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Maternal high-sugar diet changes offspring vulnerability to reinstatement of cocaine-seeking behavior: Role of melanocortin-4 receptors

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Abstract

Maternal diet significantly influences the proper development of offspring in utero. Modifications of diet composition may lead to metabolic and mental disorders that may predispose offspring to a substance use disorder. We assessed the impact of a maternal high-sugar diet (HSD, rich in sucrose) consumed during pregnancy and lactation on the offspring phenotype in the context of the rewarding and motivational effects of cocaine and changes within the central melanocortin (MC) system. Using an intravenous cocaine self-administration model, we showed that maternal HSD leads to increased relapse of cocaine-seeking behavior in male offspring. In addition, we demonstrated that cocaine induces changes in the level of MC-4 receptors in the offspring brain, and these changes depend on maternal diet. These studies also reveal that an MC-4 receptor antagonist reduces the reinstatement of cocaine-seeking behavior, and offspring exposed to maternal HSD are more sensitive to its effects than offspring exposed to the maternal control diet. Taken together, the results suggest that a maternal HSD and MC-4 receptors play an important role in cocaine relapse.

KEYWORDS

cocaine self-administration, high-sugar diet, maternal diet, MC-4R, rat offspring

1 | INTRODUCTION

According to the concepts presented in The Developmental Origins of Health and Disease (DOHaD), exposure to external factors such as stress, infections or maternal diet in the critical period—pregnancy and early childhood—significantly affects the physical and mental development of offspring and is a factor that induces many diseases in later life.[1] Epidemiological and experimental studies have shown the relationship between the composition of the mother's diet (eg, diets rich in fat and sugar or restriction of nutrients) and the increased occurrence metabolic syndrome, obesity, diabetes, and hypertension in offspring.[2,3] Other clinical evidence highlights the risk of presenting behavioral disorders or mental illnesses (increased impulsivity, depression, anxiety, psychosis, memory disorders, and attention-deficit hyperactivity disorder) in children of mothers who consumed high-calorie foods during pregnancy.[4-7] Animal models and recent clinical studies have confirmed that the in utero diet triggers emotional disturbances in adulthood (depression symptoms, anxiety, and resident

Abbreviations: AgRP, agouti-related protein; CD, control diet; DOHaD, Developmental Origins of Health and Disease; FR, fixed ratio; HSD, high-sugar diet; MC-4, melanocortin-4; PND, postnatal day; PR, progressive ratio; α-MSH, α-melanocyte-stimulating hormone.

[Correction added on June 9, 2020, after first online publication: Double quotes has been made consistent through out the article.]

behavior), and the above diseases can contribute to the development of substance use disorders.[8] In subsequent preclinical experiments, separate reports indicated the impact of a maternal diet rich in fats on the enhancement of alcohol and nicotine use[9-11] or the consumption of high-calorie food[12] in offspring. Together, these studies support a positive relationship between maternal diet and a drug-prone phenotype in offspring; however, there is no information on cocaine use disorder and maternal high-sugar diet (HSD).

In recent years, it was shown that the melanocortin (MC) system, particularly melanocortin-4 (MC-4) receptors, is involved in the neurobiological effects of substance use disorders. This is supported by the neurochemical data on MC-4 receptors, which are localized to the reward system (eg, the ventral tegmental area and nucleus accumbens)[13,14]: pharmacological studies that have confirmed the participation of MC-4 receptors in the control of alcohol consumption,[15-18] relapse of stress-induced nicotine exploration behaviors,[19] and development of cocaine sensitization[20,21] and cocaine-induced conditioned place preference.[21] Furthermore, neurochemical studies have shown that the expression of MC-4 receptors in the hypothalamus was higher in animals permanently consuming alcohol and in animals phenotypically selected for alcohol abuse than in control animals.[22,23]

In light of the above information, the main goal of this study was to evaluate the impact of a maternal HSD rich in disaccharides (sucrose) during pregnancy and lactation and its influence on phenotypic changes in rat male offspring regarding cocaine reward and MC-4 receptor expression in the brain. To assess the susceptibility of offspring to the acquisition and maintenance of cocaine reinforcement, we used an intravenous cocaine self-administration model in two separate procedures: (a) an increasing fixed ratio (FR) reinforcement schedule with a stable dose of cocaine and (b) increasing doses of cocaine under a stable FR. In addition, we evaluated the rats' motivation in the progressive ratio (PR) paradigm and the strength of cocaine-seeking behavior in response to drug-associated conditioning and to cocaine priming. Moreover, molecular analyses were performed in a separate cohort of rats after cocaine self-administration and extinction training to evaluate brain MC-4 receptor levels, while pharmacological analyses were performed following blockade of MC-4 receptors during reinstatement tests.

2 | MATERIALS AND METHODS

2.1 | Animals and diets

All experiments were performed in accordance with the guidelines of the European Directive 2010/63/EU and were approved by the Ethical Committee at the Maj Institute of Pharmacology Polish Academy of Sciences. Every effort was made to minimize suffering and the number of animals used.

Wistar rats from Charles River (Sulzfeld, Germany) were housed in standard plastic rodent cages in a room maintained at 22 ± 2°C and 55 ± 10% humidity under a 12 hour light-dark cycle (lights on at 6.00 AM). Unless otherwise specified, animals had free access to water and food. The animals were drug naïve, and each experimental group consisted of 6-12 rats.

Nulliparous female rats (200-240 g), after the acclimatization period and during the proestrus phase (smears from females were collected daily to determine the estrous cycle phase), were mated with males overnight, and pregnancy was confirmed by examining vaginal smears for the presence of sperm. Pregnant females were then individually housed and randomly assigned to two groups: control diet (CD; 65% carbohydrate: 4.64% sucrose, 13% fat, 22% protein (by energy); 3.4 kcal/g; catalog number: VRF1; Special Diets Services, Witham, UK) or specific HSD (70% carbohydrate: rich in disaccharides—44% sucrose, 12% fat, 18% protein (by energy), 3.77 kcal/g; catalog number: C1010; purchased from Altromin, Lage, Germany). Dams were maintained ad libitum on these diets during the gestation (21 days) and lactation periods (21 days). After weaning, male offspring were housed six per cage and fed CD ad libitum. The experimental design of the study is illustrated in Figure 1.

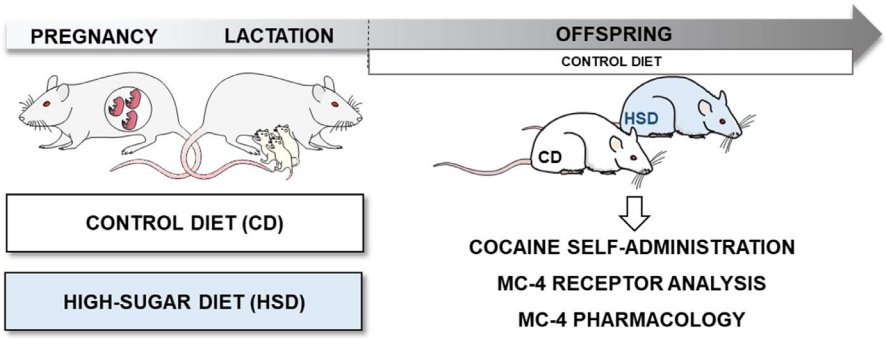

FIGURE 1 Study design. Dams were fed a control (CD) or high-sugar (HSD) diet during pregnancy and lactation. Adult male offspring at postnatal day 63 were assessed for cocaine self-administration and locomotor activity tests. The level of melanocortin-4 (MC-4) receptors after cocaine self-administration and extinction training was evaluated in the brains of offspring, while reinstatement of cocaine-seeking tests were performed using a pharmacological tool.

2.2 | Behavioral procedures

2.2.1 | Drugs

Cocaine hydrochloride (Toronto Research Chemicals, North York, ON, Canada) was dissolved in sterile 0.9% NaCl. The cocaine solution was administered *i.v*. or *i.p*. in a volume of 0.1 mL/infusion or 1 mL/kg, respectively.

ML 00253764 hydrochloride (Tocris Bioscience, Abingdon, UK) was dissolved in 0.5% DMSO and administered *s.c*. in a volume of 1 mL/kg.

2.2.2 | Cocaine self-administration procedure

Catheter implantation and initial lever-press training

At postnatal day (PND) 53, male rats (n = 12 for each group) were anesthetized with ketamine HCl (75 mg/kg; Bioketan; Biowet, Puławy, Poland) and xylazine (5 mg/kg; Sedazin; Biowet) and chronically implanted with a silastic catheter in the external jugular vein, as described previously.[24] After catheter implantation, meloxicam *s.c*. (0.5 mg/kg; Metacam; Boehringer Ingelheim Vetmedica GmbH, Ingelheim am Rhein, Germany) was administered, and rats were kept individually in standard rat home cages (with free access to water and food). Catheters were flushed every day with 0.1 mL of saline solution containing heparin (70 U/mL; Polfa, Warszawa, Poland) or 0.1 mL of cephazolin solution (10 mg/mL; Biochemie GmbH, Kundl, Austria). After 7 days of recovery, animals were food-deprived for 18-20 hours (with free access to water) and were then trained for 2 days to press a lever in 2-h daily sessions under the FR1 schedule with sweetened milk reinforcement. During these 2 training days, food was limited to 70% of the free-feeding amount provided to the rats.

Apparatus

Cocaine self-administration experiments were conducted in standard operant chambers (Med-Associates, Fairfax, VT, USA). Each chamber was equipped with a 24-V house light mounted on the ceiling, two retractable levers on one wall, a water-filled dispenser mounted equidistant between the levers, a white circular stimulus light illuminated by a 24-V bulb above each lever and a tone generator. Pressing on one of the levers (defined as "active") resulted in drug delivery to the animal when schedule requirements were met, whereas pressing on the other lever, the so-called "inactive" lever, was recorded but not reinforced. The completion of each FR1-, FR3-, or FR5-induced intravenous infusions of cocaine through a swivel (Instech Laboratories, Plymouth Meeting, PA, USA) via an infusion pump (Model 3.33 RPM, Med-Associates). The positions of the "active" and "inactive" levers remained unchanged throughout the study. The house light remained on during the experimental sessions. The operant chambers were enclosed in ventilated, sound-attenuating cubicles (Med-Associates), and controlled by an IBM-compatible computer using the Med-Associates MED-PC software package.

Cocaine self-administration

At PND 63, male rats began cocaine reinforcement lever pressing during 2-h daily sessions performed 6 days/week (maintenance) for a total of 3 weeks, and from that time, they were given food ad libitum throughout the remainder of the experiment. The rats were trained to self-administer cocaine according to two experimental protocols: (a) under a stable dose of cocaine (0.5 mg/kg/infusion) and an increased schedule of reinforcement (FR1, FR3, and FR5) or (b) under increased cocaine doses (0.25-1 mg/kg/infusion) and a stable FR1 schedule of reinforcement. Each completion of the schedule by pressing the "active" lever, resulted in a 5 seconds infusion of cocaine and a 5 seconds presentation of the stimulus complex, which consisted of activation of the white stimulus light directly above the "active" lever and a tone from the generator (2000 Hz; 15 dB above the ambient noise level). Following each infusion, there was a 20 seconds time-out period during which the response was recorded but no programed consequences occurred. Pressing the "inactive" lever never resulted in cocaine delivery.

Yoked self-administration procedure

To establish a proper control group for biochemical experiments, the yoked procedure was employed. The rats were tested in parallel in two groups: rats actively self-administering cocaine were paired with rats passively receiving saline ("yoked" saline). Unlike the self-administering rats, the lever pressing by the "yoked" rats was recorded but resulted in no programed consequences.

Progressive ratio test

After the final cocaine experimental session described above, the rats were tested for the self-administration of cocaine under the PR schedule of reinforcement, and this session lasted 4 hours. During the PR session, the delivery of *i.v*. cocaine was contingent on an increasing number of responses in response to the following progression: 1-603.[25] Breakpoints were defined as the number of completed ratios in the series over 4 hours.

Extinction

After cocaine self-administration (see above), the rats previously used for the two different cocaine self-administration protocols were used in extinction training/reinstatement tests. During the 10 extinction sessions, the animals had 1-h daily sessions with neither a cocaine delivery (exchanged to saline) nor presentation of the conditioned stimulus.

Reinstatement of cocaine-seeking

After 10 days of extinction training, the rats were tested for the response reinstatement induced by either a conditioned cue (the tone and light previously paired with cocaine self-administration) or the noncontingent presentation of a self-administered reinforcement (2.5 and 10 mg/kg cocaine, *i.p.*). The order of the cocaine priming injections was counterbalanced according to a Latin square design, and the test sessions were separated by at least 2 to 3 baseline days of the extinction sessions. During the reinstatement tests (2-h sessions), active lever presses resulted in an *i.v.* injection of saline only.

Pharmacological tests

Thirty minutes before each reinstatement test session induced by either a conditioned cue or 10 mg/kg cocaine, *i.p.,* the rats in the CD and HSD groups (n = 9 rats/group) were acutely pretreated with ML 00253764 (2.5, 10 and 15 mg/kg, *s.c.*). The doses were selected based on previous reports.[26,27] Each rat underwent only one type of reinstatement procedure in which either cocaine (10 mg/kg, *i.p.*) or the cue was presented in a maximum of four tests.

2.2.3 | Locomotor activity

The spontaneous locomotor activity of each drug-naïve animal from the subset was recorded individually at PND 63 in Opto-Varimex cages (Columbus Instruments, Columbus, OH, USA) linked online to an IBM-compatible PC. Each cage (43 cm × 44 cm) was equipped with 15 infrared emitters located on the *x* and *y* axes, 3 cm above the floor and with the same number of receivers on the opposite walls of the cage. The behavior of rats was analyzed using Auto-Track software (Columbus Instruments). Locomotor activity was defined as the breakage of three consecutive photobeams. Locomotor activity was defined as horizontal activity and presented as the distance traveled in cm during 5-, 30- and 120-minutes trials.

2.3 | Biochemical analysis

2.3.1 | Brain tissue collection

For biochemical analysis, immediately after 18 sessions of cocaine self-administration or 10 days of abstinence, subsets of "yoked" saline and cocaine animals were sacrificed by rapid decapitation, and the brains were promptly removed. Selected brain structures were dissected according to the rat brain atlas,[28] isolated on ice-cold glass plates, immediately frozen on dry ice and stored at −80°C for enzyme-linked immunosorbent assay (ELISA) analyses.

2.3.2 | Melancortin-4 receptor expression

MC-4 receptor expression was measured in the synaptosomal fraction isolated according to the methods described previously by Kamat et al[29]. The brain tissue samples were homogenized using a sonicator (EpiShear Probe Sonicator; Active Motif, Carlsbad, CA, USA) in 10% (w/v) of 0.32 M sucrose HEPES buffer (containing 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES) with a protease inhibitor cocktail (Complete, Roche Diagnostics, Mannheim, Germany). Later, suspended tissue was homogenized with a Dounce tissue grinder. The homogenate was centrifuged at 4°C for 10 minutes at 600*g*. The supernatant was then diluted 1:1 with 1.3 M sucrose HEPES buffer to obtain a suspension with a final concentration of 0.8 M sucrose. This suspension was further centrifuged twice in a series of washes with HEPES buffer at 4°C for 15 minutes at 12000*g*. The supernatant was discarded each time. The pellet was suspended in RIPA buffer (containing protease inhibitor, PMSF and 0.2% Triton X-100) and centrifuged at 4°C for 30 minutes at 20000*g*. The supernatant containing the synaptosomal fraction was frozen overnight at −20°C and was used for further analyses the following day.

The levels of MC-4 receptors in the synaptosomal fraction of the prefrontal cortex (including the infralimbic, prelimbic and cingulate cortices; bregma: 5.2-2.7 mm), dorsal striatum, nucleus accumbens, ventral tegmental area, hypothalamus, and amygdala were measured using an ELISA method (catalog number: E11964R; Wuhan EIAab Science Co., Wuhan, China) according to the manufacturer's instructions. Duplicate aliquots of 100 μL of each sample and of the along with MC-4 receptor standards (0, 0.312, 0.625, 1.25, 2.5, 5, 10, and 20 ng/mL) was transferred to precoated 96-well ELISA plates. The absorbance was measured at a wavelength of $\lambda = 450$ nm using a Multiskan Spectrum spectrophotometer (Thermo LabSystems, Philadelphia, PA, USA). The concentration of MC-4 receptors was calculated from a standard curve and expressed as ng/mg of protein. For protein measurement, bicinchoninic acid assay (BCA) protein assay kits (Thermo Scientific, Rockford, IL, USA) were used (Pierce BCA Protein Assay Kit for the prefrontal cortex, dorsal striatum and nucleus accumbens and Micro BCA Protein Assay kit for the ventral tegmental area, hypothalamus and amygdala).

2.4 | Statistical analysis

Animals that had problems with catheters during the recovery or experimental periods were excluded from the data analysis. All data are expressed as the mean (±SEM). Statistical analyses were performed with Student's *t* test or one- or multiple-way analysis of variance (ANOVA), with the terms

of the repeated measure analysis dependent on the experiment, using Statistica version 12 software (StatSoft, Tulsa, OK, USA). Post hoc Newman-Keuls tests were used to analyze differences between group means. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Maternal HSD does not change cocaine self-administration and extinction training

We studied the impact of maternal HSD during pregnancy and lactation on cocaine self-administration in male rat offspring in different experimental protocols. To exclude the potential impact of locomotor activity at PND 63 (the day that the cocaine self-administration procedure was started), appropriate measurements were performed on a drug-naïve cohort of animals. The spontaneous locomotor activity, recorded for 5, 30, and 120 minutes, did not differ between the offspring from mothers that consumed different diets (Supporting Table S1).

To determine the effect of maternal HSD on the rewarding properties of cocaine, in each subsequent week, additional effort was required to obtain a fixed dose of cocaine. Figure 2A shows the number of active and inactive lever presses (*upper panels*) and the number of infusions (*lower panels*) during 3 sequential weeks with increasing FR1-5 schedules of reinforcement and a stable dose of cocaine (0.5 mg/kg/infusion) for the CD and HSD groups. Together with the FR increase, we observed an increase in the number of active lever presses; however, in the CD and HSD offspring groups, during the 18 days of cocaine self-administration, the numbers of active and inactive lever presses were similar ($F_{(17, 697)} = 0.773$, $P = .726$). In addition, we did not find differences between the effect of maternal diet on the number of cocaine infusions during three weeks of self-administration ($F_{(17, 357)} = 0.618$, $P = .878$).

With weekly increasing doses of the drug (0.25, 0.5 and 1 mg/kg/infusion) under the stable FR1 reinforcement schedule, we found that the active and inactive lever presses of the HSD group did not differ significantly from those of the CD group ($F_{(17, 646)} = 0.740$, $P = .763$) (Figure 3A). Maternal diets rich in sugar also did not change the sensitivity to the drug, which resulted in a lack of significant changes in the number of cocaine infusions between groups of animals ($F_{(17, 323)} = 0.399$, $P = .985$). In both the CD and HSD groups, the smallest number of cocaine infusions was recorded in the last week of self-administration with the highest dose of cocaine (1 mg/kg/infusion).

Furthermore, the rats in the CD and HSD groups subjected to the PR reinforcement schedule for a dose of cocaine 0.5 mg/kg/infusion (Figure 2B) or 1 mg/kg/infusion (Figure 3B) showed no differences in achieved break point ($t = -0.072$, $df = 20$, $P = .943$ and $t = -0.275$, $df = 19$, $P = .786$, respectively).

Extinction training was introduced to all rats trained to self-administrate cocaine. After substituting saline for cocaine, a progressive decrease in lever responses was observed over the extinction sessions for all diet groups. In the offspring self-administered cocaine (0.5 mg/kg/infusion) under the increasing FR schedule of reinforcement, we did not observed significant effects of maternal HSD on active and inactive lever pressing ($F_{(9, 306)} = 1.103$, $P = .360$) (Figure 2C). Rats of mothers that were fed a modified diet did not differ in their lever pressing response ($F_{(9, 324)} = 1.083$, $P = .375$) (Figure 3C) following cocaine administration (0.25-1 mg/kg/infusion) under an FR1 reinforcement schedule. Despite the lack of statistically significant differences in the extinction phase, the HSD rat groups was characterized by a decreased reduction in the number of presses on the active lever compared to the CD group, which was especially visible during the first 4 days of extinction training (Figures 2C and 3C).

3.2 | Maternal HSD enhances the reinstatement of cocaine-seeking behavior

Following 10 days of extinction training, we tested all groups of rats for response reinstatement induced by the cocaine-associated cue or by cocaine (2.5 or 10 mg/kg, *i.p.*).

The number of active and inactive lever presses by offspring that were previously subjected to self-administered cocaine (0.5 mg/kg/infusion) under the increased FR schedule of reinforcement is shown in Figure 2D. We observed that the cue-induced (light + sound) relapse was significantly higher in the HSD group than in the control group ($F_{(1, 34)} = 6.181$, $P < .05$) as evidenced by the significantly increased number of presses on the active lever. Furthermore, there was no statistically significant difference in the strength of the recurrence of cocaine-seeking behavior between the CD and the experimental diet for cocaine doses of 2.5 mg/kg and 10 mg/kg ($F_{(1, 68)} = 0.485$, $P = .489$). It is worth emphasizing, however, that at the 10 mg/kg dose, rats exposed to the HSD were characterized by an increase in the number of presses on the active lever.

Figure 3D shows the reinstatement response to conditional and unconditional stimuli in animals that were previously trained to self-administer increasing cocaine doses (0.25-1 mg/kg/infusion) under a stable FR1 schedule of reinforcement. We noted that the re-exposure of cocaine-associated cues significantly increased cocaine-seeking behavior in HSD rats compared to the control animals ($F_{(1, 36)} = 8.433$, $P < .01$). Furthermore, maternal HSD did not significantly change the relapse strength after the administration of 2.5 mg/kg or 10 mg/kg cocaine ($F_{(1, 72)} = 1.077$, $P = .303$). Despite the lack of statistical significance, we observed that the offspring from mothers fed the HSD generated more presses on the active lever during cocaine-induced reinstatement than the offspring from mothers fed the CD.

3.3 | Cocaine self-administration increases the expression of MC-4 receptors in CD, but not HSD, rat brain

To determine whether cocaine self-administration and extinction training influence the expression of MC-4 receptors in rat brain structures associated with the reward system and drug-seeking,[30] we trained animals to self-administrate cocaine (0.5 mg/kg/infusion) with an increasing schedule of reinforcement (FR1-5) using the "yoked" paired procedure according to the scheme shown in Figure 4A. The impact of a modified mother's diet on the number of active and inactive

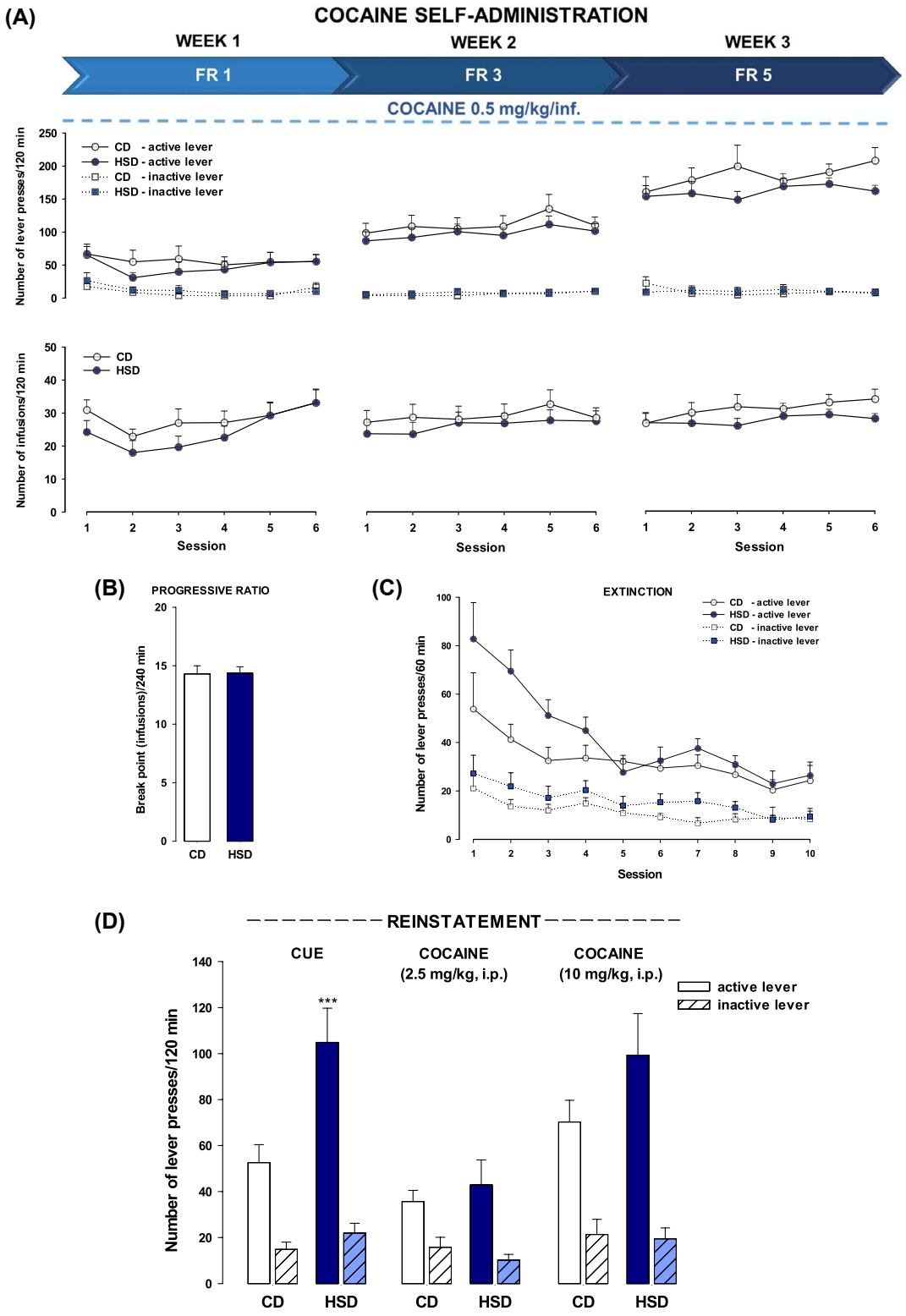

FIGURE 2 Behavioral characteristics of high-sugar diet (HSD) offspring under cocaine self-administration and cocaine-seeking behavior. A, Numbers of active and inactive lever presses (*upper panel*) and infusions (*lower panel*) during the acquisition/maintenance of cocaine (0.5 mg/kg/infusion) self-administration under increasing schedules of reinforcement (FR1-5) in rat offspring. The number of animals in each group was as follows: control diet (CD) (n = 11), HSD (n = 12). B, Break points (number of infusions of cocaine) under a progressive ratio schedule in the CD and HSD offspring groups the day after the last session of cocaine self-administration under an increasing schedule of reinforcement (FR1-5) and a stable dose of cocaine (0.5 mg/kg/infusion). The number of animals in each group was as follows: CD (n = 10), HSD (n = 12). C, Numbers of active and inactive lever presses during extinction from cocaine self-administration under an increased schedule of reinforcement (FR1-5). The number of animals in each group was as follows: CD (n = 9), HSD (n = 10). D, Effects of a maternal HSD during pregnancy and lactation on the reinstatement of the cocaine-seeking behavior induced by the cue (CUE; tone + light) and drug in male rat offspring. Drug-induced reinstatement was triggered by the administration of cocaine (2.5 or 10 mg/kg, *i.p.*). Tests were performed after cocaine (0.5 mg/kg/infusion) self-administration under the increasing schedule of reinforcement (FR1-5) and 10 days of extinction training. The number of animals in each group was as follows: CD (n = 9), HSD (n = 10). Data were analyzed by two-way ANOVA and the post hoc Newman-Keuls test. $^{***}P < .001$ vs the control diet (CD) group. The data are expressed as the mean (±SEM).

lever presses ($F_{(17, 2244)} = 1.106$; $P = .341$) and cocaine intake ($F_{(17, 1122)} = 1.377$; $P = .140$) during self-administration and withdrawal ($F_{(9, 900)} = 0.286$; $P = .978$) confirmed the previous observations described above (Supporting Figure S1).

Studies on the synaptosomal fraction isolated from the brain structures of the CD and HSD rat groups immediately after the last cocaine (0.5 mg/kg/infusion) self-administration session showed significant changes in the level of MC-4 receptors in the prefrontal cortex ($F_{(1, 24)} = 28.414$; $P < .001$), nucleus accumbens ($F_{(1, 24)} = 37.513$; $P < .001$), dorsal striatum ($F_{(1, 24)} = 32.768$; $P < .001$) and ventral tegmental area ($F_{(1, 24)} = 5.913$; $P < .05$) (Figure 4B). At the same time, statistical analyses showed no significant differences in the hypothalamus ($F_{(1, 24)} = 0.778$; $P = .386$) and amygdala ($F_{(1, 24)} = 3.964$; $P = .058$). We observed increased levels of MC-4 receptors in rats self-administering cocaine in the prefrontal cortex, nucleus accumbens, dorsal striatum and ventral tegmental area compared with "yoked" animals, only in the CD rat groups. Moreover, cocaine reduced the expression of MC-4 receptors in the nucleus accumbens in offspring exposed to the HSD during pregnancy and lactation compared with "yoked" rats.

In addition, we compared the expression of MC-4 receptors between the HSD and CD groups. In the "yoked" rats from the HSD group, we recorded a higher level of MC-4 receptors in the nucleus accumbens and the ventral tegmental area than in the "yoked" CD group. Moreover, rats exposed to the maternal HSD and self-administered cocaine were characterized by a decreased level of synaptosomal MC-4 receptors in the prefrontal cortex, nucleus accumbens, and dorsal striatum compared with cocaine self-administered controls.

3.4 | Extinction training reinstates the basal brain MC-4 receptor levels in the CD, but not HSD, group

To assess the effect of abstinence from cocaine on changes in the levels of MC-4 receptors previously observed after cocaine self-administration (0.5 mg/kg/infusion) in rats exposed to maternal CD and HSD, we also performed analyses on isolated brains after extinction training. After a 10-day drug abstinence period with extinction training, we observed significant changes in the number of MC-4 receptors in the synaptosomal fraction in the nucleus accumbens ($F_{(1, 24)} = 7.213$; $P < .05$), ventral tegmental area ($F_{(1, 24)} = 4.629$; $P < .05$) and amygdala ($F_{(1, 24)} = 4.505$; $P < .05$) (Figure 5). At the same time, no differences were noted in the prefrontal cortex ($F_{(1, 24)} = 0.019$; $P = .893$), dorsal striatum ($F_{(1, 24)} = 1.216$; $P = .281$) or hypothalamus ($F_{(1, 24)} = 3.630$; $P = .069$). We found that extinction training normalized the increased expression of MC-4 receptors in rats in the CD group after cocaine intake (see Figure 4B). Thus, in animals previously self-administering cocaine and control "yoked" rats, similar levels of MC-4 receptors were measured in the brain structures. Reduced expression of MC-4 receptors in the nucleus accumbens and ventral tegmental area and significantly increased expression in the amygdala were observed in rats exposed to the HSD in utero and in early life after cocaine abstinence compared to saline "yoked" rats (Figure 5).

Maternal HSD consumption during pregnancy and lactation caused an increase in the levels of MC-4 receptors in the ventral tegmental area in saline "yoked" rats. At the same time, in HSD rats trained to self-administrate cocaine, after the last extinction session, an increase in the number of MC-4 receptors was found in the amygdala compared to the rats from the CD group, while a reduction was observed in the nucleus accumbens (Figure 5).

3.5 | Pharmacological blockade of MC-4 receptors attenuates cocaine-seeking behavior

To verify the hypothesis about the impact of the MC-4 receptor on the reinstatement of cocaine-seeking behaviors, we assessed the effect of acute, peripheral administration of ML 00253764, the MC-4 receptor antagonist with brain-penetrant properties,[27] on the reinstatement of cocaine-seeking behaviors induced by conditional or unconditional stimuli (Figure 6A).

Figure 6B shows the effects of ML 00253764 administration on the number of active and inactive lever presses during cocaine reinstatement tests. We noted that the administration of the MC-4 receptor antagonist significantly changed the cue-induced response in the HSD ($F_{(3, 64)} = 10.413$; $P < .001$) and CD ($F_{(3, 64)} = 3.597$; $P < .05$) groups. In CD animals, only the highest dose (15 mg/kg) of the MC-4 receptor antagonist significantly (by 42%) reduced the reinstatement of cocaine-seeking as evidenced by a decrease in active-lever presses. In contrast, HSD rats responded to the whole range

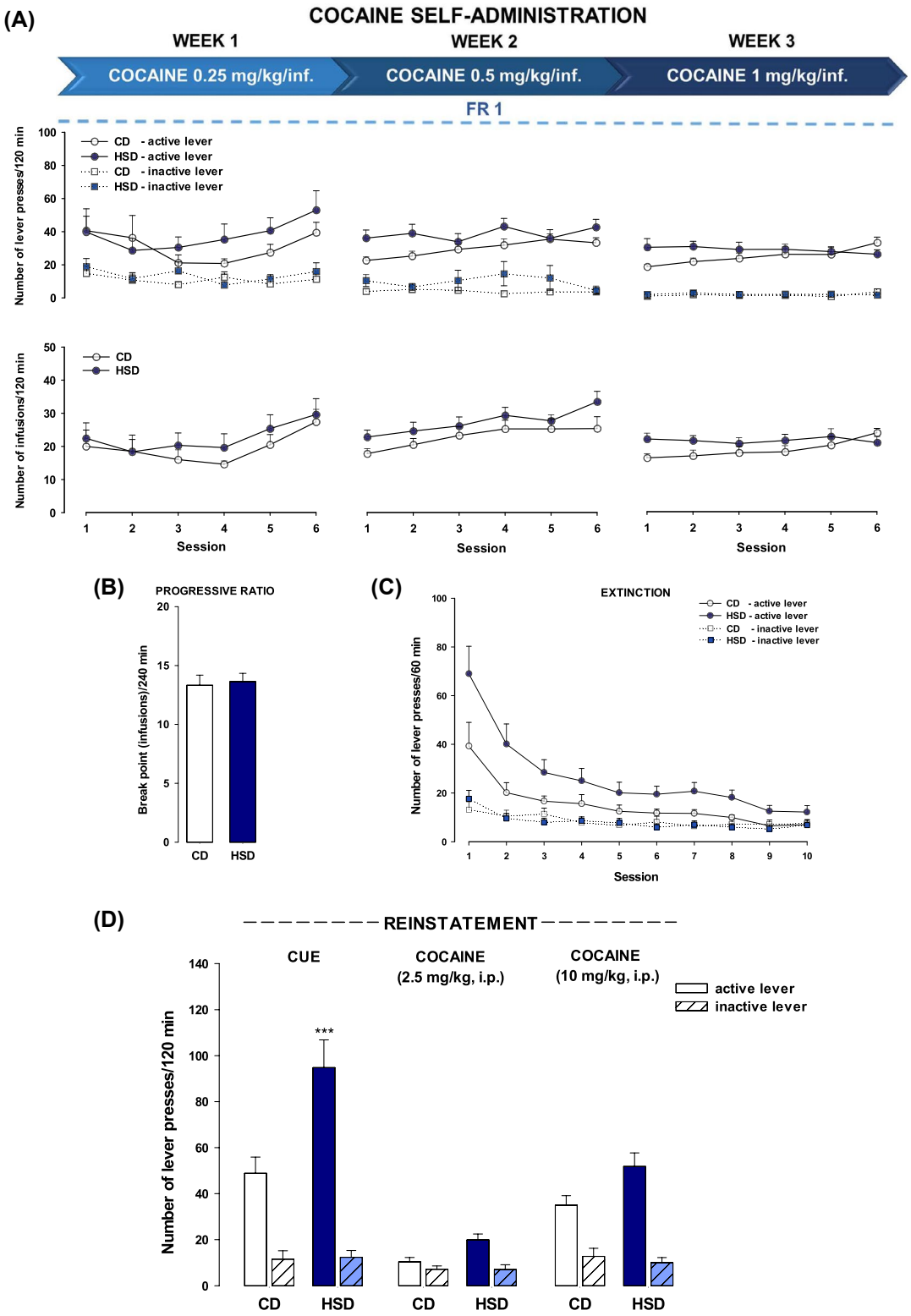

FIGURE 3 Behavioral characteristic of high-sugar diet (HSD) offspring under cocaine self-administration and cocaine-seeking behavior. A, Numbers of active and inactive lever presses (*upper panel*) and infusions (*lower panel*) during the acquisition/maintenance of cocaine (0.25-1 mg/kg/infusion) self-administration under a stable FR1 schedule of reinforcement in rat offspring. The number of animals in each group was as follows: control diet (CD) (n = 10),HSD (n = 11). B, Break points (number of infusion of cocaine) under a progressive ratio schedule in the CD and HSD offspring groups the day after the last session of cocaine self-administration under a stable FR1 schedule of reinforcement and an increasing dose of cocaine (0.25-1 mg/kg/infusion). The number of animals in each group was as follows: CD (n = 10), HSD (n = 11). C, Numbers of active and inactive lever presses during extinction from cocaine self-administration under an increased doses of cocaine (0.25-1 mg/kg/infusion). The number of animals in each group was as follows: CD (n = 9), HSD (n = 11). D, Effects of a maternal HSD during pregnancy and lactation on the reinstatement of the cocaine-seeking behavior induced by the cue (CUE; tone+light) and drug in male rat offspring. Drug-induced reinstatement was triggered the administration of cocaine (2.5 or 10 mg/kg, *i.p.*). Tests were performed after cocaine (0.25-1 mg/kg/infusion) self-administration under a stable FR1 schedule of reinforcement and 10 days of extinction training. The number of animals in each group was as follows: CD (n = 9), HSD (n = 11). Data were analyzed by two-way ANOVA and the post hoc Newman-Keuls test. $^{***}P < .001$ vs the control diet (CD) group. The data are expressed as the mean (±SEM).

of ML 00253764, with 44%, 55% and 62% reductions in active lever presses at 2.5 mg/kg, 10 mg/kg and 15 mg/kg, respectively.

ML 00253764 significantly altered the reinstatement of cocaine-seeking behaviors induced by cocaine priming at 10 mg/kg (*i.p.*) in the CD ($F_{(3, 64)} = 3.118$; $P < .05$) and HSD ($F_{(3, 64)} = 8.219$; $P < .001$) groups. In CD rats, ML 00253764 potently decreased the reinstatement of cocaine-seeking behaviors at its higher (10-15 mg/kg) doses, with no effects observed at the dose of 2.5 mg/kg. In contrast, the HSD group was more sensitive to the action of the MC-4 receptor antagonist (2.5-15 mg/kg) than the CD group; all doses significantly (~30%-70%) reduced the number of active lever presses during cocaine reinstatement tests.

4 | DISCUSSION

This paper reports that modification of the composition of the maternal diet, particularly an increased in the amount of sugars (mainly sucrose) consumed during fetal development in utero and in early childhood, resulted in an increased relapse of cocaine-seeking behavior and changes in MC-4 receptor levels in male offspring brain structures after exposure to cocaine. Moreover, pharmacological intervention with an MC-4 receptor antagonist potently reduced cocaine-seeking behavior in both the control and HSD groups.

The obtained results confirm the DOHaD theory that external factors, including maternal diet (eg, rich in carbohydrates), may play an important role in the risk of behavioral changes in offspring.[8] Although we found here that the maternal HSD did not influence cocaine reward and motivation in male rat offspring, as both groups showed similar responses to different cocaine doses and schedules of reinforcements (FR or PR), the maternal HSD evoked a higher reinstatement response following re-exposure to the cue associated with previous cocaine infusions. Thus, the offspring in the HSD group showed an increase in reinstatement to the drug-associated conditional stimulus, while some (not statistically significant) increase was also observed after cocaine priming. Very recent findings from our group indicate that the above observations are not sex-dependent, as an increase in cocaine-seeking behavior induced by a conditional or unconditional stimulus was also noticed in female offspring exposed to maternal HSD, while the modified maternal diet did not affect the acquisition and maintenance phases of cocaine self-administration (Gawliński et al, in press). At the same time, there was no change in inactive lever responses, which suggests the specificity of the above behavioral responses. In addition, during extinction training, there were no statistically significant differences in the level of response in the CD and HSD groups over the entire 10-day drug-free period. Interestingly, in addition to in utero and early childhood, intermittent or prolonged access to sucrose reward in adult rats promoted cocaine seeking[31] or enhanced cocaine sensitization to behavioral responses.[32] As elegantly reported by Lenoir et al,[33] the effect of intense sweetness surpassed that of cocaine.

Many similarities between a HSD and cocaine have been shown in human and animal offspring. At the behavioral level, in utero cocaine exposure led to earlier and more frequent use of stimulants (eg, cocaine) by adolescent and young adult human offspring,[34-37] while rodent findings indicated that prenatal exposure to cocaine causes increased cocaine self-administration in offspring.[38-40] Similarly, in rats, enhanced responses for sugar[41] or increased responses for cues related to sugar intake[42] were observed after previous sucrose self-administration and a period of abstinence. At the neurochemical level, the consumption of sugars (mainly sucrose) produces—similar to most drugs of abuse—increased dopamine levels,[43-46] changing the expression of dopamine D_1-D_3 receptors and dopamine transporters[47-50] or leading to an adaptive reduction in dopamine concentration after chronic exposure[51] in the reward system structures.

We report here that the neurochemical mechanism that may explain the behavioral responses of offspring to cocaine is associated with changes in the MC system induced by a modified maternal diet. In fact, the offspring in the HSD group showed increased relapse after cocaine self-administration and drug abstinence compared to the CD

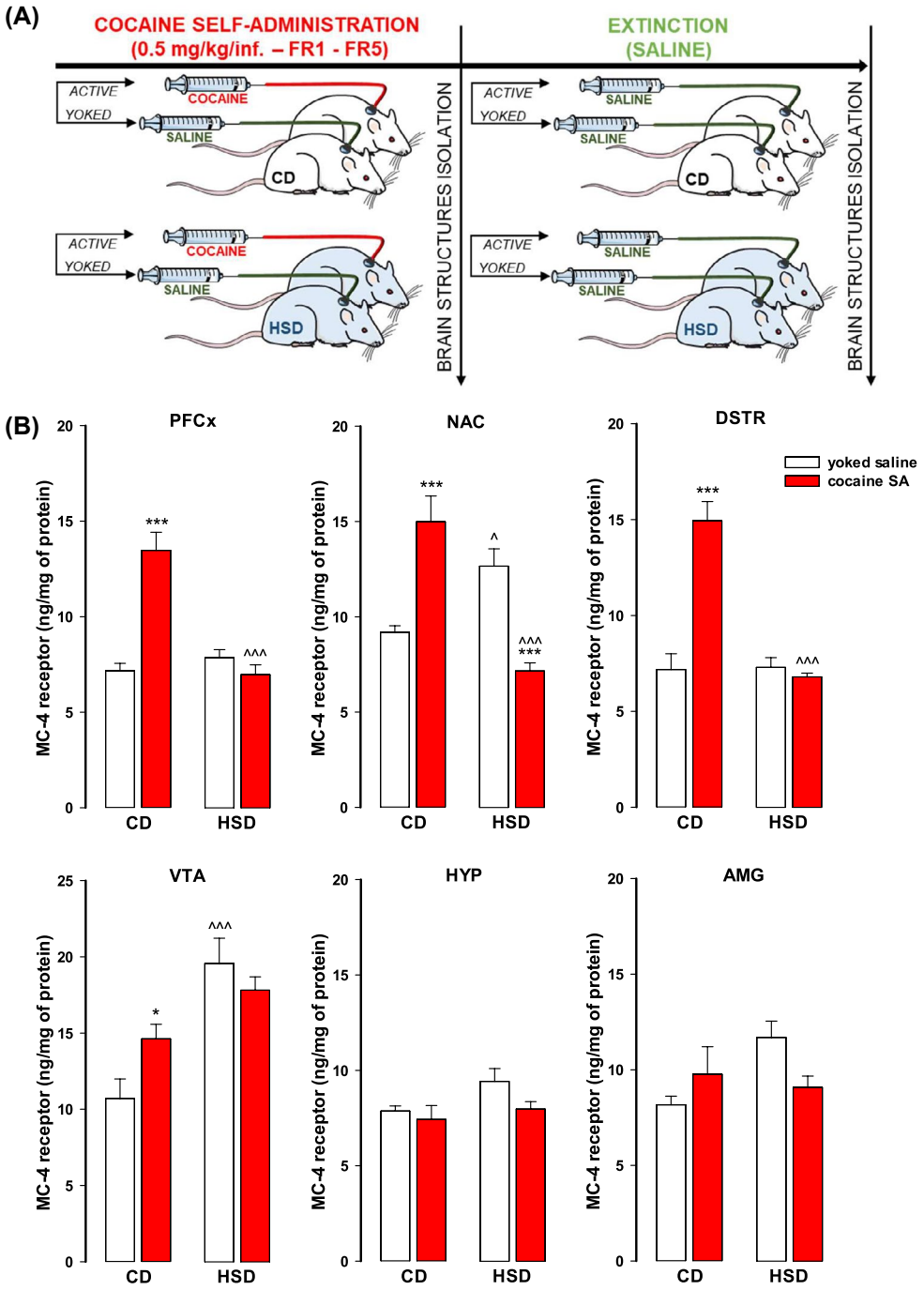

FIGURE 4 Melanocortin-4 (MC-4) receptor levels in offspring brain structures after cocaine self-administration. A, Scheme showing cocaine self-administration and extinction training as well as "yoked" saline control rats and time points of brain isolation. B, The levels of MC-4 receptor in the synaptosomal fraction of the prefrontal cortex (PFCx), nucleus accumbens (NAC), dorsal striatum (DSTR), ventral tegmental area (VTA), hypothalamus (HYP), and amygdala (AMG) after 18 days of cocaine self-administration and in "yoked" saline controls. The number of animals in each group was as follows: active cocaine (n = 8; cocaine SA), "yoked" saline (n = 6). Significance was determined using two-way ANOVA and the post hoc Newman-Keuls test. $^{*}P < .05$, $^{***}P < .001$ vs the saline control rats; $^{\wedge}P < .05$, $^{\wedge\wedge\wedge}P < .001$ vs the control diet (CD) group. *HSD – high-sugar diet*

group. While chronic cocaine use resulted in increased amounts of MC-4 receptors in the prefrontal cortex, nucleus accumbens, dorsal striatum and ventral tegmental area in rats in the CD group, such changes were absent in the HSD group, except for a decrease in receptor expression in the nucleus accumbens. In addition, cocaine abstinence resulted in the restoration of basic levels of MC-4 receptors in the CD group, unlike males in the HSD group, in which we recorded a decrease in the number of MC-4 receptors in the nucleus accumbens and ventral tegmental area and a large increase in

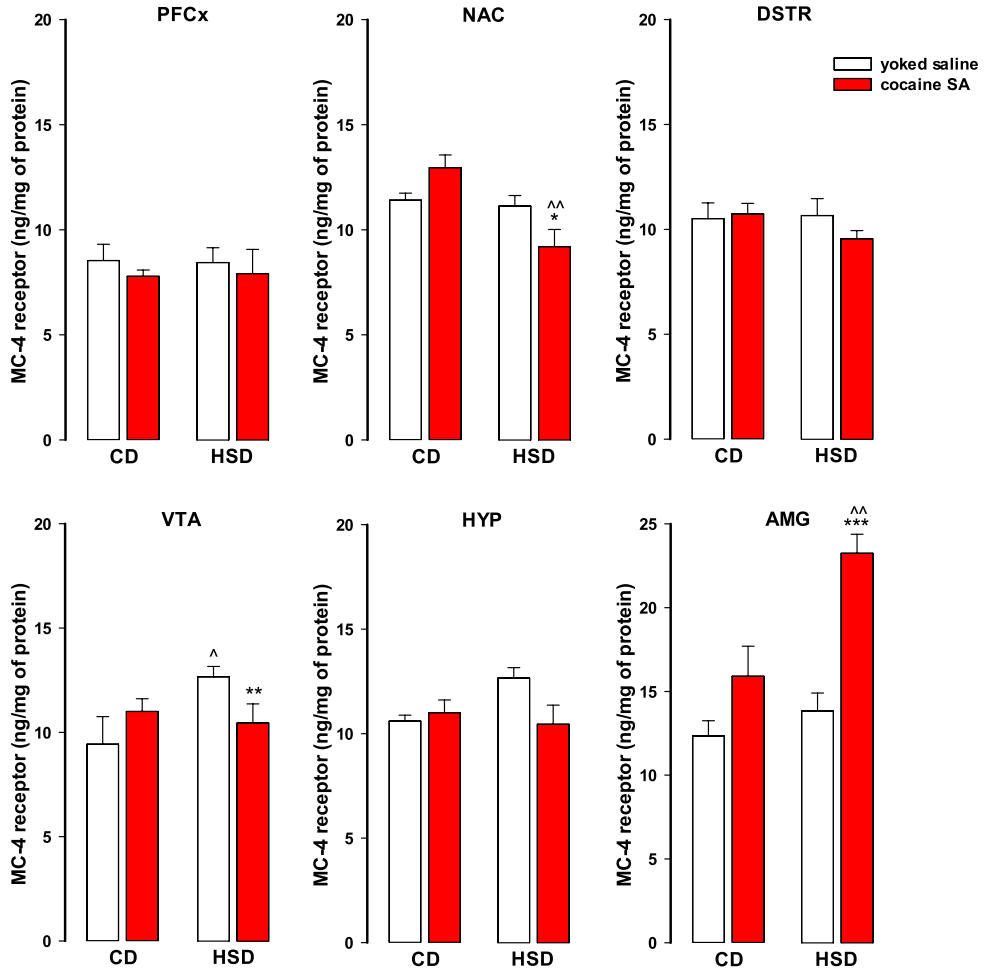

FIGURE 5 Melanocortin-4 (MC-4) receptor levels in offspring brain structures after cocaine abstinence with extinction training. The levels of MC-4 receptor in the synaptosomal fraction of the prefrontal cortex (PFCx), nucleus accumbens (NAC), dorsal striatum (DSTR), ventral tegmental area (VTA), hypothalamus (HYP), and amygdala (AMG) after 10 days of extinction training and in "yoked" saline controls. The number of animals in each group was as follows: active cocaine (n = 8; cocaine SA), "yoked" saline (n = 6). Significance was determined using two-way ANOVA and the post hoc Newman-Keuls test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs the saline control rats; $^{\wedge}P < .05$, $^{\wedge\wedge}P < .01$ vs the control diet (CD) group. *HSD – high-sugar diet*

MC-4 receptor expression in the amygdala. It is also worth noting that maternal HSD led to an increase in the nucleus accumbens and ventral tegmental area in male offspring (in "yoked" rats not exposed to cocaine). The above differences between HSD and CD offspring regarding the MC-4 receptors were observed in brain structures involved in cocaine-, stress- and cue-induced relapse. In fact, it was found that the interconnections of the ventral tegmental area—medial prefrontal cortex—nucleus accumbens core—ventral pallidum—amygdala with dopaminergic and glutamatergic projections represent the brain circuit underlying cocaine seeking.[52-57] Thus, MC-4 receptor expression under the influence of maternal HSD in the nucleus accumbens and amygdala may lead to changes in the neuronal response of these structures after cue exposure, resulting in observed behavioral changes in the strength of relapse. Confirming the significance of the above areas of the brain, reports have indicated a key role of the amygdala-nucleus accumbens projection in cocaine craving and relapse after withdrawal.[58,59]

Furthermore, our present observation extends recent findings on the changes in the activity of the MC system in the nucleus accumbens after cocaine. Thus, the intra-accumbens administration of the MC-4 receptor antagonist SHU-9119 impaired cocaine-induced self-administration, conditioned place preference, and conditioned reinstatement or locomotor sensitization.[21] In addition, intracerebral ventricular Agouti-related protein (AgRP), an MC receptor inverse agonist/competitive antagonist,[60] blocked acute and sensitized locomotor responses to cocaine.[20] Similarly, the loss of AgRP neurons increased cocaine-induced locomotion and place preference.[61] Findings from genetics research showed that MC-4 receptor +/- mice partially showed and that MC-4 KO mice did not show cocaine-induced locomotor-activating effects.[21] Moreover, chronically administered cocaine resulted in an

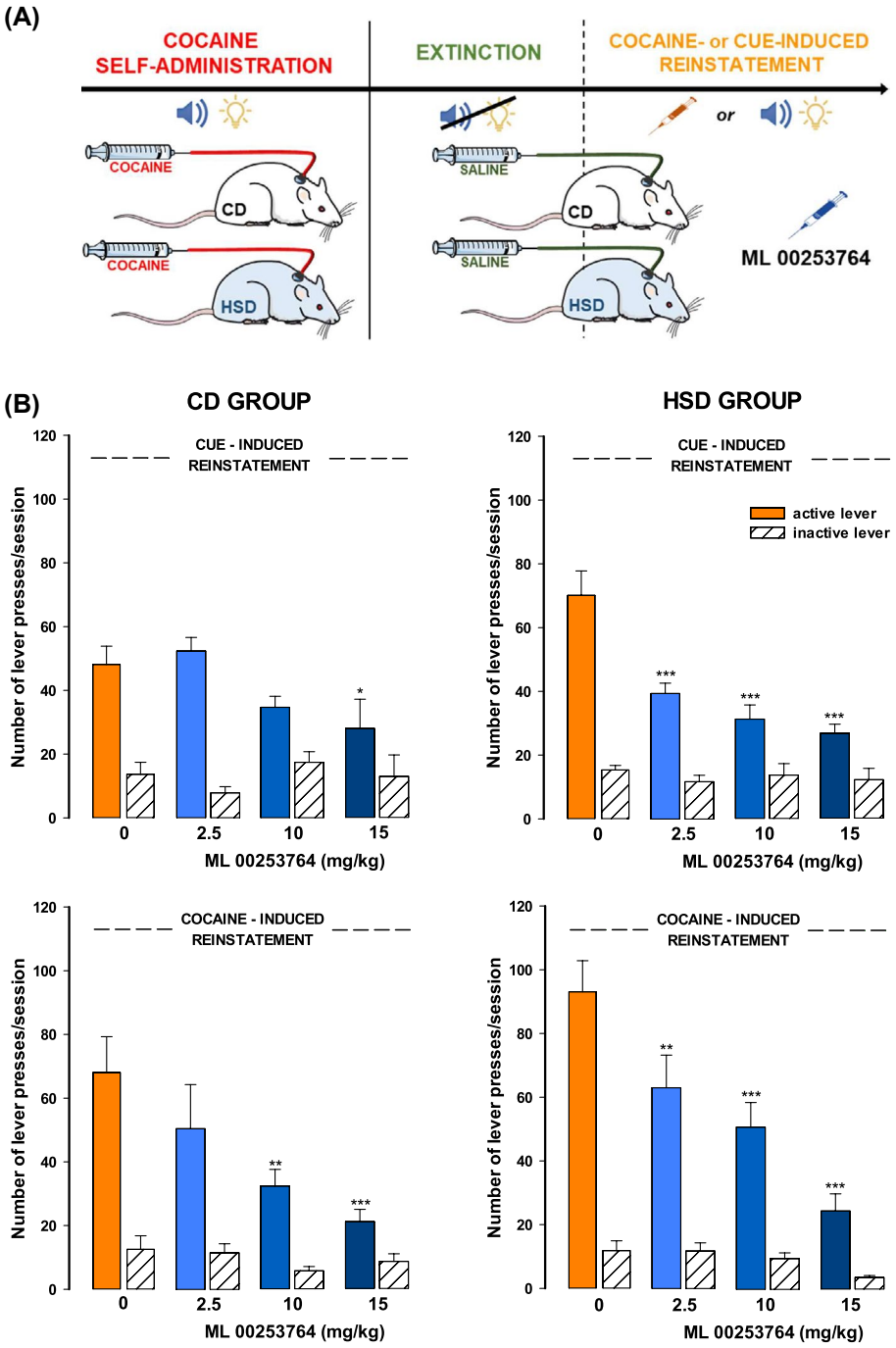

FIGURE 6 Effect of ML 00253764 on cocaine-seeking behavior in the control (CD) and high-sugar (HSD) diet offspring groups. A, Scheme of pharmacological experiment with ML 00253764 during cocaine- or cue-induced reinstatement in offspring. B, Effects of the melanocortin-4 (MC-4) receptor antagonist (ML 00253764; *s.c.*) on active and inactive lever presses during reinstatement of the cocaine-seeking behavior induced by the cue (tone + light; *upper panel*) or cocaine administration (10 mg/kg, *i.p.; lower panel*). Tests were performed after cocaine (0.5 mg/kg/infusion) self-administration under the increasing schedule of reinforcement (FR1-5) and 10 days of extinction training. The number of animals in each group was as follows: CD (n = 9), HSD (n = 9). Significance was determined using two-way ANOVA and the post hoc Newman-Keuls test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs the nontreated rats.

increase in MC-4 receptor mRNA levels in the striatum, hippocampus and hypothalamus.[21,62] In addition, research indicates that the MC system and MC-4 receptors are involved not only in behavior control related to drugs of abuse but also in natural, sweet rewards, which confirms the possibility of cross-regulation/sensitization. Thus, the injection of the MC-3/4 receptor agonist α-melanocyte-stimulating hormone (α-MSH) or AgRP into the nucleus accumbens shell resulted in a decrease or increase in motivation for sucrose, respectively.[63] In the case of rats fed two types of diets—a corn

starch diet vs a sucrose diet—AgRP only affected the intake of the sucrose-containing diet.[64]

To support the neurochemical data, we performed a pharmacological analysis with the peripheral administration of a nonpeptide brain-penetrant MC-4 receptor antagonist.[26,27] The administration of ML 00253764 to rats before the drug-associated cue or cocaine priming significantly reduced the number of active lever responses in the CD and HSD groups. It is worth noting, however, that in rats exposed to the maternal HSD, all doses of ML 00253764 showed anti-reinstatement activity, whereas in the CD group, only higher doses of ML 00253764 reduced the number of presses on the active lever. The greater sensitivity of the HSD offspring group to the test compound may be due to the differences in the number of synaptosomal MC-4 receptors in the nucleus accumbens and amygdala observed after drug abstinence. This is further evidence of an interaction in which the MC system is responsible for the behavioral aspects of cocaine, while cocaine itself modifies the MC system. As mentioned above, in the control of rewarding behaviors and the pathophysiology of drug addiction and hedonic overeating, the dopamine mesocorticolimbic pathways play a key role.[65] Previous studies highlight the functional interaction between dopaminergic neurotransmission and the MC system.[66] Destruction or degradation of AgRP neurons in addition to the behavioral changes mentioned above results in an increase in extracellular dopamine in the nucleus accumbens.[61] Moreover, an increase in the accumbal dopamine level results from the administration of α-MSH to the ventral tegmental area,[66,67] and this effect is blocked by a selective antagonist of MC-4 receptors.[66] Research using genetic animal models has indicated that the selective restoration of MC-4 receptors within dopamine D_1 receptor-expressing neurons normalized chronic cocaine-induced development of locomotor sensitization disturbed in mouse MC-4-/- mutants.[68] Moreover, modifications of the excitatory synapses in dopamine D_1 receptor-expressing neurons can be triggered by the administration of α-MSH to the nucleus accumbens,[69] while the chronic administration of its analog changes the expression of dopamine D_1 and D_2 receptors in the striatum and ventral tegmental area.[70] The above data connect MC-4 receptors to dopamine transmission and thus may provide a basis for understanding the observed changes in the evaluated offspring in the relapse of cocaine-seeking behavior in response to maternal diet and the mechanism of ML 00253764 action.

In conclusion, our findings underlie the important role of maternal diet during pregnancy and lactation in the development of offspring into adulthood. A maternal diet with excessive sugar increased the relapse of cocaine-seeking behavior in offspring. At the same time, differences in the expression of MC-4 receptors observed in offspring exposed to maternal HSD after cocaine may be responsible for the enhanced cocaine seeking, while this behavior was reduced following the administration of an antagonist of MC-4 receptors. Finally, based on neurochemical and behavioral analyses, MC-4 receptors can be a potential target for new drugs to prevent cocaine relapse in human addicts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

D. Gawliński designed and performed the study, analyzed the data, and wrote the manuscript. K. Gawlińska and M. Frankowska performed the study, analyzed the data, and contributed to writing the manuscript. M Filip conceived, designed, and coordinated the study and contributed to writing the manuscript. All authors revised the manuscript and approved its final version.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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