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Paternal cocaine taking elicits epigenetic remodeling and memory deficits in male progeny

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Abstract

Paternal environmental perturbations including exposure to drugs of abuse can produce profound effects on the physiology and behavior of offspring via epigenetic modifications. Here we show that adult drug-naïve male offspring of cocaine-exposed sires have memory formation deficits and associated reductions in NMDA receptor-mediated hippocampal synaptic plasticity. Reduced levels of the endogenous NMDA receptor co-agonist D-serine were accompanied by increased expression of the D-serine degrading enzyme d-amino acid oxidase (*Dao1*) in the hippocampus of cocaine-sired male progeny. Increased *Dao1* transcription was associated with enrichment of permissive epigenetic marks on histone proteins in the hippocampus of male cocaine-sired progeny, some of which were enhanced near the *Dao1* locus. Finally, hippocampal administration of D-serine reversed both the memory formation and synaptic plasticity deficits. Collectively, these results demonstrate that paternal cocaine exposure produces epigenetic remodeling in the hippocampus leading to NMDA receptor-dependent memory formation and synaptic plasticity impairments only in male progeny, which has significant implications for the male descendants of chronic cocaine users.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MEW, LAB, BAG and RCP designed the experiments. MEW, LAB, LAG, ACA, BF, HS, SHD and YH performed experiments. BF, MEW, LAB, SHD and RCP analyzed the data and prepared the figures. MEW and RCP wrote the paper with edits from all authors.

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INTRODUCTION

Illicit drug use is a significant public health problem and constitutes a substantial economic and societal burden worldwide.¹ A growing number of clinical and animal studies indicate that parental exposure to drugs of abuse can have deleterious consequences for progeny, including negative birth outcomes, increased rates of anxiety and depression as well as impaired cognition.^{2,3} According to recent estimates, the fathers of over 5 million children under the age of 18 years met the criteria for past year substance abuse.² However, few studies examined the influence of paternal drug use on offspring,^{4–7} with the vast majority of studies in this area focusing on maternal drug exposure. In a clinical setting, studying the paternal line presents many challenges, including identifying and locating fathers who abuse drugs, which can be extremely difficult and costly. Therefore, pre-clinical models may be best suited to address the dearth of research in this area. To that end, we recently developed an animal model of paternal cocaine exposure and demonstrated that cocaine exposure in sires reprogrammed the germline epigenome and produced alterations in mood and drug-related reward processing in male progeny.^{4,5} Although lower doses of psychostimulants are commonly used clinically to enhance attention and cognition, chronic cocaine abuse is often associated with global cognitive impairments including deficits in attention and declarative memory.^{8,9} We hypothesized that paternal cocaine taking might also influence cognitive function in subsequent generations.

MATERIALS AND METHODS

Animals and housing

Sires and dams (F0 generation) were Sprague-Dawley rats weighing 250–300 g obtained from (Taconic Biosciences, Hudson, NY, USA). Sires were housed individually except for 1 week of pair housing during the mating period. Food and water were available *ad libitum* and rats were kept on a 12 h–12 h light–dark cycle. All experiments were performed during the light phase. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of the Perelman School of Medicine at the University of Pennsylvania and conducted in accordance with the National Institute of Health guidelines.

Cocaine self-administration

Animals were anesthetized using a ketamine/xylazine cocktail (80 and 12 mg kg^{−1}, respectively). Silastic catheters were implanted into the right jugular vein, sutured in place and mounted on the shoulder blade using a mesh backmount. Catheters (CamCaths, Cambridge, UK) were flushed daily with an antibiotic (Timentin, Spectrum Chemical, New Brunswick, NJ, USA; 0.93 mg ml^{−1}) dissolved in heparinized saline and sealed using plastic obturators, when not in use. Animals were given a minimum recovery period of 7 days prior to the onset of cocaine self-administration. Cocaine was gifted from the US National Institute on Drug Abuse and dissolved in bacteriostatic saline.

Self-administration was conducted in Med-Associates operant chambers comprised of two response levers (active and inactive), house light and injection pumps. The self-administration equipment was enclosed in ventilated, sound attenuating chambers. Sires

were placed in the operant chambers and allowed to lever press for cocaine infusions (0.25 mg cocaine per 59 μ l saline per infusion over 5 s) on a fixed ratio 1 schedule. Each infusion was followed by a 20 s time-out period. Rats were limited to a maximum of 75 infusions for each daily 2-h self-administration session for 60 days.

Breeding

Twenty-four hours after the last self-administration session, sires were pair housed with drug-naïve females. Vaginal plugs were checked daily and females were separated the day that plugs were found. Animals remained co-housed for a maximum of 7 days. The resulting F1 offspring were weaned at 21 days and remained group housed (2–3 per cage) until the onset of experiments (beginning at ~ 60 days of age). For each experiment 1–2 rats per litter (either male or female) were used such that no litter was over-represented in any of the behavioral or molecular biological assessments. The assignment of animals to a particular experiment was random. The sample size for each experiment was based on a combination of power analyses and previous publications from our lab and others.

Object location memory task

Prior to the onset of training, animals were habituated to the training context during two 5-min sessions on two separate days. On the day of training, rats were placed in the training arena with two identical objects for a total of three 5-min sessions with an intersession interval of 1–2 min, during which animals were returned to their home cage. The objects used were a glass Erlenmeyer flask, an aluminum L-bracket or a plastic falcon tube (~5.5 in (height), ~ 2 in (width), ~ 2.5 in (depth)) and counterbalanced across experiments. The objects were fixed to the floor using double sided tape. A picture of the apparatus is shown in Supplementary Figure 1. The objects and the arenas were wiped with 70% ethanol before each session. Either 30 min or 24 h after training, animals were placed back in the training context for 5 min with 1 object displaced to a new location (displaced object; DO), while the other object was not moved (non-displaced object; NDO). All sessions were videotaped and time spent exploring each object was scored by researchers blind to the experimental groups. Exploration of the objects was defined as the amount of time rats were oriented toward an object with their nose within ~ 1 cm of the object. Grooming near the object was not considered exploration. Percent preference was calculated as time spent exploring the DO relative to the total time spent exploring both objects ($\text{preference} = \text{DO}/(\text{NDO} + \text{DO}) \times 100$).¹⁰

Novel object recognition task

Object recognition memory experiments were performed in the same training arenas and using the same objects as described above. Animals were pre-exposed to the training context for 10 min per day on 5 days prior to the onset of training. Twenty-four hours after the last context exposure, rats were placed in the training arena with 2 identical objects for 10 min. Thirty minutes or 24 h after training, animals were placed back in the training arena for 10 min with 1 familiar object and 1 novel object in the same locations used during training. Exploration time was measured as described above. Percent preference was calculated as time spent exploring the novel object relative to the total time exploring both objects (that is, $\text{novel}/(\text{familiar} + \text{novel}) \times 100$).¹⁰ In one experiment (shown in Figure 1f), the rats were first

trained on object location memory (described above) prior to the novel object recognition task. There was a minimum of 10 days between tasks, at which point the animals no longer show a preference for the displaced object.¹¹ Different objects were used in this group such that rats were never exposed to the same set of objects twice.

Locomotor activity

Locomotion was assessed during habituation on the object location memory task described above. The training arena was divided into nine zones. Crossing into another zone was defined as both paws and the nose of the animal going over the demarcation of that zone. The number of crosses were scored offline by an experimenter blind to treatment group.

Electrophysiology and LTP

Animals were anesthetized using a brief isoflurane exposure, then killed by decapitation. Three hundred micrometer thick coronal hippocampal slices were prepared as previously described,^{12,13} placed in an interface chamber and continuously perfused with oxygenated artificial cerebral spinal fluid (aCSF) while they equilibrated for at least 30 min. A bipolar stimulating electrode placed in stratum radiatum was used to stimulate the Schaffer collateral pathway. An aCSF-filled glass microelectrode with resistance approximately 2–3 M Ω placed in the stratum radiatum region of CA1 was used to record the resulting field-potentials (fEPSPs). Data were acquired using Clampex (Molecular Devices, Sunnyvale, CA, USA), and analyzed using Clampfit (Molecular Devices). Stimulations occurred every 20 s and three traces were averaged to yield a single data point per minute. A minimum of a 10-min baseline was recorded for each experiment, recordings continued for at least 1 h after long-term potentiation (LTP) induction. Initial fEPSP slopes were normalized against the average of the baseline traces. Theta burst stimulation was 40 ms in duration, 100-Hz bursts delivered at 5 Hz for 3 s (15 bursts of 4 pulses per burst, for a total of 60 pulses). In all LTP figures, representative sample sweeps are shown for each experiment. The black sweeps represent the average of 5 baseline sweeps, and the red sweeps represent traces recorded after LTP induction. The amplitude of the presynaptic fiber volley was measured in all experiments. Slices for which the amplitude of the fiber volley varied by more than 10% were not used in the analysis. For rescue experiments, D-serine (Sigma-Aldrich, St Louis, MO, USA) was dissolved in aCSF at a saturating dose^{14,15} of 100 μ M and bath applied during the entire recording period for saline- and cocaine-sired slices.

HPLC-MS, amino-acid profiling and D-serine measurements

Hippocampi from naive F1 offspring were dissected and flash frozen using liquid nitrogen. A neutralized perchloric acid extract was prepared from frozen hippocampus tissue. D-Serine and high-pressure liquid chromatography (HPLC) measurements were performed by The CHOP Metabolomics Core directed by Dr Itzhak Nissim (<http://www.research.chop.edu/cores/metabolomic/>). HPLC analysis was performed as previously described.¹⁶ D-Serine measurements were performed as previously described with minor adaptations.¹⁷ Briefly, samples and standards were derivatized with Marfey's reagent (Sigma-Aldrich; cat#71478) Na-(2,4-Dinitro-5-fluorophenyl)-l-alaninamide. Serine isomers were separated with the use of LC-MS technique as in (see Nissim *et al.*¹⁶). Quantification of D-Serine was done with ¹³C-Serine (Sigma-Aldrich) using an isotope dilution approach.

RNA extraction and real-time quantitative PCR

We extracted RNA using the RNeasy kit (Qiagen, Germantown, MD, USA) according to manufacturer's instructions. RNA concentration and purity were quantified by NanoDrop spectrophotometry (ThermoFisher Scientific, Waltham, MA, USA). Generation of cDNA was carried out using the RETROscript kit (ThermoFisher Scientific) with 1 µg of RNA as template. Reactions were prepared in 96-well optical reaction plates (ThermoFisher Scientific) with optical adhesive covers (ThermoFisher Scientific) using Taqman Fast Universal PCR Master Mix (Life Technologies, Grand Island, NY, USA). Three technical replicates were used for each animal. Reactions were carried out in the Step One Plus with an initial incubation at 95 °C for 10 min, and 40 subsequent cycles of 95 °C for 15 s, 60 °C for 30 s. The Taqman probes used in these experiments are listed in Supplementary Table 1.

C_t values were corrected using housekeeping gene expression levels for each sample and fold change was calculated as $2^{\exp(-C_t)}$.¹⁸ The data presented are the calculated mean for the biological replicates with n being equal to the number of biological replicates (that is, the number of rats examined).

Western blotting

Tissue from the hippocampus was processed for western blotting as described previously.¹⁹ For all samples, protein concentration was quantified using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Equal amounts of protein (10–20 µg) were loaded and separated in 10% Tris-Glycine gels (Life Technologies, Carlsbad, CA, USA) by SDS-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes using the iBlot dry transfer system (Life Technologies), which were then pre-blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% bovine serum albumin for 1 h before overnight incubation with the primary antibody to NR1 (Santa Cruz Biotechnology, Dallas, TX, USA, 1:1000). Membranes were concurrently incubated with mouse monoclonal anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA; 1:2000) as a loading control. Primary antibody incubation was followed by three washes (10 min each with rocking, room temperature) in Tris-buffered saline containing 0.1% Tween 20. Membranes were then incubated for 1 h at room temperature with secondary antibodies (IRDye 800 goat anti-mouse and IRDye 680 goat anti-rabbit, 1:5000) in Odyssey blocking buffer 0.05% Tween 20 (LI-COR Biosciences, Lincoln, NE, USA). Antibody/protein complexes were visualized and quantified using the Odyssey IR imaging system and software (LI-COR Biosciences). For data analysis, all bands were normalized to GAPDH.

Quantitative high-throughput characterization of single histone post-translational modification

Hippocampi were dissected from naive F1 male offspring and histones were acid-extracted according to standard protocols.²⁰ Briefly, nuclei were isolated by suspending cells into nuclei isolation buffer (15 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 250 mM sucrose and 0.2% NP-40) including the following inhibitors: 1 mM DTT, 0.5 mM AEBSF and 10 mM sodium butyrate. Nuclei were separated by centrifugation (1000 *g* for 10 min). Two milliliter of cold 0.4 N H₂SO₄ was added on the nuclei pellet and sample was incubated at 4 °C with shaking for 2 h. The nuclei were pelleted at 3400 *g* for 5

min. The supernatant was collected, trichloroacetic acid was added at a final concentration of 33% trichloroacetic acid (w/v), and the sample was incubated overnight at 4 ° C. Afterwards, precipitated histones were pelleted by centrifugation (3400 *g*) for 10 min. Pellet was washed from leftover acid using ice-cold acetone. Purified histones were processed with two cycles of chemical derivatization (propionic anhydride), trypsin digestion, and desalting as previously described.²¹ Sample was dissolved in 30 µl of 50 mM NH₄HCO₃, pH 8.0. Derivatization reagent was prepared by mixing propionic anhydride with acetonitrile in the ratio 1:3 (v/v) and added to the histone sample in the ratio of 1:2 (v/v) for 20 min at room temperature. The reaction was performed twice to obtain complete labeling. Histones were then digested with trypsin with an enzyme:sample ratio of 1:20 (w:w) overnight at room temperature in 50 mM NH₄HCO₃, pH 8.0. After digestion, the derivatization reaction was performed again twice to derivatize peptide N-termini. Samples were analyzed by using a nanoLC-MS/MS setup. nanoLC was configured with a 75 µm ID × 17 cm Reprosil-Pur C18-AQ (3 µm; Dr Maisch, Ammerbuch-Entringen, Germany) nano-column using an EASY-nLC nanoHPLC (Thermo Scientific, San Jose, CA, USA). The HPLC gradient was 2–35% solvent B (A = 0.1% formic acid; B = 95% MeCN, 0.1% formic acid) over 30 min and from 34% to 100% solvent B in 30 min at a flow-rate of 300 nl/min. LC was coupled with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Full scan MS spectrum (*m/z* 290 – 1400) was performed in the Orbitrap with a resolution of 60 000 (at 400 *m/z*) with an AGC target of 10⁶. The acquisition method contained both data-dependent and targeted scans. The targeted signals were the histone H3 and H4 peptides in isobaric forms, if any. MS/MS was performed with collision induced dissociation with normalized collision energy of 35, an AGC target of 10⁴ and a maximum injection time of 100 ms. MS/MS data were collected in centroid mode. Precursor ion charge state screening was enabled and all unassigned charge states as well as singly charged species were rejected. The selected modified peptides were quantified using label-free based extracted ion chromatography. EpiProfile was used for the purpose²² with a peak extraction mass tolerance set to 10 p.p.m. The peptide relative ratio was calculated by considering the peak area of all peptides that share the same amino acid sequence as total peptide abundance, and estimating the percentage of each individual species by dividing the peak area by the total peptide abundance.

Chromatin immunoprecipitation

We adapted the chromatin immunoprecipitation (ChIP) technique to the analysis of brain tissue. Briefly, tissue samples were fixated for 10 min at room temperature in 1% formalin to cross-link DNA to associated proteins, histones included. Cross-linking was stopped by addition of 100 µl of 1M glycine and further incubation at room temperature for 5 min. Brain tissue was washed three times with ice-cold phosphate-buffered saline and then re-suspended in cell lysis buffer 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 50 mM Tris-HCl, pH 8.1) in the presence of protease inhibitors (Complete Mini protease inhibitor cocktail tablets, Roche, Indianapolis, IN, USA). Tissues were homogenized by mechanical action and centrifuged at 5500 *g* to pellet the intact nuclei. The supernatant was removed and the pellet of nuclei was incubated for 10 min on ice in nuclear lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris pH = 8.1). Brain nucleic lysates were then sonicated to shear DNA into fragments of an appropriate length for ChIP (200–350 bp). After centrifugation to remove

cellular debris, 1% of the resulting homogenates were set aside as 'Input' DNA. Another 10% were used for quality control, including concentration assessment and gel electrophoresis. Lysates that passed quality control were subsequently used for IP. Two microgram of chromatin was used for each IP experiment. Negative control was an IgG IP (Jackson, West Grove, PA, USA). Each sample was incubated overnight (4 °C) with 1 µg of the appropriate antibody or IgG. After immunoprecipitation, 100 µl of washed protein G-agarose beads 50% slurry were added to samples, and immunocomplexes were collected for 1 h at 4°C on a rocking platform. The chromatin-antibody/protein G-agarose complexes then underwent a 4 min wash in low-salt buffer (0.1% SDS, % Tx-100, 2 mM EDTA, 20 mM Tris-HCl pH = 8, 150 mM NaCl), a 4- min wash in high salt buffer (0.1% SDS, % Tx-100, 2 mM EDTA, 20 mM Tris-HCl pH = 8, 500 mM NaCl), a 4-min wash in a LiCL buffer, and a final wash in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Elution was performed by incubation in 250 µl of freshly made elution buffer (1% SDS, 0.1 M NaHCO₃) for 15 min at room temperature; elution was repeated and eluates combined. Cross-links were reversed for both immunoprecipitated samples and input DNA by addition of 20 µl 5 M NaCl to the pooled eluates and heating of the mixture to 65 °C overnight. After cross-link reversal, all samples (ChIP and Input) were be digested with 20 µg proteinase K (1 h, 45 °C), and the DNA was extracted, precipitated and re-suspended in 25 µl of sterile water. Nine microliter of ChIP-derived DNA were used as template in 25 µl real-time PCR reactions with gene-specific primers in triplicate reactions. The primers used can be found in Supplementary Table 2. Threshold amplification cycle numbers (T_c) using iCycler software were used to calculate IP DNA quantities as percentage of corresponding inputs. Finally, DNA quantities (normalized to Input) were compared for immunoprecipitated vs mock-immunoprecipitated samples; only when immunoprecipitated samples contained >1.5 times as much DNA were they considered to have sufficient DNA for analysis.

Cannulae implant surgery and microinjection procedure

F1 offspring were mounted in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). Guide cannulae (10 mm, 24 gauge) for microinjections were implanted bilaterally 1 mm dorsal to CA1 in the dorsal hippocampus. Guide cannulae were cemented in place by affixing dental acrylic to stainless steel screws secured in the skull. The coordinates for the guide cannulae, relative to bregma were: +3.6 mm A/P; ± 2.2 mm M/L and – 2.7 mm D/V. An obturator (10 mm, 33 gauge) was inserted into each guide cannula in order to prevent occlusion. Animals were infused with either aCSF or D-serine (Sigma-Aldrich) dissolved in aCSF at a concentration of 1 mM.²³ Prior to microinjections, the obturators were removed from the guide cannulae and 33 gauge, 11 mm stainless steel microinjectors were inserted. Bilateral infusions were performed simultaneously over 1 min in a total volume of 0.3 µl per hemisphere. Following infusion, microinjectors were left in place for an additional minute in order to allow for diffusion of the drug solution away from the injection site. In every case, each animal received a minimum treatment of one injection (D-serine or vehicle). Some of the animals underwent two treatments, interspaced by a minimum of 1 week. In such cases, the order of treatment was counterbalanced and different objects were used for each object location memory experiment. After completion of all microinjection experiments, brains were collected into 10% formalin and coronal sections (100 µm) were taken at the level of the dorsal hippocampus with a Vibratome (Leica, Buffalo Grove, IL, USA). The sections

were mounted on slides and a researcher blind to experimental groups determined cannula placements using light microscopy. Animals with placements outside of the hippocampus were excluded from the analysis.

Statistical Analyses

For both object location memory and novel object recognition experiments, the percent preference for displaced or novel object was analyzed using repeated measures analysis of variance (RM-ANOVA) to compare saline- and cocaine-sired groups. We used session (training vs test) as the within subject factor and siring (saline- vs cocaine-sired) as the between subject factor. For experiments using D-serine treatment, mixed models ANOVAs were used and the factors were drug (vehicle vs D-serine), siring and session. Bonferroni *post hoc* tests were used when appropriate. For LTP experiments, RM-ANOVAs were used with time as the within subject factor and sire as the between subject factor. Bonferroni *post hoc* tests were performed in case of significant interactions. ANOVAs were used to compare fold change in all qPCR experiments, for amino acids profiling and to compare fold enrichment of epigenetic marks near the *Dao1* gene. Mann–Whitney *U*-tests were used to analyze the high-throughput characterization of single histone post-translation modifications (PTMs). Unpaired *t*-tests were used to compare levels of D-serine, lactate and fold enrichment of epigenetic marks near the synaptophysin gene.

RESULTS

Sex-specific spatial learning deficits in the offspring of cocaine-exposed sires

Male rats were allowed to self-administer cocaine daily for 60 days, encompassing the duration of spermatogenesis, and control animals received saline passively.^{4,5} Sires were then bred with drug-naïve females resulting in cocaine-sired and saline-sired first generation (F1) offspring (see Figure 1a). We bred 16 saline-exposed sires to 22 naïve females and 16 cocaine-exposed sires to 24 naïve females, which produced 46 total litters. Paternal cocaine taking had no effect on litter size, sex ratio or growth curves (Supplementary Figure 2). Memory formation was examined in both male and female adult drug-naïve offspring. To assess memory, we used a hippocampus-dependent object location memory task, where animals were exposed to two identical objects. Twenty-four hours after training, adult F1 offspring were returned to the training arena in which one of the objects was in a novel location (Figure 1b). Male offspring of saline-treated sires spent more time exploring the displaced object during this test, indicating intact long-term object location memory. In contrast, cocaine-sired male progeny explored both objects equally during the 24-h test indicating impaired long-term memory (Figure 1c). Following the acquisition phase of memory formation, short-term labile memories are converted to longer-lasting traces through a gene- and protein synthesis-dependent consolidation process.²⁴ Short-term memory was assessed 30 min after training to determine which phase of memory formation was affected in cocaine-sired progeny. The male offspring of cocaine-exposed sires showed no preference for the displaced object during the short-term retrieval test compared to controls (Figure 1d), suggesting that paternal cocaine taking impairs the ability of male offspring to form hippocampus-dependent object location memories.

To determine the anatomical specificity of this memory impairment, we subjected a separate cohort of animals to a hippocampus-independent object recognition task (Figure 1e). Rats were trained in familiar arenas lacking spatial cues, two factors that have been shown to render object recognition independent of hippocampal function.^{11,25,26} Both saline- and cocaine-sired male offspring showed a preference for the novel object during long-term (Figure 1f) and short-term (Figure 1g) retrieval tests, indicating that object recognition is not influenced by paternal cocaine exposure. Importantly, the memory deficits that we observed were not due to generalized deficits in exploration and the total time spent exploring objects in all of the aforementioned experiments was not altered by sire cocaine exposure (Supplementary Table 3). We next examined object location memory in naive adult female offspring (Figure 2a). Both cocaine- and saline-sired female progeny showed a preference for the displaced object during a 24-h retrieval test (Figure 2b). Female offspring also showed normal object recognition memory during a long-term memory test (Figures 2c and d). These data indicated that the memory deficits related to paternal cocaine exposure were sex specific.

Male, but not female progeny of cocaine-exposed sires had synaptic plasticity deficits and reduced levels of D-serine

Memories are encoded by changes in synaptic strength via physiological mechanisms such as LTP.²⁷ We measured synaptic plasticity in a subset of the offspring that were tested for object location memory no less than 2 weeks after the behavioral tests, when rats no longer show any trace of the memory.¹¹ These rats were randomly selected and are representative of performance as a whole (Supplementary Table 4). Hippocampus slices were collected and theta burst-induced LTP was measured in the Schaffer collateral pathway. Male offspring of cocaine-exposed sires showed reduced LTP compared with saline-sired controls (Figure 3a). We found no differences in input-output curves between the two groups, suggesting that postsynaptic excitability is unaffected by paternal cocaine exposure (Supplementary Figure 3a). Furthermore, theta burst-induced LTP was unaffected in female F1 offspring, which is consistent with our behavioral observations (Supplementary Figure 3b). Interestingly, there was a positive correlation between performance on the object location task and LTP induction (linear regression, $R^2 = 0.4737$, $P = 0.0192$) in male offspring, suggesting that these memory and synaptic plasticity impairments in progeny may be caused by similar underlying mechanisms.

Glutamate signaling through *N*-methyl-D-aspartate receptors (NMDARs) in the hippocampus is critically important for memory and synaptic plasticity. Both glutamate and its endogenous co-agonist D-serine are required for LTP induction and memory formation.^{28–30} Even brief exposures to cocaine can have long-lasting effects on glutamate and D-serine levels in the brain.^{31,32} This raises the possibility that glutamatergic and/or D-serine signaling may be impaired in male progeny of cocaine-exposed sires. To address this question, we used HPLC and electrochemistry (HPLC-EC) to establish the amino acid profile in the hippocampus of naive adult F1 male offspring. Of the amino acids that are known neurotransmitters, only total glutamate levels were diminished in the hippocampus of cocaine-sired progeny (Figure 3b). Interestingly the glutamate precursor glutamine³³ was also reduced by paternal cocaine exposure (Supplementary Table 5). Although no changes in

serine were observed, HPLC-EC did not distinguish D-serine from its inactive enantiomer L-serine and only permitted the examination of total serine (D+L). Using HPLC in tandem with mass spectrometry and isotope dilution to separate the two serine enantiomers, we found that hippocampal D-serine was reduced in cocaine-sired male progeny relative to controls (Figure 3c). This effect was specific to the hippocampus in that D-serine levels were comparable in saline- and cocaine-sired male offspring in the prefrontal cortex and cerebellum (Supplementary Figure 4a). Furthermore, measurements of D-serine in the hippocampus of F1 female progeny revealed no changes related to paternal cocaine exposure (Supplementary Figure 4b).

Paternal cocaine exposure was associated with increased expression of *Dao1* and enriched permissive epigenetic marks near the *Dao1* locus in the hippocampus of male offspring

Serine racemase synthesizes D-serine in neurons and astrocytes, while the enzyme D-amino-acid oxidase catalyzes the oxidative deamination of D-serine^{29,30} (Figure 4a). We measured the gene expression of molecules involved in the production and degradation of D-serine as well as the subunits that make up NMDARs. mRNA expression levels of NMDAR subunits and serine racemase were not changed by cocaine sire exposure. However, the gene that encodes D-amino acid oxidase, *Dao1*, was over-expressed in the hippocampus of male cocaine-sired progeny (Figure 4b). We also examined the mRNA expression of enzymes involved in the synthesis of glutamate that may contribute to the reported changes in total glutamate levels. Siring had no effect on the expression of glutamate dehydrogenase or glutaminase (Supplementary Figure 4c). Gene expression of the AMPA receptor subunit GluA1 (*Gria1* gene) and protein levels of the NR1 subunit were also unchanged in the hippocampus of F1 cocaine-sired offspring (Supplementary Figures 4c and d). Taken together, these data demonstrated that paternal cocaine exposure increased *Dao1* mRNA expression, which is associated with reduced D-serine levels in the hippocampus.

We hypothesized that the changes in gene expression in male progeny would emanate from epigenetic remodeling in the brain. PTMs on histone proteins that package DNA are critically involved in the regulation of gene expression.³⁴ A mass-spectrometry-based assay was first employed to examine alterations in histone PTMs genome-wide in the hippocampus of F1 male progeny. A total of six epigenetic marks were found to be significantly enriched in cocaine-sired male offspring (Figure 4c and Supplementary Table 6). Interestingly, most of the enriched histone PTMs are associated with active gene expression. For example, acetylation of lysine 9 on histone H3 (H3K9ac) is usually found at active transcriptional sites and single methylation of lysine 4 on histone H3 (H3K4me1) is typically enriched near enhancers. We next examined whether these changes in histone PTMs were located near the *Dao1* gene that encodes D-amino-acid oxidase. We first focused our attention on global acetylation of histone H3 (H3ac), which includes acetylation of lysine 9 (H3K9ac) and has long been known to be functionally relevant for promoting gene expression in the brain.³⁵ Acetylation of histone H3 was higher at the transcription start site and downstream of the *Dao1* gene in cocaine-sired male progeny (Figure 4d). These findings suggest that lysine residues of histone H3 were increasingly acetylated near the *Dao1* locus in cocaine-sired male progeny. We also used an antibody specific for H3K9ac to determine

whether the broad changes in acetylation on H3 were driven solely by H3K9ac. Cocaine siring had no significant effect on the presence of H3K9ac near the *Dao1* locus (Supplementary Figure 5a), suggesting compound increases at several H3 acetylation sites at the *Dao1* locus. These results also indicate that the genome-wide increases in H3K9ac caused by paternal cocaine exposure in our spectrometry-based global assay are not present at the specific *Dao1* locus. Finally, H3K4me1 was also enriched near the *Dao1* transcription start site (Figure 4d). To test the target specificity of both remodeling events, we examined H3ac and H3K4me1 levels near the actively expressed synaptophysin neuronal gene (*Syp*) in the hippocampus of F1 male progeny (Supplementary Figure 5b). Sire cocaine exposure had no effect on either mark. Thus, the epigenetic remodeling elicited by paternal cocaine exposure seems to be at least somewhat specific to the *Dao1* locus, in that the same epigenetic changes are not found at other actively transcribed loci. Taken together, these findings indicate that cocaine taking in sires can elicit epigenetic reprogramming associated with open chromatin states or active gene transcription in F1 male progeny and that some of these alterations of the epigenetic landscape are present specifically at the *Dao1* locus.

Restoring D-serine levels in cocaine-sired male offspring rescued both memory formation and synaptic plasticity impairments

We next tested the hypothesis that impaired synaptic plasticity and memory in cocaine-sired males was due to reduced hippocampal D-serine. Theta burst-induced LTP was measured while D-serine was bath-applied to hippocampus slices collected from F1 male offspring. Exogenous D-serine rescued LTP in offspring of cocaine-exposed sires (Figure 5a). We next examined whether microinjecting D-serine directly into the dorsal hippocampus of cocaine-sired offspring would ameliorate the memory impairments that we observed (Figure 5b). Consistent with our previous results, cocaine-sired offspring that received vehicle showed object location memory deficits (Figure 5c) compared to controls. Pre-treatment with D-serine restored memory in male progeny of cocaine-exposed sires (Figure 5c). Taken together, these data demonstrated that decreased D-serine in the hippocampus of cocaine-sired male rats was functionally relevant since replacing the depleted hippocampal D-serine levels was sufficient to restore normal synaptic plasticity and object location memory.

DISCUSSION

The once controversial idea that parental experiences, such as stress or diet, can shape the physiology and behavior of their offspring via epigenetic mechanisms has become a vibrant area of research.^{4,36–42} Here we hypothesized that hippocampus-dependent memory, which deteriorates following chronic cocaine abuse in adults,^{8,9} would also be changed in the progeny of cocaine-taking fathers. Our results show that the memory deficits caused by paternal cocaine exposure were specific to a hippocampus-dependent object location memory task. Both short- and long-term cognitive performances were diminished in cocaine-sired male offspring, indicating that the acquisition phase of memory formation is impaired in these animals. We chose object-based assays, which are known to produce little anxiety in rodents,⁴³ to avoid the potentially confounding effects of increased anxiety-like behavior in cocaine-sired offspring.⁴⁴ The normal performance on the novel object recognition test in cocaine-sired progeny also suggested that the changes in performance on

object location memory are specific to the spatial aspect of this task. Taken together, our results demonstrated that paternal cocaine exposure impairs hippocampus-dependent object location memory while sparing traces that do not engage the hippocampus.

It is noteworthy that paternal cocaine taking impacts memory formation, anxiety⁵ and drug reward⁴ selectively in male offspring. Consistent with our behavioral results, paternal cocaine exposure also did not alter D-serine content or LTP in the hippocampus of female progeny. Several physiological mechanisms could underlie the sex specificity of these phenotypes. Gonadal steroids are implicated in the development of sexually dimorphic structures in the brain, which molds not only behavioral outcomes but can also influence the brain's response to environmental insults during development. In particular, males secrete testosterone during late gestation and early neonatal periods, resulting in sexually dimorphic brain nuclei and adult behavioral traits.⁴⁵ Exposure to testosterone in males is thought to confer some protection against the development of psychiatric disorders, such as depression and anxiety, particularly through its actions in the hippocampus.⁴⁶ Furthermore, one of the critical events for appropriate branching and the proper growth of synaptic inputs in the hippocampus of males is exposure to testosterone during development.⁴⁷ Therefore, interactions between the intergenerational effects of cocaine on gene expression and subsequent hormonal influences in developing pups may contribute to the differential behavioral outcomes in male and female progeny.

Chromatin remodeling is an important regulatory component of gene transcription that influences a wide range of behaviors, including drug addiction and memory formation.^{34,35} Here we used a mass spectrometry-based technique to broadly interrogate many histone PTMs simultaneously, which circumvents many of the limitations associated with antibody-based approaches. Paternal cocaine taking elicited chromatin remodeling events in male progeny that favored the enrichment of several permissive epigenetic marks associated with open chromatin states, such as actively transcribed promoters (H3K9ac, H4K16ac) and poised enhancers (H3K4me1). Interestingly, H3 acetylation and lysine 4 single methylation of H3 were specifically enriched near the *Dao1* gene that encodes D-amino-acid oxidase. The observation that enhancing acetylation leads to increased gene expression has led to the idea that H3ac is functionally relevant for driving expression of neighboring genes.^{34,35} We posit that epigenetic remodeling near the *Dao1* locus contributes to the overexpression of *Dao1* in the hippocampus of cocaine-sired male progeny, which results in decreased levels of D-serine.

Our previous research showed that a cocaine self-administration regimen identical to the current study produced reprogramming of the germline epigenome in the sires.⁴ The mechanisms underlying the development and transmission of phenotypes in progeny following an environmental insult such as drug exposure in sires is an active and highly debated area of research. It is clear that some of the sperm epigenetic signatures can influence early embryonic development and potentially have long-lasting consequences for the adult progeny.^{48,49} The exact mechanisms by which paternal experiences are transmitted to future generations is still not fully understood. Methylation of DNA in the germline has long been known to be an important regulator of development and an important carrier of information across generations.^{50,51} During spermatogenesis, the majority of histone

proteins are replaced by protamines, which facilitates dense packaging of DNA.⁵² Interestingly, the remaining histones may influence the patterns of DNA methylation in sperm in that methylated regions are highly associated with histone retention in the germline.⁵³ Furthermore, retained histones may mark genes and chromosomal domains for gene expression during early embryonic development.⁵² Therefore, the remaining histones and their accompanying chemical changes are another potential epigenetic mechanism of transmission between sires and their offspring. Finally, the micro RNA content of sperm can also influence gene expression in the early stages of development and represent another plausible mechanism underlying the transmission of drug experience in sires to their progeny.⁴⁹

In our experiments, the potential impact of cocaine withdrawal-induced changes in sire behavior during breeding to naive dams is an important consideration. We previously showed that sire cocaine exposure had no impact on maternal behavior.⁴ We posit that drug exposure, which lasts 2 months, has more consequences on sperm epigenetic reprogramming than any withdrawal-mediated effect that lasts for a few days. However, more research is needed to address this important possibility.

We previously found that the reinforcing efficacy of cocaine is reduced in male cocaine-sired progeny due, in part, to alterations in the function of the medial prefrontal cortex (mPFC).⁴ Interestingly, spatial working memory requires the retention of visuospatial information for short periods of time and depends on the circuit comprised of the hippocampus and the mPFC.⁵⁴ Therefore, the current and previous findings are consistent with disruptions in the hippocampal-mPFC circuitry in cocaine-sired progeny that result in object location memory deficits. It would be of great interest to examine performance in additional memory tasks engaging components of the hippocampus-mPFC pathways to further dissect the exact circuits impacted by paternal cocaine exposure in male progeny.

Drug-induced cognitive impairments are often predictive of treatment retention and outcome.^{55–57} Some of the therapeutic strategies for treating addiction have focused on ameliorating drug-related neurocognitive decline and there is substantial interest in using cognitive enhancers to treat addiction.^{58–60} The memory formation and plasticity impairments that we observed were accompanied by reduced levels of the NMDAR endogenous co-agonist D-serine. Restoring D-serine levels in the brain of cocaine-sired male offspring reversed the memory and synaptic plasticity deficits that we observed. Drugs that modulate NMDARs at the glycine site, such as D-serine or D-cycloserine, cause few side effects in humans and are a promising category of novel pharmacotherapies,^{59,60} which highlights a potential translational aspect of the current results.

The present results indicate that paternal cocaine exposure in rats elicits memory formation impairments in male offspring, a finding with clear clinical implications both in terms of progeny learning disabilities in addition to the fact that cognitive impairments are associated with higher rates of drug relapse. Interestingly, from a translational perspective, our results indicate that the memory and synaptic plasticity impairments in cocaine-sired male rats were reversed by the administration D-serine. Finally, our results show that paternal cocaine exposure resulted in epigenetic changes in the hippocampus of male progeny associated with

open chromatin states, including modifications near the *Dao1* gene that encodes the enzyme responsible for the degradation of D-serine. Collectively, these data add to the small but growing literature indicating that paternal environmental disruption can result in specific and profound changes in the physiology and behavior of offspring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Paternal cocaine self-administration impaired spatial memory formation in male offspring. (a). Sires (F0 generation) were allowed to self-administer cocaine (2 h, daily) for 60 days or received yoked saline infusions. The day following the last self-administration session, males were paired with drug-naïve females. Memory formation was assessed in the resulting adult drug-naïve first generation (F1) offspring. (b). Animals were exposed to two identical objects and time exploring each item was recorded. After a delay of either 24 h (long-term memory test) or 30 min (short-term test), rats were placed back in the same arena and one of the objects was displaced to a new location. (c). The preference index for all object-based tasks was computed as follows: (time exploring displaced object/total time exploring both objects) $\times$ 100. Saline-sired offspring spent equal time exploring both objects during training and show a preference for the displaced object during the long-term memory test. In contrast, progeny of cocaine-exposed sires spent equal time exploring both objects during training and during the 24-h memory test (Interaction between training and siring, $F_{1,17} = 5.178$, $P = 0.0361$, $n = 9-10$). (d). Progeny of saline-treated sires showed a preference for the

displaced object 30 min after training. Cocaine-sired offspring spent equal time with both objects during the short-term memory test (Interaction between training and siring, $F_{1,16} = 7.457$, $P = 0.0148$, $n = 9$). (e). A separate cohort of rats was pre-exposed to the training context lacking spatial cues for 5 days prior to training with two identical objects. A novel object was introduced during the object recognition memory test either 24 h or 30 min after training the training session. (f, g). Both groups of animals spent equal time exploring both objects during training and showed a preference for the novel object during the long-term (f, $F_{1,18} = 26.91$, $P < 0.0001$, $n = 10$) and short-term ((g), $F_{1,22} = 19.60$, $P = 0.0002$, $n = 11-13$) memory tests. For all panels, $*P < 0.05$ comparing train vs test, Bonferroni *post hoc* test.

Figure 2.

Paternal cocaine exposure did not influence spatial memory or object recognition in female offspring. (a) Adult drug-naïve female offspring were exposed to two identical objects and time exploring each item was recorded. After a delay of 24 h, animals were placed back in the same arena and one of the objects was displaced to a new location. (b) Both saline- and cocaine-sired progeny show a preference for the displaced object during the long-term memory test (For training, $F_{1,17} = 20.22$, $P = 0.0003$, for interaction between training and sire, $F_{1,17} = 0.0444$, $P = 0.8354$, $n = 8-11$). (c) A separate cohort of rats was pre-exposed to the training context lacking spatial cues for 5 days prior to training with two identical objects. A novel object was introduced during the object recognition memory test 24 h after training the training session. (d) Both groups of female offspring spent equal time exploring both objects during training and showed a preference for the novel object during the long-term memory test ($F_{1,16} = 9.169$, $P = 0.0080$ for training; $F_{1,16} = 0.1791$, $P = 0.6798$ for interaction between training and sire, $n = 8-10$). * $P < 0.05$ train vs test.

Figure 3.

Offspring of cocaine-exposed sires showed impaired long-term potentiation (LTP) and reduced D-serine levels. **(a)** Theta burst-induced LTP in Schaeffer collateral pathway of the hippocampus was impaired in cocaine-sired offspring compared to controls ($F_{69,1242} = 2.737$, $P < 0.0001$, $n = 7$ for saline-sired and $n = 13$ for cocaine-sired). **(b)** An amino-acid profile screen revealed that total glutamate levels are reduced in the hippocampus of naive adult cocaine-sired offspring compared to controls ($F_{15,180} = 4.012$, $P < 0.0001$, $n = 6$). **(c)** D-serine levels are diminished in progeny of cocaine-experienced sires ($t(10) = 2.515$, $P = 0.0307$, $n = 6$). TBS, Tris-buffered saline.

Figure 4.

Male offspring of cocaine-exposed sires had increased mRNA expression of *Dao1* and permissive epigenetic marks were enriched near the *Dao1* locus in the hippocampus. (a) D-serine is an endogenous co-agonist that binds to the glycine site of tetrameric *N*-methyl-D-aspartate receptors (NMDARs). Serine racemase converts L-serine to D-serine in neurons and astrocytes. The enzyme D-amino acid oxidase (DAAO) catalyzes the oxidative deamination of D-serine. We measured the gene expression of the molecules related to NMDAR signaling in the hippocampus of F1 progeny. (b) Gene expression of *Grin1*, *Grin2a*, *Grin2b* and *srr* was not changed by paternal cocaine exposure ($P > 0.05$, $n = 7-8$). In sharp contrast, the expression of *dao1* was increased in the hippocampus of cocaine-sired offspring compared to controls ($F_{4,64} = 4.579$, $P = 0.0026$, $n = 7-8$, $*P < 0.05$, Bonferroni *post hoc* test). (c) Globally, 6 epigenetic marks were enriched in the hippocampus of cocaine-sired F1 male offspring (shown in blue above the dotted line; $n = 6$). (d) Chromatin immunoprecipitation against acetylated histone H3 (H3Ac) and single methylated lysine 4 on histone H3 (H3K4me1) revealed enrichment of both marks near the *Dao1* gene (H3Ac, $F_{2,18} = 5.326$, $P = 0.0152$; H3K4me1, $F_{2,20} = 6.741$, $P = 0.0058$. $*P < 0.05$ comparing saline- and cocaine-sired fold enrichments, Bonferroni *post hoc* test; $n = 5-6$). TSS, transcription start site.

Figure 5.

D-Serine rescued plasticity and learning deficits related to paternal cocaine self-administration. **(a)**. D-Serine was bath-applied during the entire recording period. Both saline- and cocaine-sired progeny showed theta burst-induced LTP (Time, $F_{69,987} = 21.83$, $P < 0.0001$, Interaction between time and sire $F_{69,1242} = 0.3630$, $P > 0.9999$; $n = 7-8$). **(b)**. D-Serine or vehicle was microinjected 30 min prior to training on the object location memory task. **(c)**. Vehicle-treated cocaine-sired offspring did not show a preference for the displaced object during short-term memory test compared to controls. All animals treated with D-serine spent more time exploring the displaced object during 30-min memory test (Interaction between training and treatment $F_{3,41} = 4.820$, $P < 0.0058$; $n = 9-14$). * $P < 0.05$ comparing train vs test, Bonferroni *post hoc* test. LTP, long-term potentiation; TBS, Tris-buffered saline.