Hyperfunction of the stress response system and novelty-induced hyperactivity correlate with enhanced cocaine-induced conditioned place preference in NCAM-deficient mice

Birgit Kähler1  |  Eva Viktoria Romswinkel2  |  Mira Jakovcevski1  |  Ashley Moses2  |  Melitta Schachner1,3,4  |  Fabio Morellini1

1Institute for Biosynthesis of Neural Structures, Center for Molecular Neurobiology Hamburg, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
2Behavioral Biology, Center for Molecular Neurobiology Hamburg, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
3Center for Neuroscience, Shantou University Medical College, Shantou, Guangdong, 515041, China
4Keck Center for Collaborative Neuroscience and Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, NJ 08854, USA

Abstract
Several studies in humans and rodents suggest an association between impulsivity and activity of the stress response on the one hand and addiction vulnerability on the other. The neural cell adhesion molecule (NCAM) has been related to several neuropsychiatric disorders in humans. Constitutively NCAM-deficient (−/−) mice display enhanced novelty-induced behavior and hyperfunction of the hypothalamic-pituitary-adrenal axis. Here we hypothesize that NCAM deficiency causes an altered response to cocaine. Cocaine-induced behaviors of NCAM−/− mice and wild-type (+/+) littermates were analyzed in the conditioned place preference (CPP) test. c-fos mRNA levels were investigated by quantitative polymerase chain reaction (qPCR) to measure neural activation after exposure to the cocaine-associated context. NCAM−/− mice showed an elevated cocaine-induced sensitization, enhanced CPP, impaired extinction, and potentiated cocaine-induced hyperlocomotion and CPP after extinction. NCAM−/− showed no potentiated CPP as compared with NCAM+/+ littermates when a natural rewarding stimulus (ie, an unfamiliar female) was used, suggesting that the behavioral alterations of NCAM−/− mice observed in the CPP test are specific to the effects of cocaine. Activation of the prefrontal cortex and nucleus accumbens induced by the cocaine-associated context was enhanced in NCAM−/− compared with NCAM+/+ mice. Finally, cocaine-induced behavior correlated positively with novelty-induced behavior and plasma corticosterone levels in NCAM−/− mice and negatively with NCAM mRNA levels in the hippocampus and nucleus accumbens in wild-type mice. Our findings indicate that NCAM deficiency affects cocaine-induced CPP in mice and support the view that hyperfunction of the stress response system and reactivity to novelty predict the behavioral responses to cocaine.

Keywords
c-fos, cocaine, conditioned place preference, corticosterone, hippocampus, mouse, NCAM, nucleus accumbens, prefrontal cortex, sensitization
1 | INTRODUCTION

It is commonly accepted that individual vulnerability towards the addictive properties of drugs of abuse is determined by environmental and genetic factors. In particular, studies in mice and humans suggest a link between specific personality and physiological traits such as novelty seeking and activity of the stress response and susceptibility to the effects of drugs of addiction. In humans, it has been reported that there is a strong correlation between impulsivity, mood disorders, novelty seeking, and substance abuse. In mice, animals with high trait anxiety and activity of the stress response develop an enhanced cocaine-induced conditioned place preference (CPP) compared with mice with low trait anxiety and activity of the stress response. Stress is considered one of the major environmental risk factors in addiction, suggesting that susceptibility to drugs of abuse depends on the activity of the hypothalamic-pituitary-adrenal (HPA) axis. Acute stressful events facilitate the expression of addictive behaviors, and chronic stress potentiates cocaine-induced locomotor activity and cue-induced relapse of cocaine self-administration in rats.

We have shown that the stress response is enhanced in mice deficient for NCAM. It has been proposed that altered NCAM function underlies an enhanced susceptibility for developing depression and anxiety disorders in mice and humans, and several studies on humans have related altered expression and function of NCAM with the occurrence of schizophrenia and bipolar neuropsychiatric disorders. NCAM belongs to the immunoglobulin superfamily of cell adhesion molecules and functions in homophilic and heterophilic cell recognition as well as proteolytic fragment-mediated cell interactions that influence signal transduction in distinct manners. More specifically, NCAM is involved in cell migration, neurite outgrowth and targeting, axonal branching, synaptogenesis, and synaptic plasticity. Noteworthy, constitutively NCAM−/− mice display two behavioral features hypothesized to predict an increased sensitivity towards the reinforcing effects of drugs of abuse, namely, enhanced novelty-induced locomotion and anxiety as compared with NCAM+/+ control littermates. Moreover, NCAM−/− mice show elevated activity of the stress response and reduced levels of glucocorticoid receptors in the hippocampus. Here, we used the NCAM−/− mouse as a model to search for a link between behavioral and physiological characteristics supposed to predispose for addiction in humans and cocaine-induced conditioned behavior.

2 | MATERIAL AND METHODS

2.1 | Animals and husbandry

NCAM-deficient (NCAM−/−) and wild-type (NCAM+/+) male littermates were generated by heterozygous breeding and transferred from a pathogen-free breeding facility into a vivarium with an inverted 12:12 light-dark cycle (light off at 7:00 AM). They were maintained in groups of two to four siblings under standard housing conditions (22 ± 1°C, 40-60% humidity, food, and water ad libitum). All mice were inbred on a C57BL/6J background (after at least 10 generations of backcrossing). C57BL/6J adult male mice were used to perform correlation analyses between their behavior in the cocaine-induced CPP test and NCAM mRNA levels in different brain regions. After two weeks of acclimatization, mice underwent the experiments that were performed between 9:30 AM and 5:30 PM under red light. Mice were between 12 and 20 weeks old at the time of the experiments. Experiments were carried out in accordance with the European Community Council Directive (86/609/EEC), and all experimental procedures were approved by the State of Hamburg. Care was taken to minimize pain or discomfort for the animals.

2.2 | Assay for cocaine-induced sensitization and CPP

The cocaine-induced CPP test assesses the rewarding properties of psychostimulants in rodents. It also allows the evaluation of sensitization defined by the further increase of drug-evoked locomotor activity after repeated administration of the drug over consecutive experimental days. The cocaine-induced CPP protocol was performed on five consecutive days comprising a preconditioning trial, three daily conditioning sessions, and a postconditioning trial (Figure 1A). The test was performed in a 50 × 50 × 40 cm box, laminated with rough, matted, light-gray resin. Each box consisted of two compartments (50 × 25 × 40 cm) separated by a white plastic wall with a cylinder containing two removable doors in the middle. Both compartments were illuminated by a white light bulb (10 lux). The two compartments differed in floor texture and landmarks on the walls. For the preconditioning trial, mice were placed in the cylinder connecting the two compartments and left exploring both compartments for 20 minutes. One NCAM+/+ mouse and one NCAM−/− mouse that spent more than 60% of the time in one compartment were excluded from further testing and analysis to avoid intrinsic preferences for one specific compartment. During the conditioning sessions on days 2 to 4, all mice underwent a trial during which they were first injected with vehicle solution (0.9% saline, ip) and then placed into the nonconditioned (CS−) compartment for 20 minutes. Four hours later, the second trial took place during which the mice were injected with either 10 or 0.5 mg/kg cocaine hydrochloride (diluted in saline solution, ip) and placed in the cocaine-conditioned (CS+) compartment for 20 minutes. During all conditioning trials, the door between the two compartments was closed to confine the mice to the compartment into which they had been placed. On day 5, mice performed the postconditioning trial to determine the time spent in the conditioned compartment as compared with the chance level (50%). Mice were placed in the cylinder and were free to explore both compartments for 20 minutes. The software EthoVision (Noldus, Wageningen, The
FIGURE 1  Enhanced cocaine-induced behavior in neural cell adhesion molecule (NCAM)−/− mice. A, experimental design of the cocaine-induced conditioned place preference (CCP) paradigm; B, distance moved (m) in the respective compartment after saline (SAL) or 10 mg/kg cocaine (10 mg) injections during the three conditioning days, **P < .001 for the comparison between genotypes for the distance moved during the saline trials (Newman-Keuls post hoc test after mixed three-way ANOVA); C, time (%) spent in the cocaine-conditioned compartment was measured before (preconditioning) and after (postconditioning) the three conditioning days using 10 mg/kg cocaine, *P < .001 (Newman-Keuls after significant effect of the interaction between genotype and trial calculated with a mixed two-way ANOVA); D, distance moved (m) in the respective compartment after saline (SAL) and 0.5 mg/kg cocaine (0.5 mg) injections during the three conditioning days, ***P < .001 for the comparison between genotypes for distance moved (Newman-Keuls post hoc test after mixed three-way ANOVA); E, time (%) spent in the cocaine-conditioned compartment as measured during the preconditioning and postconditioning trials using 0.5 mg/kg cocaine, **P < .01 (Newman-Keuls after significant effect of the interaction between genotype and trial calculated with a mixed two-way ANOVA). The sample size was 15 NCAM+/+ and 13 NCAM−/− mice in B and C and 13 mice per genotype in D and E. §P < .05 for the comparison between cocaine-induced locomotion on days 3 and 1 regardless of genotype (Newman-Keuls post hoc test after significant effect of treatment analyzed with a mixed three-way ANOVA).
Netherlands) was used to track the position of the mice and calculate distance moved and time spent in each compartment.

2.3 | Extinction and reconditioning to cocaine after extinction

A cohort of mice underwent the cocaine-induced CPP as described above followed by an extinction protocol. The extinction protocol comprised eight postconditioning trials (i.e., mice were not injected and had access to both compartments) of 10 minutes, and each trial was performed on eight consecutive days. Whether mice could re-express the CPP after extinction was tested after one conditioning day as described in the following: On the morning after the last extinction trial, mice underwent one conditioning trial during which they received a cocaine injection (5 mg/kg, p) before being placed into the CS+ compartment without access to the CS− compartment. Four hours later, mice were injected with saline and confined to the CS− compartment. On the following day, mice were subjected to a 20-minute test trial during which they were free to explore both compartments. As a control, NCAM+/+ and NCAM−/− mice that had never been conditioned before (defined as “naive”) underwent a preconditioning trial and a 1-day conditioning session with one conditioning trial with 5 mg/kg cocaine and one conditioning trial with saline, followed by a postconditioning trial 24 hours later.

2.4 | Female-induced CPP test

For this test, the maze was similar to the one described for the CPP test with the only difference being that a transparent plastic beaker was placed in one corner of each compartment. Several holes were drilled into the beaker 1 cm from the bottom. Mice did three conditioning sessions as described for the CPP test. However, this time, the mice did not receive any injection, and an unfamiliar female mouse was placed in the beaker of one of the two compartments during the conditioning trial. Thus, one compartment was always associated with the presence of a female mouse. On day 5, mice underwent the postconditioning trial to determine the time spent in the conditioned compartment as compared with chance level (50%): Mice were placed in the cylinder and were free to explore both compartments for 20 minutes. The extinction protocol consisted of five trials performed on five consecutive days as described for the postconditioning trial, but this time, the duration was 10 minutes.

2.5 | Open field test

The open field was an enclosed arena (50 × 50 × 40 cm) made of wood laminated with light-gray resin and illuminated with white light (50 lux). Mice were placed in one corner of the arena and allowed to freely move for 20 minutes. Total distance moved, mean velocity, mean distance to the wall (a parameter for thigmotaxis) and time spent in the center (an imaginary inner square of 20 × 20 cm) were analyzed with EthoVision. A trained observer scored the behavior of the animals during the first 5 minutes of the test using the software The Observer (Noldus) as described.24

2.6 | Tissue preparation

Blood was collected during the middle of the dark phase from the facial vein in heparinized collection tubes (Sarstedt, Nümbrecht, Germany), centrifuged at 2000 g for 20 minutes at 5°C. Plasma was then collected and stored at −80°C until the level of corticosterone in the plasma was measured. For c-fos mRNA measurements after the CPP test, mice were killed 10 minutes after completion of the last trial. Thereafter, brains were removed, the nucleus accumbens and the prefrontal cortex were dissected by using a mouse brain matrix for 1-mm coronal slices (Item#: RBMA-200C World Precision Instruments, Berlin, Germany), and the cerebellum and hippocampus were collected. Brain structures were immediately frozen in liquid nitrogen within 5 minutes after decapitation and stored at −80°C until use.

2.7 | Real time reverse transcription polymerase chain reaction

Total RNA was isolated using the RNAeasy Mini Kit including DNase digestion (Qiagen, Hilden, Germany). The concentration of RNA was measured spectrophotometrically. Equal amounts of RNA were reverse transcribed into cDNA using Superscript II Reverse Transcriptase Kit (Invitrogen, Karlsruhe, Germany) and random hexamer primers. The following primers were used: c-fos (forward 5′-TTC CAC CCC AGA GTC TGA GGA-3′; reverse 5′-GCT CCA CGT TGC TGA TGC TC-3′; Genbank accession number: NM_010234), NCAM (forward 5′-TGC AGT TTG ATG AGC CAG-3′; reverse 5′-CAC TCC AGC GCC TCT TGG CTT TTA-3′; Genbank accession number: NM_01081445), and, for reference, hypoxanthine-guanine phosphoribosyltransferase (Hprt) (forward 5′-TGG CTT TGC TGA CCT GCT GGA-3′; reverse 5′-TCC CCC GTG GCC ATG TCA TT-3′; Genbank accession number: NM_013556.1). Oligonucleotides were synthesized by Metabion (Martinsried, Germany). Amplicons for all primers were sequenced analyzed to verify specific amplification. Quantitative polymerase chain reaction (PCR) was performed in a total reaction volume of 20 μL using a SYBR green master mix (qPCR Core Kit for SYBRGreen; Eurogentec, Seraing, Belgium) with 1 μL (5 pmol) each of forward and reverse primers and 1 μL of the 1:10 diluted cDNA sample. Amplification conditions were as follows: 45 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Duplicates of each sample were measured and analyzed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Real-time PCR data analysis was performed using the comparative 2−ΔΔC(T) method25 with Hprt as an endogenous reference. For the graphical presentation and statistical analysis, the mRNA amount for each sample was expressed as a
2.8 | Corticosterone ELISA

Levels of corticosterone in the plasma were quantified using a commercially available kit (rat/mouse Corticosterone ELISA; Cusabio Technology LLC, Wuhan, China) according to the manufacturer’s instructions. For the assay, the plasma samples were diluted 1:20 using the sample diluent provided in the kit. Concentrations are expressed as nanogram per milliliter.

2.9 | Statistical analysis

Paired measurements were tested with mixed two- or three-way ANOVA tests, always having "genotype" (NCAM+/+ and NCAM−/−) as the between-groups factor. The within-groups factors were "treatment" (saline and cocaine) and "day" (days 1-3) for distance moved during the conditioning trials, "preconditioning versus post-conditioning trial" in the cocaine-induced CPP test, and "trial" (trials 1-8 or 1-5) for preference for the conditioned compartment during the extinction protocols. Distance moved in the open field test performed after the extinction protocol for assessing reinstatement was analyzed with mixed two-way ANOVA having treatment (saline and cocaine) as the within-groups factor. Expression of c-fos mRNA was analyzed with two-way ANOVA having "genotype" and "compartment" (saline and cocaine) as the between-groups factors. All multifactorial ANOVAs were followed by Newman-Keuls post hoc analyses when appropriate. Depending on the data sets, the t test or Mann-Whitney test were used to compare NCAM+/+ and NCAM−/− groups in monofactorial designs. Correlation analyses were performed with the nonparametric Spearman test. Data analyzed with parametric statistics are represented as means plus standard error of the mean, whereas data analyzed with nonparametric statistics are represented as scatterplots with the median. All tests were two tailed, and the level of significance was set at $P < .05$.

3 | RESULTS

3.1 | Enhanced cocaine-induced behavior in NCAM−/− mice

Cocaine-induced sensitization and CPP were tested using 10 mg/kg cocaine, a dose well accepted to induce reliable CPP in mice. NCAM−/− mice covered longer distances than NCAM+/+ littermates during the trials after injection of saline as vehicle control, in line with a previous study showing enhanced novelty-induced locomotion in NCAM−/− mice12 (effect of the interaction between genotype and treatment, $F(1, 26) = 4.29, P = .048$; Figure 1B). Cocaine led to hyperlocomotion in both NCAM+/+ and NCAM−/− groups as indicated by the enhanced distance moved after injection of cocaine as compared with the control group (effect of treatment, $F(1, 26) = 42.30, P < .0001$; Figure 1B). Moreover, both genotypes showed sensitization to cocaine as indicated by the observation that cocaine-induced locomotion was enhanced on conditioning day 3 as compared with conditioning day 1 (effect of the interaction between treatment and day, $F(1, 26) = 36.50, P < .001$). Cocaine-induced CPP was tested after the three conditioning trials by measuring the time spent in the CS+ compartment during a postconditioning trial of 20 minutes. The mixed two-way ANOVA indicated an effect of the interaction between genotype and trial, $F(1, 26) = 5.19; P = .03$: Post hoc analyses showed that both genotypes spent more time in the conditioned compartment during the postconditioning trial compared with the preconditioning trial (Figure 1C). However, CPP was enhanced in NCAM−/− versus NCAM+/+ mice (Figure 1C). Next, we performed an additional experiment with a new cohort of mice using 0.5 mg/kg cocaine as a concentration that, in preliminary experiments, was not sufficient to induce conditioned place preference in C57BL/6J male mice (data not shown). At 0.5 mg/kg, cocaine-enhanced hyperlocomotion was observed in NCAM−/− versus NCAM+/+ mice (effect of the interaction between genotype and 0.5 mg/kg cocaine: $F(1, 24) = 5.93, P = .023$, Figure 1D). Also, 0.5 mg/kg cocaine-induced CPP was observed only in NCAM−/− mice that spent more time in the CS+ compartment during the postconditioning trial as compared with NCAM+/+ mice and as compared with the time they spent in the compartment during the preconditioning trial (effect of the interaction between genotype and trial, $F(1, 24) = 4.46; P = .04$; Figure 1E). These data indicate that NCAM−/− mice are more susceptible to develop cocaine-induced CPP when compared with their NCAM+/+ littermates.

3.2 | Delayed extinction and enhanced cocaine-induced sensitization and CPP after extinction in NCAM−/− mice

We then tested whether NCAM deficiency affects extinction in mice injected with 10 mg/kg cocaine in the CPP protocol. Preference for the CS+ compartment declined over eight trials of this protocol, although NCAM−/− mice required more trials to extinguish the conditioned preference compared with NCAM+/+ mice (effect of the interaction between genotype and trial, $F(7, 126) = 3.74$, $P < .001$; Figure 2A). Also, NCAM+/+ and NCAM−/− mice that had completed the extinction protocol ("NCAM+/+ post-extinction" and "NCAM−/− post-extinction") showed enhanced susceptibility to develop CPP after a single conditioning trial using 5 mg/kg cocaine when compared with mice that had not been conditioned before ("NCAM+/+ naïve" and "NCAM−/− naïve"). The three-way ANOVA (with "genotype" and "previous experience" as the between-groups factors and "trial" as the within-groups factor) detected a tendency for the interaction between genotype, previous experience, and trial on distance moved during the saline and cocaine conditioning trials, $F(1, 26) = 2.76, P = .09$. Post hoc analyses showed that all groups
moved a greater distance after cocaine injections compared with saline injections. Moreover, NCAM−/− post-extinction mice displayed enhanced cocaine-induced hyperlocomotion as compared to NCAM+/+ post-extinction and NCAM−/− naïve mice (Figure 2B). Also, the conditioning trial induced long-term CPP only in NCAM−/− post-extinction mice that spent more time in the CS+ compartment when tested 24 hours after conditioning as compared with NCAM+/+ post-extinction and NCAM−/− naïve mice (Figure 2C).

These data indicate that 3-day-long cocaine-induced conditioning leads to long-lasting effects on NCAM−/− mice, as shown by their enhanced hyperlocomotion and CPP performance, induced by a single conditioning trial after extinction when compared with NCAM+/+ mice and naïve NCAM−/− mice.

3.3 Conditioned responses and extinction are unaltered in NCAM−/− mice when a natural reward is used

To test whether NCAM−/− mice develop enhanced CPP compared with NCAM+/+ mice independently of cocaine, the behavior was tested in a spatial learning test as described.26,27 In this test, mice develop a preference for a compartment that had been associated with the presence of an unfamiliar female. Preference for the female compartment induced by three learning trials (t_{12} = 0.30; P = .77; Figure 3A) as well as extinction of the conditioned preference (effect of the interaction between genotype and trial, F(4, 48) = 0.39, P = .81; Figure 3B) were similar in both genotypes. Thus, conditioned responses induced by natural positive stimuli are not affected by NCAM deficiency, suggesting that the behavioral alterations of NCAM−/− mice observed in the CPP tests are specific to cocaine and not to natural, unconditioned stimuli.

3.4 Enhanced c-fos expression induced by the conditioned compartment in NCAM−/− mice

To examine whether neural activation is associated with cocaine-induced behavior, c-fos expression was examined in the prefrontal cortex, nucleus accumbens, and cerebellum of NCAM+/+ and NCAM−/− mice. Mice underwent the CPP test using 10 mg/kg cocaine. One week after CPP, groups of NCAM+/+ and NCAM−/− mice were exposed for 30 minutes to the CS+ compartment (associated with 10 mg/kg cocaine injection during CPP) and another group to the CS− compartment (associated with saline injection during CPP) and sacrificed 10 minutes after termination of the test to measure c-fos mRNA expression by quantitative reverse transcription PCR (Figure 4A). Overall, mice exposed to the CS+ compartment showed enhanced c-fos expression in the prefrontal cortex and nucleus accumbens compared with mice exposed to the CS− compartment (Figure 4B,C). Two-way ANOVA detected a tendency for an effect of the interaction between genotype and compartment for c-fos expression in the nucleus accumbens, F(1, 18) = 4.13 P = .057 and a significant effect in the prefrontal cortex, F(1, 18) = 4.49 P = .048. Post hoc analyses indicated that c-fos mRNA levels, induced by exposure to the CS+ compartment, were higher in NCAM−/− compared with NCAM+/+ mice (Figure 4B,C). No effect was detected for c-fos mRNA levels in the cerebellum (Figure 4D).

3.5 Cocaine-induced hyperlocomotion and CPP correlate with novelty-induced locomotion and corticosterone levels

To test the hypothesis that enhanced cocaine-induced CPP of NCAM−/− mice relates to their elevated novelty-induced behavior
and activity of the HPA axis, we sought to correlate cocaine-induced behaviors with basal plasma corticosterone levels and behavior in an open field test performed 1 week before cocaine-induced CPP. NCAM−/− mice covered a greater distance and jumped more often as compared with NCAM+/+ mice (Figure 5A,B). Moreover, plasma corticosterone levels were enhanced in NCAM−/− versus NCAM+/+ mice (Figure 5C). Correlation analyses indicated that cocaine-induced CPP positively correlated with distance moved (Spearman $r = .72$, $P < .0001$), jumping (Spearman $r = .59$, $P < .001$), and corticosterone levels (Spearman $r = .64$, $P < .001$). When correlation analyses were performed separately within each genotype, correlations were significant only for the NCAM−/− mice (Figure 6D-F), although tendencies were detected for the NCAM+/+ mice (Figure 6A-C).

NCAM mRNA levels in the hippocampus and nucleus accumbens negatively correlate with cocaine-induced hyperlocomotion and CPP in C57BL/6J mice.

To examine whether cocaine-induced behavior correlates with NCAM expression in specific brain regions of wild-type mice, C57BL/6J mice underwent cocaine-induced CPP with 10 mg/kg as described above and, as expected, showed cocaine-induced hyperlocomotion and sensitization during the conditioning trials (effect of the interaction between treatment and day, $F(1, 18) = 5.06$, $P = .018$; Figure 7A) and developed CPP for the cocaine-associated compartment as tested in the postconditioning trial (paired $t$ test $t_9 = 3.30$, $P = .009$; Figure 7B). Cocaine-induced locomotion during the third conditioning trial negatively correlated with NCAM mRNA levels in the hippocampus (Spearman $r = -.73$, $P = .01$; Figure 8A).

**FIGURE 3** Female-induced conditioned place preference (CPP) is similar in both genotypes. A, time (%) spent in the female-conditioned compartment was measured before (Before Cond.) and after (After Cond.) the three conditioning days. $^*^*P < .01$ as compared with chance level (50%, indicated by a dotted line) (Wilcoxon signed-rank test). B, time (%) spent in the female-conditioned compartment during the extinction protocol (ie, five test trials after the three conditioning days). The sample size was seven mice per genotype.
and nucleus accumbens (Spearman $r = -.64$, $P = .047$; Figure 8C). Cocaine-induced CPP (measured as time spent in the CS+ compartment during the postconditioning trial) negatively correlated with NCAM mRNA levels in the hippocampus (Spearman $r = -.65$, $P = .04$; Figure 8B), and a similar tendency was observed for the nucleus accumbens, although this correlation was not significant (Spearman $r = -.55$, $P = .10$; Figure 8D). No correlation was detected between behavior in the cocaine-induced CPP and NCAM mRNA levels in the prefrontal cortex (Figure 8E,F).

**FIGURE 5** Enhanced novelty-induced activity and basal plasma corticosterone level in neural cell adhesion molecule (NCAM)−/− mice. A, NCAM−/− mice cover longer distances in the open field test versus NCAM+/+ mice; B, jumping was frequently observed for NCAM−/− mice, but not for NCAM+/+ mice during the open field test; C, plasma corticosterone concentration is enhanced in NCAM−/− compared with NCAM+/+ mice. $P < .05$, $^{**}P < .01$, and $^{***}P < .001$ comparison between NCAM+/+ and NCAM−/− mice using an unpaired t test (A,C) or Mann–Whitney test (B). The sample size was 15 mice per genotype.

**FIGURE 6** Cocaine-induced conditioned place preference (CPP) positively correlates with novelty-induced behaviors and plasma corticosterone concentration in neural cell adhesion molecule (NCAM)−/− mice. Correlation analyses between cocaine-induced CPP and distance moved (A,D), jumping (B,E), and plasma corticosterone levels (C,F) as measured 1 wk before CPP. A-C, correlations for NCAM+/+ mice; D-F, correlations for NCAM−/− mice. The sample size was 15 mice per genotype.

4 | DISCUSSION

The present study shows that constitutive NCAM ablation in mice leads to enhanced cocaine-induced responses as tested in the cocaine-induced conditioned place preference paradigm. No differences between genotypes were observed when an unfamiliar female mouse was used as a natural unconditioned stimulus, indicating that the enhanced cocaine-conditioned responses of NCAM−/− mice are not because of improved ability of these mice to associate the cocaine-conditioned compartment with a rewarding stimulus.
Cocaine-induced CPP was observed in NCAM−/− mice in parallel with enhanced cocaine-induced hyperlocomotion and sensitization, at a subthreshold dose, which was ineffective in NCAM+/+ mice. Altogether, these data indicate an enhanced sensitivity to the behavioral consequences of cocaine administration under NCAM deficiency. Moreover, NCAM−/− mice required more days than NCAM+/+ littermates to extinguish the cocaine-induced conditioned response. After the extinction of CPP, a single conditioning trial with 5 mg/kg cocaine was sufficient to reactivate the conditioned response in NCAM−/− mice but not in NCAM+/+ littermates. Importantly, naïve NCAM−/− mice that had not been conditioned before did not develop a preference for the cocaine-associated compartment after a single conditioning trial. These results indicate that the long-term effects of cocaine on behavior are more pronounced in NCAM−/− mice as compared with NCAM+/+ mice. In support of this idea, cocaine priming after extinction showed potentiated effects on NCAM−/− mice that displayed an enhanced cocaine-induced hyperlocomotion not observed in NCAM+/+ and naïve NCAM−/− mice, indicating that, despite the extinction protocol, sensitization to cocaine continued to be present in NCAM−/− mice.

Studies in humans and animals indicate that cocaine-induced CPP and extinction are modulated by the prefrontal cortex and nucleus accumbens, brain regions known to play an important role in relapse. Based on these observations, we measured whether re-exposure to the previously cocaine-conditioned compartment leads to functional activation of these regions, using the cerebellum as a negative control. Our data show that exposure to the cocaine-associated compartment induced higher c-fos mRNA expression in the nucleus accumbens and prefrontal cortex when compared with the exposure to the saline-associated compartment. Since mice had been equally often exposed to both the saline- and cocaine-conditioned compartments, the enhanced c-fos mRNA expression induced in mice exposed to the “conditioned compartment” compared with mice exposed to the “saline compartment” appears to be caused by the association to the previous cocaine injections. Thus, not only cocaine injection, as reported, but also a cocaine-associated stimulus appears to be sufficient to functionally activate the nucleus accumbens and prefrontal cortex, similarly to the ventral tegmental area. Our results are in agreement with previous studies showing that inactivation of the prelimbic cortex and nucleus accumbens mitigates reinstatement of cocaine-conditioned responses. Similarly, reinstatement of cocaine seeking is impaired upon optogenetic inhibition of the prelimbic cortex or nucleus accumbens. It appears that, despite the extinction of the conditioned behavioral response, the network underlying the cocaine-induced behavior continues to be responsive to the conditioned context. Based on the present study, we propose the existence of neural interactions that predispose to a prompt reactivation of cocaine-induced conditioned behaviors during reinstatement or relapse. In this context, it is worth mentioning that enhanced c-fos mRNA expression in the NCAM−/− prefrontal cortex and nucleus accumbens may explain the potentiated reinstatement observed in NCAM−/− mice, supporting the view that NCAM deficiency predisposes to enhanced susceptibility to drugs of abuse. Since we cannot exclude that the observed effects are caused by developmental alterations caused by the constitutive NCAM deficiency, we tested whether NCAM levels correlate with cocaine-induced behavior in wild-type mice. The observation that the amount of NCAM mRNA in the hippocampus and, to a lesser extent, in the nucleus accumbens negatively correlates with cocaine-induced hyperlocomotion and CPP in wild type C57BL/6J mice suggests that the long-term effects of cocaine on brain function, and thereby behavior, is regulated by NCAM in the hippocampus and nucleus accumbens independently of developmental abnormalities caused by constitutive NCAM deficiency. The lack of a correlation between cocaine-induced behaviors and the amount of NCAM mRNA in the prefrontal cortex suggests that the enhanced cocaine-induced CPP of NCAM−/− mice is not...
caused by NCAM deficiency in the prefrontal cortex. This hypothesis would imply that the elevated amount of c-fos mRNA induced by the conditioned compartment in the prefrontal cortex of NCAM−/− mice was a secondary effect of NCAM depletion in the hippocampus and nucleus accumbens. Alternatively, NCAM in the prefrontal cortex modulates, indeed, cocaine-induced behavior, but no correlation could be found because NCAM is weakly expressed in the prefrontal cortex and almost exclusively in interneurons.35,36

Although the aim of the present study was not to investigate the molecular and cellular mechanisms by which NCAM deficiency leads to enhanced behavioral responses to cocaine, several mechanisms can be brought forward based on previous studies. It has been suggested that cell adhesion molecules, such as SynCAM 137 or NrCAM,38 regulate addiction liability because of their pivotal role in synaptic plasticity.39 Similarly, NCAM had long been known to affect synaptic plasticity and ablated NCAM impairs hippocampal long-term

**FIGURE 8** Cocaine-induced sensitization and conditioned place preference (CPP) negatively correlate with NCAM mRNA levels in the hippocampus and nucleus accumbens of C57BL/6J mice; neural cell adhesion molecule (NCAM) mRNA expression in the hippocampus negatively correlates with cocaine-induced hyperlocomotion on conditioning day 3 (A) and time spent in the CS+ compartment during the postconditioning trial (B). NCAM mRNA expression in the nucleus accumbens negatively correlates with cocaine-induced hyperlocomotion on conditioning day 3 (C). A tendency was detected for a negative correlation between c-fos mRNA levels in the nucleus accumbens with time spent in the CS+ compartment during the postconditioning trial (D). No correlation was detected between NCAM mRNA levels in the prefrontal cortex (PC) and cocaine-induced hyperlocomotion on conditioning day 3 (E) and time spent in the CS+ compartment during the postconditioning trial (F). NCAM mRNA expression is indicated as the arbitrary unit (au) calculated for each data point as the relative difference to the group mean for each brain region. The sample size was 10 mice.
potentiation.\textsuperscript{58} Also, a deficit in the polysialylated form of NCAM (polysialylated NCAM) increases GluN2B-mediated transmission and Ca\textsuperscript{2+} transients in hippocampal CA1 indicating that polysialylated NCAM contributes to glutamatergic neurotransmission.\textsuperscript{40} Because considerable evidence indicates that synaptic plasticity and glutamate transmission play an important role in cocaine-induced and addictive behaviors,\textsuperscript{41,42} we propose that the behavioral effects in NCAM−/− mice described in this study are caused by impaired long-term potentiation in regions involved in addiction such as the hippocampus, ventral tegmental area, and/or nucleus accumbens, possibly affecting addiction-related learning and cognitive flexibility. Alternatively, because dopamine D2 receptor expression is enhanced in NCAM−/− mice,\textsuperscript{43} the observed enhanced cocaine-induced behavior of NCAM−/− mice might be caused by altered expression and/or function of dopamine receptors.

It is possible that the same molecular mechanisms underlying the enhanced cocaine responses of NCAM−/− mice, for instance an altered expression of dopamine receptors, are also responsible for the enhanced novelty-induced behavior and elevated activity of the HPA axis\textsuperscript{44} that have been described for NCAM−/− mice.\textsuperscript{12-14} For instance, it has been shown that corticosterone affects dopamine neurotransmission specifically when the dopamine transporter is pharmacologically blocked by cocaine, thus enhancing the effects of cocaine.\textsuperscript{35,46} Also, a 30-minute restraint enhances cocaine-induced CPP due to cholinergic and glutamatergic activation of dopaminergic neurons in the ventral tegmental area.\textsuperscript{47} Since stress is known to affect glutamatergic transmission,\textsuperscript{48,49} it is plausible to assume that several molecular mechanisms are altered under NCAM deficiency, leading to a complex behavioral phenotype affecting the stress response, novelty-induced behavior, and susceptibility to drugs of addiction. Our present data indicate that activity of the HPA axis and novelty-induced behaviors correlate with cocaine-conditioned addictive-like responses at the individual and group levels, supporting the view that subjects with an enhanced novelty-seeking and activity of the stress response are more at risk to develop maladaptive addictive behaviors upon exposure to drugs.

In conclusion, the present study has uncovered a correlational link between NCAM expression, coping strategy in response to novelty and stressors, and risk to develop addictive-like behavior in mice. Since polymorphisms in the NCAM gene have been related to risk for alcoholism in humans\textsuperscript{50} and low expression of polysialylated NCAM has been associated with the risk for alcoholism in mice,\textsuperscript{51} it is tempting to propose that NCAM deficiency or low levels of NCAM expression lead to a general predisposition to the effects of several drugs acting on different molecular targets, such as gamma-aminobutyric acid receptors for alcohol, and serotonin-norepinephrine-dopamine reuptake for cocaine. Since stress downregulates NCAM expression in the brain,\textsuperscript{10} the enhanced susceptibility to drugs of addiction observed in stressed humans\textsuperscript{5,6} might be caused, at least in part, by a stress-induced decrease in NCAM expression. Thus, NCAM-deficient mice can serve as a valid model to investigate the cellular and molecular mechanisms underlying susceptibility to drugs.

ACKNOWLEDGMENTS
The authors thank the State of Hamburg Excellence Initiative (LFF-FV27b, project P4 to FM) and the Center for Molecular Neurobiology Hamburg (ZMNH) for financial support. A. M. was supported by the Presidential Scholarship Program of Florida State University. M. S. thanks the Li Kashing Foundation for financial support. The authors are grateful to Eva Kronberg for excellent mouse husbandry and Achim Dahlmann for genotyping.

AUTHOR CONTRIBUTIONS
B. K., M. J., and F. M. designed the experiments. B. K., M. J., F. M., and E. R. performed experiments. B. K., M. J., E. R., A. M., and F. M. analyzed data. F. M. wrote the manuscript. M. S. proposed the idea to test the link between addiction and aggression in NCAM mutant mice. M. J., A. M., and M. S. edited and critically revised the manuscript for intellectual content. All authors reviewed and approved the final version for publication.

ORCID
Eva Viktoria Romswinkel https://orcid.org/0000-0002-9494-7777
Mira Jakovcevski https://orcid.org/0000-0003-2262-823X
Melitta Schachner https://orcid.org/0000-0002-3316-0778
Fábio Morellini https://orcid.org/0000-0002-5185-3850

REFERENCES
1. Di Segni M, Andolina D, Coassin A, et al. Sensitivity to cocaine in adult mice is due to interplay between genetic makeup, early environment and later experience. Neuropharmacology. 2017;125:87-98.
2. Homberg JR, Karel P, Verheij MM. Individual differences in cocaine addiction: maladaptive behavioral traits. Addict Biol. 2014;19(4):517-528.
3. Evren C, Evren B, Yancar C, Erkiran M. Temperament and character model of personality profile of alcohol- and drug-dependent inpatients. Compr Psychiatry. 2007;48(3):283-288.
4. Murray JE, Dilleen R, Pelloux Y, et al. Increased impulsivity retards the transition to dorsolateral striatal dopamine control of cocaine seeking. Biol Psychiatry. 2014;76(1):15-22.
5. Jakovcevski M, Schachner M, Morellini F. Susceptibility to the long-term anxiogenic effects of an acute stressor is mediated by the activation of the glucocorticoid receptors. Neuropharmacology. 2011;61(8):1297-1305.
6. Preston KL, Epstein DH. Stress in the daily lives of cocaine and heroin users: relationship to mood, craving, relapse triggers, and cocaine use. Psychopharmacology (Berl). 2011;218(1):29-37.
7. Shaham Y, Erb S, Stewart J. Stress-induced relapse to heroin and cocaine seeking in rats: a review. Brain Res Brain Res Rev. 2000;33(1):13-33.
8. Montagud-Romero S, Aguilar MA, Maldonado C, Manzanecho C, Mihatro J, Rodriguez-Arias M. Acute social defeat stress increases the conditioned rewarding effects of cocaine in adult but not in adolescent mice. Pharmacol Biochem Behav. 2015;135:1-12.
9. Manvich DF, Stowe TA, Godfrey JR, Weisnerken D. A method for psychosocial stress-induced reinstatement of cocaine seeking in rats. Biol Psychiatry. 2016;79(11):940-946.
10. Haile CN, GrandPre T, Kosten TA. Chronic unpredictable stress, but not chronic predictable stress, enhances the sensitivity to the behavioral effects of cocaine in rats. Psychopharmacology (Berl). 2001;154:213-220.
11. Glynn RM, Rosenkranz JA, Wolf ME, et al. Repeated restraint stress exposure during early withdrawal accelerates incubation of cue-induced cocaine craving. *Addict Biol*. 2018;23(1):80-89.

12. Brandewiede J, Jakovcevski M, Stork O, Schachner M. Role of stress system disturbance and enhanced novelty response in spatial learning of NCAM-deficient mice. *Stress*. 2013;16(6):638-646.

13. Bisaz R, Schachner M, Sandi C. Causal evidence for the involvement of the neural cell adhesion molecule, NCAM, in chronic stress-induced cognitive impairments. *Hippocampus*. 2011;21(1):56-71.

14. Brennan LM, Maness PF. NCAM in neurodevelopmental and neurodegenerative disorders. *Adv Exp Med Biol*. 2010;663:299-317.

15. Sandi C, Bisaz R. A model for the involvement of neural cell adhesion molecules in stress-related mood disorders. *Neuroendocrinology*. 2007;85(3):158-176.

16. Hidese S, Hattori K, Sasayama D, et al. Cerebrospinal fluid neural cell adhesion molecule levels and their correlation with clinical variables in patients with schizophrenia, bipolar disorder, and major depressive disorder. *Prog Neuropsychopharmacol Biol Psychiatry*. 2017;76:12-18.

17. Piras F, Schiffr M, Chiapponi C, et al. Brain structure, cognition and negative symptoms in schizophrenia are associated with serum levels of polysialic acid-modified NCAM. *Transl Psychiatry*. 2015;5:e658.

18. Sytnyk V, Leshchyns’ka I, Schachner M. Neural cell adhesion molecules of the immunoglobulin superfamily regulate synapse formation, maintenance, and function. *Trends Neurosci*. 2017;40(5):295-308.

19. Belin D, Deroche-Gamonet V. Responses to novelty and vulnerability to cocaine addiction: contribution of a multi-symptomatic animal model. *Cold Spring Harb Perspect Med*. 2012;2:a001940.

20. Piazza PV, Deminière JM, Le Moal M, Simon H. Factors that predict individual vulnerability to amphetamine self-administration. *Drug Neurosci*. 2001;25(4):402-408.

21. KÄHLER ET AL.

22. Druhan JP, Wilent WB. Effects of the competitive N-methyl-D-aspartate receptor antagonist, CPP, on the development and expression of conditioned hyperactivity and sensitization induced by aspartate receptor antagonist, CPP, on the development and expression of conditioned hyperactivity and sensitization induced by cocaine. *Behav Brain Res*. 1999;102(1-2):195-210.

23. Olson VG, Zabetian CP, Bolanos CA, et al. Regulation of drug reward systems and addiction-related behaviors. *Addict Biol*. 2014;19(3):343-350.

24. Muskievicz DE, Uhl GR, Hall FS. The role of cell adhesion molecule genes regulating neuroplasticity in addiction. *Neural Plast*. 2018;2018:386-391.

25. Kochlamazashvili G, Senkov O, Grebenyuk S, et al. Neural cell adhesion molecule-associated polysialic acid regulates synaptic plasticity and learning by restraining the signaling through GluN2B-containing NMDA receptors. *J Neurosci*. 2010;30(11):4171-4183.

26. Lüscher C. The emergence of a circuit model for addiction. *Annu Rev Neurosci*. 2016;39:257-276.

27. Jones S, Banci A. Synaptic plasticity and drug addiction. *Curr Opin Pharmacol*. 2005;5(1):20-25.

28. Xiao MF, Xu JC, Tereshchenko Y, Novak D, Schachner M, Kleene R. Neural cell adhesion molecule modulates dopaminergic signaling and behavior by regulating dopamine D2 receptor internalization. *J Neurosci*. 2009;29(47):14752-14763.

29. Duric V, Banasr M, Franklin T, et al. Cariprazine exhibits anxiolytic and dopamine D3 receptor-dependent antidepressant effects in the chronic stress model. *Int J Neuropsychopharmacol*. 2017;20(10):798-796.

30. Wheeler DS, Ebben AL, Kortoug B, et al. Corticosterone regulates both naturally occurring and cocaine-induced dopamine signaling by selectively decreasing dopamine uptake. *Eur J Neurosci*. 2017;46:2638-2646.

31. Graf EN, Wheeler RA, Baker DA, et al. Corticosterone acts in the nucleus accumbens to enhance dopamine signaling and potentiate reinstatement of cocaine seeking. *J Neurosci*. 2013;33(29):11800-11810.

32. Shinohara F, Asaoka Y, Kamii H, Minami M, Kaneda K. Stress augments the rewarding memory of cocaine via the activation of brainstem-reward circuitry. *Addict Biol*. 2019;24(3):509-521.
and cognitive functions. Int J Neuropsychopharmacol. 2017;20:948-955.

49. Holmes A, Wellman CL. Stress-induced prefrontal reorganization and executive dysfunction in rodents. Neurosci Biobehav Rev. 2009;33:773-783.

50. Yang BZ, Kranzler HR, Zhao H, Gruen JR, Luo X, Gelernter J. Association of haplotypic variants in DRD2, ANKK1, TTC12 and NCAM1 to alcohol dependence in independent case control and family samples. Hum Mol Genet. 2007;16(23):2844-2853.

51. Barker JM, Torregrossa MM, Taylor JR. Low prefrontal PSA-NCAM confers risk for alcoholism-related behavior. Nat Neurosci. 2012;15(10):1356-1358.

How to cite this article: Kähler B, Romswinkel EV, Jakovcevski M, Moses A, Schachner M, Morellini F. Hyperfunction of the stress response system and novelty-induced hyperactivity correlate with enhanced cocaine-induced conditioned place preference in NCAM-deficient mice. Addiction Biology. 2021;26:e12887. https://doi.org/10.1111/adb.12887