingomyelinas: enzymology and membrane activity. FEBS Lett. 2002;531:38-46.

4. Schneider M, Levant B, Reichel M, Gulbins E, Kornhuber J, Müller CP. Lipids in psychiatric disorders and preventive medicine. Neurosci Biobehav Rev. 2017;76:336-362.

5. Brodowicz J, Przegalinski E, Müller CP, Filip M. Ceramide and its related neurochemical networks as targets for some brain disorder therapies. Neurotox Res. 2018;32:474-484.

6. Kornhuber J, Medlin A, Bleich S, et al. High activity of acid sphingomyelinas in major depression. J Neural Transm. 2005;112:1583-1590.

7. Reichel M, Greiner E, Richter-Schmidinger T, et al. Increased acid sphingomyelinas activity in peripheral blood cells of acutely intoxicated patients with alcohol dependence. Alcohol Clin Exp Res. 2010;34:46-50.

8. Reichel M, Beck J, Mühlé C, et al. Activity of secretory sphingomyelinas is increased in plasma of alcohol-dependent patients. Alcohol Clin Exp Res. 2011;35:1852-1859.

9. Mühlé C, Weinland C, Gulbins E, Lenz B, Kornhuber J. Peripheral acid sphingomyelinas activity is associated with biomarkers and phenotypes of alcohol use and dependence in patients and healthy controls. Int J Mol Sci 2018;19 pii: E4028.

10. Gracia-Garcia PD, Rao V, Haughey NJ, et al. Plasma ceramides are elevated in depression. J Neuropsychiatry Clin Neurosci. 2011;23:1-5.

11. Gulbins E, Palmada M, Reichel M, et al. Acid sphingomyelinas-ceramide system mediates effects of antidepressant drugs. Nat Med. 2013;19:934-938.

12. Zocais I, Reichel M, Gulbins E, Kornhuber J. Role of acid sphingomyelinas in the regulation of social behavior and memory. Plos One. 2016a;11:e0162498.

13. Müller CP, Kalinichenko LS, Tiesel J, et al. Paradoxical antidepressant effects of alcohol are related to acid sphingomyelinas and its control of sphingolipid homeostasis. Acta Neuropathol. 2017a;133:463-483.

14. Kornhuber J, Tripal P, Reichel M, et al. Functional inhibitors of acid sphingomyelinas (FIASMAs): a novel pharmacological group of drugs with broad clinical applications. Cell Physiol Biochem. 2010;26:9-20.

15. Kalinichenko LS, Hammad L, Reichel M, et al. Acid sphingomyelinas controls dopamine activity and responses to appetitive stimuli in mice. Brain Res Bull. 2019;146:310-319.
21. Kornhuber J, Zoicas I. Neuropeptide Y prolongs non-social fear in adult male mice. Behav Brain Res. 2016;287:17-22.

22. Rhodes JS, Best K, Bellknap JF, Finn DA, Cranberry JC. Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. Physiol Behav. 2005;84:53-63.

23. Huber SE, Zoicas I, Reichel M, et al. Prenatal androgen receptor activation determines adult alcohol and water drinking in a sex-specific way. Addict Biol. 2018;23:904-920.

24. Kornhuber J, Huber SE, Zoicas I. Effects of conditioned social fear on ethanol drinking and vice-versa in male mice. Psychopharmacology (Berl). 2019;236:2059-2067.

25. Zoicas I, Neumann ID. Maternal separation facilitates extinction of social fear in adult male mice. Behav Brain Res. 2016b;297:323-328.

26. Toth I, Neumann ID, Slattery DA. Social fear conditioning: a novel and specific animal model to study social anxiety disorder. Neuropsychopharmacology. 2012;37:1433-1443.

27. Lukas M, Toth I, Reber SO, Slattery DA, Veenema AH, Neumann ID. The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rats and mice. Neuropsychopharmacology. 2011;36:2159-2168.

28. Pum ME, Carey RJ, Huston JP, Müller CP. Role of medial prefrontal, entorhinal, and occipital 5-HT in cocaine-induced place preference and hyperlocomotion: evidence for multiple dissociations. Psychopharmacology (Berl). 2008;201:391-403.

29. Contreras C, Gonzalez-Garcia I, Martinez-Sánchez N, et al. Central ceramide-induced hypothalamic lipotoxicity and ER stress regulate energy balance. Cell Rep. 2014;9:366-377.

30. Ramirez S, Martins L, Jacas J, et al. Hypothalamic ceramide levels regulated by CPT1C mediate the orexigenic effect of ghrelin. Diabetes. 2013;62:2329-2337.

31. Agoglia AE, Herman MA. The center of the emotional universe: alcohol, stress, and CRF1 amygdala circuitry. Alcohol. 2018;72:61-73.

32. Ono M, Kikusui T, Sasaki N, Ichikawa M, Mori Y, Murakami-Murofushi K. Early weaning induces anxiety and precocious myelination in the anterior part of the basolateral amygdala of male Balb/c mice. Neuroscience. 2008;156:1103-1110.

33. Willner P. The chronic mild stress (CMS) model of depression: history, evaluation and usage. Neurobiol Stress. 2016;6:78-93.

34. Zhou XD, Shi DD, Zhang ZJ. Antidepressant and anxiolytic effects of the proprietary Chinese medicine Shexiang Baoxin pill in mice with chronic unpredictable mild stress. J Food Drug Anal. 2019;27:221-230.

35. Oliveira TG, Chan RB, Bravo FV, et al. The impact of chronic stress on the rat brain lipidome. Mol Psychiatry. 2016;21:80-88.

36. Sousa N, Lukoyanov NV, Madeira MD, Almeida OF, Paula-Barbosa MM. Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement. Neuroscience. 2000;97:253-266.

37. Sousa N, Almeida OF. Disconnection and reconnection: the morphological basis of (mal)adaptation to stress. Trends Neurosci. 2012;35:742-751.

38. Pinto V, Costa JC, Morgado P, et al. Differential impact of chronic stress along the hippocampal dorsal-ventral axis. Brain Struct Funct. 2015;220:1205-1212.

39. Gulbins E, Grassmé H, Hoehn R, et al. Role of Janus-kinases in major depressive disorder. Neurosignals. 2016;24:71-80.

40. Holleran KM, Winder DG. Preclinical voluntary drinking models for alcohol abstinence-induced affective disturbances in mice. Genes Brain Behav. 2017;16:8-14.

41. Lee KM, Coehlo M, McGregor HA, Waltermire RS, Szumlinski KK. Binge alcohol drinking elicits persistent negative affect in mice. Behav Brain Res. 2015;291:385-398.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.