Cocaine attenuates acid sphingomyelinase activity during establishment of addiction-related behavior—A translational study in rats and monkeys

Małgorzata Frankowska¹ | Fernando M. Jesus² | Christiane Mühle³ | Jéssica V.N. Pacheco² | Rafael S. Maior⁴,⁵ | Anna Sadakierska-Chudy¹ | Irena Smaga¹ | Marcin Piechota⁶ | Liubov S. Kalinichenko³ | Erich Gulbins⁷,⁸ | Johannes Kornhuber³ | Małgorzata Filip¹ | Christian P. Müller³ | Marilia Barros²,⁵

¹Department of Drug Addiction Pharmacology, Polish Academy of Sciences, Maj Institute of Pharmacology, Krakow, Poland
²Department of Pharmacy, School of Health Sciences, University of Brasilia, Brasilia, Brazil
³Department of Psychiatry and Psychotherapy, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Erlangen, Germany
⁴Department of Physiological Sciences, University of Brasilia, Brasilia, Brazil
⁵Primate Center, Institute of Biology, University of Brasilia, Brasilia, Brazil
⁶Department of Molecular Neuropharmacology, Polish Academy of Sciences, Maj Institute of Pharmacology, Krakow, Poland
⁷Department of Molecular Biology, University of Duisburg-Essen, Essen, Germany
⁸Department of Surgery, University of Cincinnati, Cincinnati, Ohio, USA

Abstract
Cocaine addiction is a severe psychiatric condition for which currently no effective pharmacotherapy is available. Brain mechanisms for the establishment of addiction-related behaviors are still not fully understood, and specific biomarkers for cocaine use are not available. Sphingolipids are major membrane lipids, which shape neuronal membrane composition and dynamics in the brain. Here, we investigated how chronic cocaine exposure during establishment of addiction-related behaviors affects the activity of the sphingolipid rheostat controlling enzymes in the brain of rats. As we detected specific effects on several enzymes in the brain, we tested whether the activity of selected enzymes in the blood may serve as potential biomarker for cocaine exposure in non-human primates (Callithrix penicillata). We found that intravenous cocaine self-administration led to a reduced mRNA expression of Cers1, Degs1 and Degs2, and Smpd1 in the prefrontal cortex of rats, as well as a reduction of Cers4 expression in the striatum. These effects reversed after 10 days of abstinence. Monkeys showed a robust cocaine-induced place preference (CPP). This coincided with a reduction in blood acid sphingomyelinase (ASM) activity after CPP establishment. This effect normalized after 15 days of abstinence. Altogether, these findings...
suggest that the establishment of cocaine addiction-related behaviors coincides with changes in the activity of sphingolipid controlling enzymes. In particular, blood ASM levels may serve as a translational biomarker for recent cocaine exposure.

**KEYWORDS**
cocaine, conditioned place preference, monkeys, rats, self-administration, sphingolipids, sphingomyelinase

1  |  INTRODUCTION

Cocaine is a widely abused and frequently instrumentalized drug. Continuous use may lead to the establishment of addiction, which is characterized by the emergence of distinct drug-related behaviors. Cocaine addiction is a severe psychiatric condition for which currently no effective pharmacotherapy is available. Brain mechanisms for the establishment of addiction-related behaviors are still not fully understood, and specific biomarkers for cocaine use are not available.

Sphingolipids are major membrane lipids in living cells. They also shape lipid landscape of neuronal membranes in the brain. The distinct sphingolipid composition of membranes controls the formation of lipid rafts at the synapse, which determines signaling protein allocation and turnover. It was believed that the sphingolipid composition of neuronal membranes was rather static and little responsive to environmental influences or behavioral activity. Only recently was it shown that they are highly dynamic in their expression and spatial location and may exert a profound influence on neuronal and network function.

Ceramides and their precursors, sphingomyelins, are prominent sphingolipids that control emotional behavior, as well as learning and memory function in the brain. They are sensitive to stress and a dysregulation may give rise to anxiety and depression. The synthesis and metabolism of both sphingolipid classes are tightly regulated by the sphingolipid rheostat, which comprises numerous enzymes that may regulate membrane concentrations in a subspecies specific way. Enzymes such as acid sphingomyelinase (ASM) may even be the target of pharmacological interventions with strong effects on subjective state and emotional behavior.

We previously reported an important role of sphingolipids and controlling enzymes in the paradoxical antidepressant effects of voluntary alcohol consumption in depressed mice and in mice with neurological disorders. Enzyme activity is dynamically regulated also in peripheral tissue. In the blood, it may serve as a biomarker for alcohol consumption and a depressive state. A particular role of sphingolipid changes in the brain was reported for extinction learning, when a naturally reinforced operant behavior was no longer rewarded and behavioral responses had to be adapted. How sphingolipid systems respond to other drugs of abuse and whether these responses play a role in the establishment and extinction of addiction-related behaviors, or whether they might serve as biomarkers for drug exposure, is currently unclear. Here, we asked whether chronic cocaine exposure and extinction from self-administration affect the expression of major sphingolipid rheostat controlling enzymes in the brain of rats. As we detected specific effects on several enzymes in the brain, we tested whether the activity of selected enzymes in the blood may serve as a potential biomarker for cocaine exposure in non-human primates.

2  |  MATERIALS AND METHODS

2.1  |  Animals: cocaine self-administration in rats

Forty-eight male Wistar rats (290–350 g; Charles River Laboratories, Germany) were used. The study was carried out in accordance with the European Union Directive 2010/63/EU and with approval from the Local Ethics Commission in the Max Institute of Pharmacology, Polish Academy of Sciences. The animals were housed under standard laboratory conditions (see Supporting Information).

2.2  |  Cocaine self-administration and extinction training

The surgical and cocaine self-administration procedures were described previously. Briefly, rats were trained initially for water reinforcement for 1 week with increasing fixed ratio (FR) requirements, from FR1 to FR5. Later, animals were implanted with indwelling jugular catheters and allowed a week for recovery. For rats self-administering cocaine, presses on the active lever under a FR5 resulted in a single 0.1-ml infusion of cocaine (0.5 mg/kg/infusion; cocaine hydrochloride, Sigma-Aldrich, USA, in sterile 0.9% NaCl) and a 5-s conditioned cue. A 20-s time-out period followed each infusion (inactive lever presses had no consequence). Rats underwent 2-h daily sessions 6 days/week for a minimum of 12 days. The extinction training sessions occurred in the same operant chambers and lasted for 2 h daily; however, presses on the previously active lever were no longer linked with cocaine delivery and presentation of the conditioned cue. To distinguish the pharmacological effects from motivation, a yoked procedure was used. Subsets of animals serving as yoked cocaine and yoked saline controls received cocaine or saline infusions, respectively, each time that their active cocaine counterpart received a cocaine infusion.

The experiment consisted of 14 days of cocaine self-administration and early (3 days) and late (10 days) extinction from cocaine self-administration. In each experiment, three groups of rats were tested: 1, active cocaine (SACoc); 2, yoked cocaine (YCoc); and
3, yoked saline (YSal). The animals were the same as in our previous study (n = 8/group; for details, see Supporting Information).  

2.3 | Tissue collection, RNA extraction, and microarray analysis

The animals were sacrificed immediately following the 2-h experimental session on the third and 10th day of extinction training. The dissected hippocampus, prefrontal cortex (PFC), and striatum were rapidly placed on dry ice and frozen at −80°C for further analyses. RNA was isolated, and the Rat 4x44K Gene Expression Array v2 (Agilent Technologies, USA), representing 39 000+ rat genes and transcripts, was used to assess gene expression in the rat brain tissues on the third and 10th days of extinction training (for details, see Supporting Information).

2.4 | Animals: conditioned place preference in monkeys

Twelve adult captive black tufted-ear marmosets (Callithrix penicillata), six males and six females, weighing 340 ± 6 g (range: 305–360 g) at the beginning of the study were used. Animal numbers and all procedures were approved by the Animal Ethics Committee of the University of Brasilia (no. 66744/2016) and carried out in accordance with the Brazilian regulations for the scientific use of laboratory animals (Lei Arouca 11.794/2008), as well as the CONCEA/Brazil and NIH/USA guidelines for care and use of laboratory animals (for details, see Supporting Information).

2.5 | Conditioned place preference apparatus

Conditioned place preference (CPP) was established and tested in a two-compartment rectangular CPP box (120 × 60 × 35 cm) as previously described (for details, see Supporting Information).

2.6 | Drugs

Cocaine hydrochloride (7 mg/kg; Sigma-Aldrich, USA) was dissolved in phosphate buffered saline and injected intraperitoneally, in a volume of 0.5 ml/kg, 5-min prior to the behavioral testing. Saline was used as vehicle control on alternating days with cocaine injections. The doses used were based on previous reports in the same species.  

2.7 | Spontaneous activity in new environment

In order to test blood ASM and neutral sphingomyelinase (NSM) activity as a trait marker for general behavioral activity, on day 1, subjects were captured in their home cages, placed in the transportation box and taken to the test room where they were released into the CPP box’s antechamber for a 15-min trial with both compartments being accessible (Figure S1). After that, a blood sample was collected, the marmosets were returned to their home cages via transportation box, and the apparatus was cleaned with 70% alcohol. Subjects were tested in random order. The sessions were held between 13:00 and 17:30 h.

2.8 | Habituation learning

In order to test whether sphingomyelinase activity in the blood would be a predictor for habituation learning and whether habituation learning would affect sphingomyelinase activity, animals were submitted on day 2 again to a test trial, which was performed identical as on day 1 (Figure 6). After behavioral testing was completed, a further blood sample was collected before animals were returned to their home cages.

2.9 | Cocaine conditioned place preference

The habituation trial on day 2 also served as a baseline activity measure for cocaine CPP (H2; Figure S1). Marmosets were assigned to one of two groups: one received 7 mg/kg of cocaine (n = 6) and the other received saline vehicle (n = 6). Locomotion, vigilance behavior, and time spent in each compartment on trial H2 were used to establish two behaviorally equivalent groups. On days 3–14, they all underwent daily conditioning training in one of the compartments, with the common sliding wall of the CPP box remaining shut at all times. On alternate days, the cocaine group was given a cocaine or saline injection (1 ml/kg) for 12 consecutive days (C1–C12). After 5 min, they were confined for 15 min in their designated cocaine- or saline-paired compartment, respectively, that is, days 3, 5, 7, 9, 11, and 13 with cocaine and days 4, 6, 8, 10, 12, and 14 pseudo-conditioned with saline. The saline group received saline injection on all 12 days, but was confined to a different compartment on alternate days, as done with the cocaine group. On day 15, all marmosets were tested for CPP establishment in a 15-min test trial (T1). The animals were left undisturbed for 14 days in their home cages and tested again (T2). On the test days, they were exposed to the entire CPP box with the sliding wall retracted and without any prior cocaine/saline injection. A blood sample was collected 24 h before the T1 and T2 trials (for details, see Supporting Information).

2.10 | ASM and NSM activity analysis

Blood samples were centrifuged, 10 min at 2000 × g with room temperature, and resulting supernatant serum was aliquoted and placed into storage at −80°C for later activity assays. The activity of ASM and NSM in serum was quantified using the fluorescent substrate
BODIPY-FL-C12-sphingomyelin (N-(4,4-difluoro-5,7-dimethyl-4-bora-
3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosyl phosphocholine, D-
7711, Thermo Fisher Scientific, Waltham, MA, USA), as described
previously26 (for details, see Supporting Information).

2.11 | Statistics

2.11.1 | Experiment I

The data are expressed as the means ± SEM. The behavioral data
were analyzed by two-way ANOVA for repeated measures (factors:
lever and session day) followed by a post hoc Newman–Keuls' test
using the Statistica v.10 software. Gene expression data for each time
point and brain structure were analyzed with one-way ANOVA (lever
factor) followed by a Bonferroni post hoc tests.

2.11.2 | Experiment II

Spontaneous activity

In order to test whether ASM/NSM activity can predict spontaneous
behavior, Pearson correlations were calculated on total sample
(n = 12).

Habituation learning

In order to assess habituation learning, behavioral and enzyme activity
changes between day 1 and day 2 were analyzed by paired t tests.
Pearson correlations were calculated for total sample (n = 12).

Cocaine CPP and sensitization

Behavioral parameters as well as enzyme activity were analyzed using
a mixed-design two-way analysis of variance (ANOVA), "treatment
group" and "experimental trial" being the independent and repeated
measure variables, respectively. A CPP effect was established by com-
paring the test trials with pre-CPP levels seen on the last habituation
trial (T1, T2, and H2, respectively). Sensitization was assessed by
comparing the first two and last two conditioning trials (C1, C2, C11,
and C12, respectively). For single time point effects, pre-planned
comparisons were performed using Fisher's LSD test with Bonferroni
correction where appropriate. Pearson correlations were calculated
within the treatment groups. Significance level for all tests was set at
p ≤ 0.05.

3 | RESULTS

3.1 | Cocaine self-administration and extinction
training in rats

The animals showed stable lever pressing during the last three self-
administration days, with less than a 10% difference in their daily
intake of cocaine. In the last self-administration session, the group
1 (Table 1) displayed 154 ± 11 and 3 ± 1 active lever and inactive
lever presses, respectively. During early (3 days) extinction training,
when cocaine was replaced by saline, the active lever presses
compared with last cocaine self-administration session decreased till
29%. In the group 2 (Table 1), the number of active and inactive lever
presses during the last self-administration session and the 10th day of
extinction training were 157 ± 14 and 7 ± 3, and 24 ± 5 and 17 ± 4,
respectively. During the last day of extinction training, the total
number of active lever presses did not differ by more than 16%. In the
yoked cocaine and saline groups, no significant difference in pressing
the active versus the inactive lever was observed (for details, see
Supporting Information).

3.2 | Sphingolipid enzyme gene expression after
cocaine self-administration and extinction training in
rats

The results for the cocaine self-administration and its abstinence on
the gene expression for sphingolipid enzymes in rat brain structures
are shown in Figures 1–5. A one-way ANOVA with Bonferroni

| TABLE 1 | Number of active and inactive lever presses in rats that self-administered cocaine (0.5 mg/kg/infusion, FR5) and underwent 3-day (group 1) or 10-day (group 2) extinction training procedure |

| Experimental group | Behavioral procedures | 3rd extinction day | 10th extinction day |
|--------------------|-----------------------|--------------------|--------------------|
| | Cocaine self-administration | | |
| | Number of lever presses | Active | Inactive | Active | Inactive | Active | Inactive |
| Group 1 | 155 ± 11 *** | 3 ± 1 | 45 ± 3 * *** | 15 ± 4 | - | - |
| Group 2 | 157 ± 14 *** | 7 ± 3 | 55 ± 9 * *** | 20 ± 4 | 24 ± 5 *** | 17 ± 4 |

Note: Number of active and inactive lever presses in rats that self-administered cocaine (0.5 mg/kg/infusion, FR5) and underwent 3-day (group 1) or 10-day (group 2) extinction training procedure. Data are presented as the mean ± SEM.

* p < 0.05.
*** p < 0.001 versus inactive lever presses (in day session).
*** p < 0.001 versus active lever presses during last cocaine self-administration session.
correction revealed significant effect of cocaine self-administration and 3-day extinction training on the Cers1 expression in the PFC and striatum, Cers2 expression in the hippocampus, Cers3 expression in the PFC and striatum, Cers6 expression in the hippocampus and striatum, Degs1 and Degs2 expression in the PFC, Enpp7 expression in the hippocampus, and Smpd1 expression in the PFC and striatum. Following a 10-day abstinence from cocaine self-administration with extinction training, a significant effect on the Cers3 expression in the hippocampus, Cers4 expression in the PFC, Cers5 expression in the hippocampus, Cers6 expression in the striatum, Degs2 expression in...
the PFC, Smpd1 expression in the hippocampus, and Smpd3 expression in the PFC was observed. Post hoc tests showed significant ($p < 0.01$) changes in the expression of sphingolipid enzyme genes for the third extinction day with decreases observed in the PFC (Cers1, Degs1, Degs2, and Smpd1) and striatum (Cers4) of rats with a history of cocaine self-administration as compared with yoked saline controls (Figures 1–5; Table 2). After 10 days of cocaine abstinence, a significant enhancement in the gene expression was demonstrated for Cers3 in the PFC, while a decline was observed for Smpd3 in the PFC (Figures 1–5; Table 2).
The self-administration by itself caused effects evident by a comparison of cocaine self-administration versus yoked cocaine controls. Post hoc tests showed significant ($p < 0.01$) changes in the expression of sphingolipid enzyme genes on the third and 10th day after last cocaine intake. SACoc, cocaine self-administration; YCoc, yoked cocaine control; YSal, yoked saline control ($^{*} p < 0.01$).

**FIGURE 3** The effects of cocaine self-administration and extinction training on brain mRNA expression of genes coding for (A) sphingolipid delta(4)-desaturase (Degs1), (B) delta(4)-desaturase, sphingolipid 2 (Degs2), and (C) serine palmitoyltransferase (Spt1). The bars illustrate the effects of cocaine intake and drug withdrawal with either extinction training or abstinence on the third and 10th day after last cocaine intake. SACoc, cocaine self-administration; YCoc, yoked cocaine control; YSal, yoked saline control ($^{**} p < 0.01$).
3.3 | Sphingomyelinase activity as marker for spontaneous behavior in monkeys

We tested whether blood ASM/NSM activity would predict spontaneous activity of monkeys in a new environment. Exploratory behavior consisted of smelling and/or licking the apparatus, whereas vigilance was the continuous sweeping upward or downward movement of the head while stationary. We found that neither ASM activity nor NSM activity correlated significantly with locomotion, exploration, or vigilance behavior of monkeys in a new environment.
4. Sphingomyelinase activity and habituation learning in monkeys

Monkeys showed habituation learning in a new environment. This was indicated by a decline in locomotion (t = 9.03, p < 0.0001) from first to second day of exposure to the new environment (Figure 7A). Exploration and vigilance behavior did not show habituation (p > 0.05; Figure 7A). Habituation learning had no effect on blood ASM or NSM activity (p > 0.05, Figure 7B). We tested whether basal ASM or NSM activity would predict the degree of habituation learning, but did not find a significant correlation (p > 0.05; Figure 7C,D). These findings suggest that blood ASM/NSM activity may have no relation to habituation learning in monkeys.

3.5 | Sphingomyelinase activity and cocaine conditioned place preference and sensitization in monkeys

Cocaine conditioning sensitized vigilance behavior (trial effect: F_{3,30} = 9.71, p = 0.001; treatment effect: F_{1,10} = 3.68, p = 0.08; interaction: F_{3,30} = 6.79, p = 0.003), but not locomotor activity (trial, treatment, and interaction effect: p > 0.05; Figure 8). Testing for place preference showed a significant preference for the conditioned compartment in the cocaine-treated monkeys compared with the saline-treated controls on test day T1 (trial effect: F_{2,20} = 3.71, p = 0.04; treatment effect: F_{1,10} = 3.68, p = 0.08; interaction: F_{2,20} = 4.73, p = 0.02; Figure 8). This preference was still significant after 15 days of consolidation, at test day T2, which suggests an effective CPP establishment and maintenance for 15 days.

ASM activity declined in the cocaine group during the CPP establishment, which was prevented in the saline-treated animals, suggesting that repeated cocaine exposure attenuates ASM activity in the blood when tested 1 day after the last cocaine administration (Figure 9A). After 15 days of abstinence, this difference was no longer seen (p > 0.05). Although a general effect for treatment (F_{1,10} = 0.2979, p = 0.5971) and trial (F_{2,20} = 1.9784, p = 0.1644) was not detected, it yielded a trend effect for the treatment × trial interaction (F_{2,20} = 2.94, p = 0.08). Pre-planned comparisons showed significantly lower ASM activity in the cocaine-conditioned compared with saline-conditioned animals only at the first CPP test (p = 0.04). A correlation analysis of ASM activity and CPP establishment in the cocaine group suggested a negative relationship (Figure 9B), which however failed to reach statistical significance (CPP establishment: r = -0.52, p > 0.05; CPP consolidation: r = -0.06, p > 0.05).

NSM activity increased during the conditioning and abstinence period, but did not differ between cocaine- and saline-conditioned animals (Figure 9C). There was no general effect for treatment (F_{1,10} = 0.18, p = 0.68) or interaction (F_{2,20} = 0.82, but a significant effect for time (F_{2,20} = 5.99, p = 0.01). However, pre-planned comparisons did not reveal significant differences between groups at single time points (p > 0.05). A correlation analysis within the cocaine group suggested a positive relationship between blood NSM activity and cocaine CPP establishment (r = 0.49, p > 0.05; Figure 9D). This was lost after the consolidation period (r = -0.18, p > 0.05).

The conditioning procedure disrupted the previously observed coupling between blood ASM and NSM activity in the cocaine- and saline-conditioned groups (cocaine: r = 0.13, p > 0.05; saline: r = -0.12, p > 0.05, Figure 9E). This relationship was re-established as a trend after the consolidation period in the saline-conditioned group (r = 0.72, p = 0.10), but not in the cocaine-conditioned group (r = 0.01, p > 0.05).

Altogether, these findings suggest that the establishment of a cocaine CPP is associated with a decline in blood ASM, but not NSM activity. Cocaine may also disrupt the coupling of ASM and NSM activity in the blood. After a period of consolidation with no drug exposure, ASM activity was comparable with non-drugged animals, but coupling of ASM-NSM activity was still disrupted.
In this study, we found that rats that self-administered cocaine show a dysregulation of the sphingolipid rheostat, as measured by the expression of genes encoding crucial enzymes of the sphingolipid regulatory pathways (Table 2). Sphingolipids are lipids composed of a long-chain sphingoid base, most commonly the unsaturated sphingosine. In the cell, they can be synthesized de novo by serine palmitoyltransferase with a condensation of the activated fatty acid palmitoyl-CoA with the amino acid serine. The sphingolipid generated is 3-ketosphinganine/3-dehydrosphinganine. It can then be converted to sphinganine, dihydroceramide, and ceramide (Cer) in three consecutive metabolic steps. They involve the action of the enzymes 3-ketosphinganine reductase, ceramide synthase (Cers) and dihydroceramide desaturase (Degs), respectively. Ceramide is considered to be the central molecule of the sphingolipid pathway with multiple actions in the brain. In addition, it may also serve as the precursor for higher order sphingolipids such as sphingomyelin or glycosphingolipids that shape the composition of plasma membranes. Ceramide can also be phosphorylated by ceramide kinase forming ceramide-1-phosphate or become hydrolyzed to sphingosine by ceramidases. Sphingosine may be phosphorylated to sphingosine-

### TABLE 2  Activity of sphingolipid regulating enzymes in the brain and blood of rats and monkeys after chronic cocaine exposure and an abstinence period

| Enzyme | Species | Hipp | PFC | Striatum | Blood |
|--------|---------|------|-----|----------|-------|
| Spt1   | Rat     | -/-  | -/- | -/-      | -/-   |
| Cers1  | Rat     | -/1  | -/- | -/-      | -/-   |
| Cers2  | Rat     | -/-  | -/- | -/-      | -/-   |
| Cers3  | Rat     | -/1  | -/- | -/-      | -/-   |
| Cers4  | Rat     | -/-  | -/- | -/-      | -/-   |
| Cers5  | Rat     | -/-  | -/- | -/-      | -/-   |
| Cers6  | Rat     | -/-  | -/- | -/-      | -/-   |
| Degs1  | Rat     | -/-  | -/- | -/-      | -/-   |
| Degs2  | Rat     | -/-  | -/- | -/-      | -/-   |
| Enpp7  | Rat     | -/-  | -/- | -/-      | -/-   |
| Smdd1/ASM | Rat | -/- | -/- | -/-      | -/-   |
| Smdd3/NSM | Rat | -/- | -/- | -/-      | -/-   |
| Smdd4  | Rat     | -/-  | -/- | -/-      | -/-   |
| ASM    | Monkey  | -/  | o   | o        | o     |
| NSM    | Monkey  | -/  | o   | o        | o     |

Note: Arrows indicate either a significant upregulation (†), downregulation (¶), or no effect (-), (o) not determined (Hipp, hippocampus; PFC, prefrontal cortex; Spt1, serine palmitoyltransferase; Cers, ceramide synthase; Degs1, dihydroceramide desaturase 1; Degs2, dihydroceramide desaturase 2; Enpp7, alkaline sphingomyelinase; Smdd1, acid sphingomyelinase (ASM); Smdd3, neutral sphingomyelinase (NSM); Smdd4, neutral sphingomyelinase-3). Signs indicate 1b – 3a days/10b – 15a after last cocaine exposure.

a mRNA expression after intravenous self-administration versus saline yoked control.
b Enzyme activity after conditioned place preference.

4 | DISCUSSION

In this study, we found that rats that self-administered cocaine show a dysregulation of the sphingolipid rheostat, as measured by the expression of genes encoding crucial enzymes of the sphingolipid regulatory pathways (Table 2). Sphingolipids are lipids composed of a long-chain sphingoid base, most commonly the unsaturated sphingosine. In the cell, they can be synthesized de novo by serine palmitoyltransferase with a condensation of the activated fatty acid palmitoyl-CoA with the amino acid serine. The sphingolipid generated is 3-ketosphinganine/3-dehydrophinganine. It can then be converted to sphinganine, dihydroceramide, and ceramide (Cer) in three consecutive metabolic steps. They involve the action of the enzymes 3-ketosphinganine reductase, ceramide synthase (Cers) and dihydroceramide desaturase (Degs), respectively. Ceramide is considered to be the central molecule of the sphingolipid pathway with multiple actions in the brain. In addition, it may also serve as the precursor for higher order sphingolipids such as sphingomyelin or glycosphingolipids that shape the composition of plasma membranes. Ceramide can also be phosphorylated by ceramide kinase forming ceramide-1-phosphate or become hydrolyzed to sphingosine by ceramidases. Sphingosine may be phosphorylated to sphingosine-
1-phosphate by sphingosine kinases. In this study, cocaine self-administration resulted in a local downregulation of the gene expression of several sphingolipid controlling enzymes, predominantly in the PFC, such as ceramide synthase (CerS1), dihydroceramide desaturase 1 and 2 (Degs1 and Degs2), and ASM (Smpd1).

CerS1 shows specificity for C18 acyl chain length ceramides. It displays a high brain expression, particularly in the hippocampus and cortex, and medium expression in the striatum of mice. It is highly expressed in most neurons of the gray matter, but significantly less in white matter. A global CerS1 deficiency was described in flincher mice. As a consequence, they showed lower total brain ceramide levels. In particular, ceramide Cer18 was reduced, while Cer16 ceramide levels were increased. At a behavioral level, this resulted in cerebellar cell loss and motor impairments. A selective deletion of neuronal CerS1 attenuated Cer18 levels and increased Cer16 and Cer22 ceramide levels in the cerebellum. The CerS1 deletion attenuated locomotor activity and impaired motor learning, anxiety-related behavior was reduced, and spatial working memory deficits emerged. Polymorphisms in the human CerS1 gene that resulted in enhanced CerS1 activity were associated with higher longevity, while CerS1 activity reducing mutations were linked to epilepsy and dementia. Polymorphisms in the human CerS1 gene that resulted in enhanced CerS1 activity were associated with higher longevity, while CerS1 activity reducing mutations were linked to epilepsy and dementia.

Degs1 encodes C4-dihydroceramide desaturase, the last enzymatic step in ceramide synthesis when dihydroceramide is converted to ceramide. In the brain, it is primarily expressed in the cerebellum, medulla, and hippocampus, with only small expression in the cortex and striatum. In humans, a homozygous missense mutation in DEGS1 was associated with a severe regressive neurological disease condition including intellectual disability, spastic paraplegia, and epilepsy. Its specific function in the brain is currently unknown, but it may also be involved in the fine regulation of the balance between sphingolipids and dihydrosphingolipids.

Degs2 shows some expression in the cortex, but little in the hippocampus and striatum. In humans, an association of polymorphisms in the Degs2 gene with the susceptibility for schizophrenia has been reported. Thus, effects on Degs2 expression may possibly mediate psychosis-like effects of high doses of cocaine. However, a downregulation of Degs2 expression was also reported in mice after chronic alcohol intake in the forebrain of alcohol-prefering P-rats.

ASM is widely expressed throughout the brain with high activity in the cortex, hippocampus, and striatum. While an ASM knockout has little effects on behavior, selective overexpression can enhance ceramide load in the hippocampus, impair neurogenesis, and induce depression. In particular, a selective increase of Cer16:0, but not Cer18:0 or Cer20:0, as it is also observed after chronic stress can induce depression-like behavior in mice. In contrast, a local increase of Cer16:0 in the basolateral amygdala can induce anxiety-like behavior. Here, we observed a decline in Smpd1 expression in the PFC after self-administering cocaine in rats. This effect may be more related to cellular plasticity, as it was also observed after extinction of a previously food-rewarded operant behavior. Extinction was paralleled by a decline in brain ASM activity. Thereby, the stronger the decline in dorsal hippocampal efficacy emerged, the more

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**FIGURE 7** Habituation learning and blood acid sphingomyelinase (ASM) and neutral sphingomyelinase (NSM) activity in monkeys (mean + SEM). (A) Habituation learning became evident by a decline in locomotor activity, but not in exploration or vigilance behavior. (B) Habituation learning was not associated with altered blood ASM or NSM activity. (C) Neither basal ASM nor NSM activity predicted the degree of habituation learning. (D) No association of changes in ASM or NSM activity during habituation learning with altered locomotor activity (*p < 0.001*)
Effective was the behavioral extinction. From this study, it was concluded that a temporal and locally restricted decline in ASM activity may facilitate behavioral plasticity. In the present study, rats were withdrawn from operant cocaine self-administration, which may also constitute an extinction condition in which no further option for a previously rewarded behavior was learned. However, a role in extinction learning was only suggested for the dorsal hippocampus, and PFC influences not measured. For cocaine addiction-related behaviors and plasticity in the adjustment of drug seeking and self-administration, the PFC may play a more important role. There was also a downregulation of ceramide synthase 4 (\( \text{CerS4} \)) expression in the striatum after cocaine self-administration in rats. \( \text{CerS4} \) shows specificity for C18 and C20 acyl chains. Similar to \( \text{CerS1} \), it is widely expressed in the brain, but involvement of \( \text{CerS4} \) in emotion and behavioral organization still has to be determined. Altogether, these results suggest that establishment and maintenance of a crucial cocaine addiction-related behavior, the self-administration of cocaine, is associated with a downregulation of sphingolipid rheostat controlling enzymes predominantly in the PFC, a brain region that is involved in reward learning and addiction related behaviors.

After 10 days of abstinence, the effects on \( \text{CerS1} \), \( \text{DegrS1} \), \( \text{DegrS2} \), \( \text{Smpd1} \), and \( \text{CerS4} \) expression returned to baseline level, which suggests fully reversible effects. However, some new effects emerged only during this time: an upregulation of \( \text{CerS3} \) expression in the hippocampus and a down-regulation of \( \text{Smpd3} \) expression in the PFC. While \( \text{CerS3} \) shows only little expression in the brain, NSM (\( \text{Smpd3} \)) is highly expressed in neurons of the hippocampus, striatum, and in the cortex. Complete \( \text{Smpd3} \) deficiency results in severe perturbation of postnatal growth and development including cognitive deficits.

During abstinence from cocaine, many of the effects on sphingolipid controlling enzymes normalize to the level of control animals. This may suggest that the observed effects are temporary results of the cocaine exposure and of acute cocaine action in the brain. They may not reflect mechanisms of neuronal plasticity and drug memories, as they are rather enduring and extinction resistant. Newly emerging effects on \( \text{CerS3} \) and NSM may, however, be long lasting adaptations that contribute to emotional state after cocaine withdrawal.

Conditioned drug-seeking behavior can be measured in a CPP paradigm. We tested whether sphingolipid rheostat controlling enzymes would respond to recent cocaine exposure and CPP learning and consolidation/abstinence in a translational approach in monkeys, thereby getting closer to the human condition. We established a cocaine CPP in monkeys as previously reported. The CPP establishment went along with a decline in blood ASM, but not NSM activity. After a CPP consolidation period, which is also an abstinence time, ASM activity returned to control levels, while NSM activity did not change. In particular, the temporal effects on ASM closely resemble those in the PFC. It may be argued that the observed temporal decline in brain ASM activity in rats may reflect cocaine self-administration extinction learning, as a similar ASM dynamic was previously reported for the extinction of a food-reinforced operant response. However, it should be noted that despite cocaine availability had ceased in both settings, the conditions in both experiments were not exactly the same. In the rats, there was an extinction training, while in the monkey study, animals were left alone without any explicit extinction learning. As such, ASM activity might possibly respond to both, extinction learning and other learning processes associated with the withdrawal from cocaine.

Chronic alcohol exposure was associated with increased peripheral ASM activity and higher concentrations of several ceramide species in humans. The observed opposite direction of effects for cocaine may reflect the distinct ways of action of the different classes of drugs.

We tested whether ASM and NSM activity in the blood can serve as trait marker during spontaneous behavioral activity and during habituation learning. Results of this analysis do not suggest
a relationship of ASM or NSM activity with spontaneous behavior. While monkeys showed habituation learning in a new environment, enzyme activity was not changed. This suggests that blood ASM and NSM are not responsive to a basal learning process with natural novelty reward. In the blood, we found a functional coupling of ASM and NSM activity, which was disrupted after cocaine exposure.

5 | CONCLUSION

The present study showed that cocaine self-administration in rats may cause complex dysregulation of the sphingolipid rheostat at single brain area level. In particular, it caused a downregulation of ASM activity in the PFC. Many of those effects reverse after protracted abstinence. The ASM adaptations could be replicated in
non-human primates in a cocaine CPP paradigm at the level of peripheral ASM activity. Altogether, these findings suggest the enzymes of the sphingolipid rheostat in the brain as a potential substrate for changes in brain function as they occur after establishment of cocaine-addiction related behaviors. In particular, blood ASM levels may serve as a translational biomarker for recent cocaine exposure.

ACKNOWLEDGEMENTS
C.M. is an associated fellow of the research training group 2162 funded by the DFG grant 270949263/GRK2162/1. F.M.J. and J.V. N.P. received study scholarship from CAPES and ProIc/CNPq, respectively, while M.B. (305525/2018-2) and R.S.M. (310719/2017-8) received research fellowships from CNPq. The authors thank Dr. A.R.S. for marmoset blood sampling and G.V.S., A.G.A., and L.R.S. for excellent animal care. This work was supported by grants from the Secretaria Nacional Anti-Drogas of Brazil to M.B. (SENAD/MJ; TED 27/2015), from the German National Science Foundation (Deutsche Forschungsgemeinschaft [DFG]), grants MU 2789/8-2, GU 335/29-2, and KO 947/15-2, a grant from the Polish National Science Centre (no. UMO-2012/06/A/NZ3/00022), and by bilateral cooperation (no. PPN/BIL/2018/1/00004) between the Polish National Agency for Academic Exchange (NAWA) and the German Academic Exchange Service (DAAD). Open access funding enabled and organized by Projekt DEAL.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHORS CONTRIBUTION
CPM, MB, and MFi initiated and planned the study. MFr, FMJ, CM, JVNP, RSM, ASC, IS, MP, LSK, and CPM performed the experiments. CPM, MB, and MFi initiated and planned the study. MFr, FMJ, CM, is an associated fellow of the research training group 2162 funded by the DFG grant 270949263/GRK2162/1. F.M.J. and J.V. N.P. received study scholarship from CAPES and ProIc/CNPq, respectively, while M.B. (305525/2018-2) and R.S.M. (310719/2017-8) received research fellowships from CNPq. The authors thank Dr. A.R.S. for marmoset blood sampling and G.V.S., A.G.A., and L.R.S. for excellent animal care. This work was supported by grants from the Secretaria Nacional Anti-Drogas of Brazil to M.B. (SENAD/MJ; TED 27/2015), from the German National Science Foundation (Deutsche Forschungsgemeinschaft [DFG]), grants MU 2789/8-2, GU 335/29-2, and KO 947/15-2, a grant from the Polish National Science Centre (no. UMO-2012/06/A/NZ3/00022), and by bilateral cooperation between the Polish National Agency for Academic Exchange (NAWA) and the German Academic Exchange Service (DAAD). The authors declare no conflict of interest.

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How to cite this article: Frankowska M, Jesus FM, Mühle C, et al. Cocaine attenuates acid sphingomyelinase activity during establishment of addiction-related behavior—A translational study in rats and monkeys. Addiction Biology. 2021;26:e12955. https://doi.org/10.1111/adb.12955