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Cocaine-induced locomotor sensitization in rats correlates with nucleus accumbens activity on manganese-enhanced MRI

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Abstract

A long-standing goal of substance abuse research has been to link drug-induced behavioral outcomes with the activity of specific brain regions to understand the neurobiology of addiction behaviors and search for drug-able targets. Here we tested the hypothesis that cocaine produces locomotor (behavioral) sensitization that correlates with increased calcium channel-mediated neuroactivity in brain regions linked with drug addiction such as, the nucleus accumbens (NAC), anterior striatum (AST) and hippocampus, as measured using manganese-enhanced magnetic resonance imaging (MEMRI). Rats were treated with cocaine for 5-days followed by a 2-day drug-free period. The following day, locomotor sensitization was quantified as a metric of cocaine-induced neuroplasticity, in the presence of manganese. Immediately following behavioral testing, rats were examined for changes in calcium channel-mediated neuronal activity in the NAC, AST, hippocampus, and temporalis muscle that was associated with behavioral sensitization using MEMRI. Cocaine significantly increased locomotor activity and produced behavioral sensitization compared to saline-treatment to control rats. A significant increase in MEMRI signal intensity was determined in the NAC, but not AST or hippocampus, of cocaine-treated rats compared to saline-treated control rats. Cocaine did not increase signal intensity in the temporalis muscle. Notably, in support of our hypothesis, behavior was significantly and positively correlated with MEMRI signal intensity in the NAC. Since neuronal uptake of manganese is regulated by calcium channels, these results indicate that MEMRI is a powerful research tool to study neuronal

activity in freely behaving animals and to guide new calcium channel-based therapies for treating cocaine abuse and dependence.

Graphical abstract

Cocaine-induced locomotor sensitization in rats correlates with nucleus accumbens activity on manganese-enhanced MRI

Manganese-enhanced MRI (MEMRI) was paired with behavioral sensitization to cocaine to determine if striatal neuroactivity could be measured in the awake, freely behaving rat. We found that MEMRI signal intensity in the nucleus accumbens, a brain region involved in cocaine action, correlated with locomotor activity. These findings show that MEMRI is a sensitive tool to measure neuronal activity in the behaving animal.

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Introduction

Enduring neuroplastic changes associated with drugs of abuse and addiction perpetuate deleterious behavioral choices (e.g., impulsivity) and render abstinent patients highly vulnerable to relapse. Preclinical and clinical studies have identified brain regions and networks that undergo structural neuroplasticity during initial drug exposure, drug seeking behavior, and drug-induced behavioral sensitization; however, it has been difficult to link the effect of abused drugs, such as cocaine, on behavior to functional neuronal activity or drug-able targets in specific vulnerable brain regions, such as the nucleus accumbens (NAC).

Here, we assessed the utility of manganese (Mn^{2+})-enhanced magnetic resonance imaging (MEMRI), a functional neuroimaging technique for detecting changes in calcium (Ca^{2+})-dependent neuroactivity, in the context of a well-established behavioral sensitization model of cocaine-induced neuroplasticity (1, 2). Behavioral sensitization is measured as an enhancement of motor stimulation after repeated administration of cocaine and is a model of drug-induced neuroplasticity with well-defined neurocircuitry that includes the NAC and anterior striatum (AST), two brain regions central to the rewarding and reinforcing effects of drugs of abuse, and the hippocampus, which plays a role in drug-related learning and memory (3–5).

The application of MEMRI has emerged as a powerful new way to understand behavioral neuroscience (6–9), although its use in the field of addiction has to-date been limited to a few proof-of-concept studies, none of which directly investigated links between psychostimulant-related behavior and MEMRI-detected changes in specific brain regions (10–12). Given that behavioral studies are performed on awake and freely moving animals, a major advantage of MEMRI is that functional brain activity can also be interrogated in the same rat that underwent drug administration and behavioral testing, thus eliminating the

confound of anesthesia exposure. Mn^{2+} is an MRI contrast agent as well as a Ca^{2+} surrogate which enters excitable cells via L-type Ca^{2+} channels (LTCCs), such as $Ca_v1.2$ (13, 14). Since LTCCs are critical modulators of normal neuronal function, increased neuronal activity is tightly linked with their activity. In the application of MEMRI, neuronal activity is associated with increased uptake and accumulation of Mn^{2+} , which can allow researchers to study Ca^{2+} signaling in the context of disease (13–16). Importantly, many psychiatric diseases and disorders including addiction are mediated by Ca^{2+} signaling (17–19), yet much remains unknown about the nature of this addiction-related Ca^{2+} channel-based behavior.

In this study, we tested the hypothesis that cocaine-induced behavioral sensitization in rats is significantly correlated to localized increased MEMRI signal intensity (i.e., accumulation of Mn^{2+} via Ca^{2+} channel activity) in neurons of the NAC, AST, and hippocampus. Cocaine-induced neuronal activity was also quantified in the temporalis muscle, which served as a negative control and region for normalization. Finally, as other studies (20, 21) have reported that Mn^{2+} administration affects the availability of striatal dopamine transporter (DAT), which could interfere with the effect of cocaine in the present study since DAT is the molecular target of cocaine, we tested whether our Mn^{2+} administration paradigm alters DAT in striatal synaptosomal membranes.

Methods

Animals, drugs, groups, and overall design

For the MEMRI experiment, fifteen male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) were housed in pairs on a normal 12-h light/dark cycle (with lights on at 7 AM). Standard housing conditions with free access to normal lab rat chow and water were provided. Rats were acclimated to the vivarium for 5 days upon arrival before starting the study. The rats weighed 277 ± 8 g (mean $\pm$ standard error of the mean) at the start of experimentation on day 1. Rats were transported to the laboratory on days 1–5 and 8 to measure locomotor activity and transported to the 7 T magnet on day 8 to measure MEMRI; otherwise, animals were housed in the vivarium. Wayne State University (WSU) maintains AAALAC accredited animal facilities under the Division of Laboratory Animal Resources. Animals are maintained in accordance with applicable portions of the Animal Welfare Act and United States Department of Health and Human Services “Guide for the Care and Use of Laboratory Animals.”

Cocaine hydrochloride (National Institute on Drug Abuse, Drug Supply Program, Bethesda, MD) was dissolved in sterile saline (0.9% NaCl). Normal sterile saline served as control (i.e., 0 mg/kg cocaine). A repeated, binge-pattern administration model of cocaine exposure was used that consisted of multiple injections per day for several days to model human consumption and produce a robust activation of behavior and neuroactivity (22–24). The number of days of administration was based on published studies that show robust behavioral sensitization to cocaine develops in male adult rats over the selected time-frames (25).

The rats were split into 2 experimental groups: cocaine (n=7) and saline (n=8). On days 1–5, cocaine (10 mg/kg) or saline was intraperitoneally (IP) injected once every hour for 3 hours and locomotor activity was measured starting 30 min before the first injection and lasting 1 hour after the last injection (for a total 3.5 h). On days 6–7 animals were left undisturbed in their home cage. On day 8, Mn^{2+} and cocaine or saline were IP injected and locomotor activity was measured, followed by MEMRI neuroimaging. All animals received systemic administration of Mn^{2+} (88 mg/kg $MnCl_2 \cdot 4H_2O$, IP; dissolved in PBS) 6 hours before neuroimaging, which was 15 min before the first cocaine or saline injection and at the beginning of locomotor activity testing. Note that systemic administration of 88 mg/kg Mn^{2+} has been shown to produce robust signal enhancement in the brain at 6 hours post-injection (26). Cocaine (3 mg/kg) or saline was IP injected 10 times once every 30 minutes starting 15 minutes after the onset of testing. This pattern was chosen to produce a robust, long-lasting cocaine effect that occurred during the 6 hour period of Mn^{2+} uptake. Locomotor activity was recorded during the entire 6-hour period on day 8. Following behavioral testing and before neuroimaging, animals were anesthetized with urethane (2.2 ± 0.2 ml/kg, 36% solution in saline) administered by IP injection. Urethane was chosen because it provides long-lasting anesthesia while maintaining largely normal systemic physiology; more importantly, urethane has no paramagnetic or diamagnetic susceptibility that would interfere with MR measurements. Note that the use of anesthesia does not complicate the link between behavior and neuroimaging because the animals were freely behaving in response to cocaine during functional neural encoding with Mn^{2+} . Animals were euthanized by decapitation while still under anesthesia following neuroimaging.

For the DAT immunoblotting experiment, sixteen male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) were housed, cared for, and acclimated as described above. The rats weighed 361 ± 4 g at the time of Mn^{2+} treatment. The rats were split into two experimental groups, Mn^{2+} (n=8) and control (n=8). On the day of treatment, rats were injected with Mn^{2+} (88 mg/kg $MnCl_2 \cdot 4H_2O$, IP; dissolved in PBS) or PBS, and 6 hours later were euthanized for brain tissue collection by rapid decapitation without anesthesia using a sharp, small-animal guillotine.

Measurement of locomotor activity and behavioral sensitization

Locomotor activity and behavioral sensitization were tested in standard infrared photobeam-style activity monitors (Opto M3 animal activity meter, Columbus Instruments). Locomotor activity was measured as counts that are dependent on a rat moving past and blocking a series of infrared photobeams. Data were collected for the entire daily sessions, which on days 1–5 lasted 3.5 hours in total but only the last 3 hours was in response to cocaine or saline and on day 8 lasted 6 hours with the cocaine or saline injection occurring 15 minutes after the onset Mn^{2+} injection and behavioral testing. This method provided locomotor data that was in response to cocaine or saline, and it yielded baseline data (prior to cocaine or saline) that on days 1–5 was in the absence of Mn^{2+} and on day 8 was in the presence of Mn^{2+} . Behavioral sensitization was measured as an increase in locomotor activity on day 5 compared to day 1 of cocaine administration in the same rats. The activity monitors were contained in sound-attenuating chambers with controlled temperature (at $\sim 25^\circ C$) and house-

lighting and were connected to a dedicated computer system for controlling the software and recording, storing, and analyzing the behavioral data.

Measurement of MEMRI

In preparation for scanning, anesthetized rats were secured using blunted ear-bars and a tooth-bar. Body temperature was maintained by a heated re-circulating water system. Scans were performed on a 7 T Bruker ClinScan system with a Siemens console (see (6, 7, 9, 27) for details of imaging methods). Briefly, a transmit-only whole body coil and a receive-only surface coil (4-element phased array for mouse brain) placed dorsal to the head of the animal were used to acquire two types of images based on a turbo-FLASH sequence: Scans acquired both with and without a slice-selective inversion pulse respectively generated magnetization prepared rapid acquisition gradient echo (MPRAGE) and proton density weighted (PDGE) images that shared almost all parameters (flip angle of 3°; TE 3.03 ms; NA 1; echo spacing 7.77 ms; matrix size 192×192×112; FOV 2.50 × 2.50 × 2.91 cm³; providing a resolution of 130 μm×130 μm×260 μm). MPRAGE imaging took 8 min and PDGE imaging took 3 min 12 s, and for each rat the sequence of taking MPRAGE and PDGE images were performed twice and was interleaved. All animals were alive when scanning was complete.

Analysis of MEMRI images

In MEMRI, differential Mn²⁺ uptake primarily affects tissue T₁; however, several other variables modify the signal intensity measured in standard T₁-weighted images, such as tissue proton density, distance from receive-coil, and B₁ inhomogeneity (28). The influence of these variables is shared by gradient echo images acquired with a small flip angle for PDGE and MPRAGE images (29). As in previous work (6, 7, 9, 27), we therefore began image analysis by registering the PDGE and MPRAGE images, and on a voxel-by-voxel basis, divided signal intensities from MPRAGE by PDGE to produce the resulting ratio image (MPRAGE/PDGE) (see Figure 1A), which is weighted heavily by tissue T₁ and largely free of non-biological influences on signal (29).

Using a whole brain 3D set of images, we quantified MEMRI signal intensities in striatum. All images (MPRAGE, PDGE, and ratio) were imported into ImageJ (free NIH software), and the ratio (T₁-weighted) image was used for all analyses. The MPRAGE and PDGE images were used to identify neuroanatomical landmarks and guide placement of regions-of-interest (ROIs). Neuroanatomical landmarks (i.e. reference points) were identified for each 3D set of images by careful comparison of MR images with schematics (slices) of the rat brain atlas of Paxinos and Watson (30). Prominent landmarks include white matter tracts (e.g. corpus callosum, anterior and posterior commissures, and fasciculus retroflexus), the overall shape of the brain and ventricle location, and unique grey matter structures (e.g. striatum and hippocampus). The NAC (core and shell), (dorsal) AST, and (anterio-dorsal) CA1 region of the hippocampus were selected as ROIs for measurement of signal enhancement. Standard ROI templates were used to make all measurements and maintain uniform sampling from one subject to the next and were created according to defined anatomy (30). After identifying the coronal MR image to measure, the user-defined template of each ROI was placed on the image and mean signal intensities were measured. The ROIs

are shown in Figure 1B and 1C. All MEMRI data, including landmarks, image selection, and placement of the template, were checked by at least two-independent scorers who were unaware of the experimental conditions, and the average measurement was used.

The MEMRI signal intensity is dependent on neuronal activity; however, intensities can be affected by variables independent of neuronal activity such as distance from receiver coil or differences in Mn^{2+} uptake among rats. Per common practice, signal intensities (ratio data) of temporalis muscle tissue located in the field of view (see Figures 1B and 1C) were used for signal intensity normalization (6, 9). In other words, signal intensities (SI) within the ROIs were normalized to average muscle tissue of the same animal (normalized ROI SI = ROI SI/muscle SI), except for muscle SI data which are reported as raw values.

Isolation of striatal synaptosomal membranes and measurement of DAT by immunoblotting

Immediately after decapitation, brains were removed and whole striatum were isolated by freehand dissection, frozen on dry ice, and stored at -80°C for ~1 week. Striatum tissue were then processed to isolate synaptosomal membranes (31). Briefly, each bilateral-pair of striatum were thawed in 5 ml of ice-cold isotonic buffer containing a protease and phosphatase inhibitor cocktail (10 mM Tris, 5 mM NaF, 1 mM Na_3VO_4 1 mM EDTA, 1 mM EGTA, and 320 mM sucrose adjusted to pH 7.4) and disrupted using a dounce homogenizer. Nuclei and large debris were removed with an initial centrifugation ($800 \times g$ 10 min), and the resulting supernatants were centrifuged again ($9,200 \times g$ 15 min). The resulting pellets (crude synaptosomal fractions) were resuspended in hypotonic (sucrose-free) buffer and allowed to stand for 30 min in order to lyse synaptosomes. A final centrifugation was done ($25,000 \times g$ 20 min), the resulting supernatant (cytosolic fraction) was recovered, and the pellet (synaptosomal membrane fraction) was washed and resuspended in sucrose-free buffer.

DAT content was analyzed in both synaptosomal membrane and cytosolic fractions from striatum. Whole tissue protein levels were determined using the bicinchoninic acid assay. Equal amounts of protein from each sample (30 μg) were resolved by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted to a nitrocellulose membrane. Blots were blocked in a solution of 5% non-fat dry milk and 0.1% Tween 20 in TBS for 1 h at room temperature ($22-25^{\circ}\text{C}$). Primary antibodies against DAT (1:1000, gift – see Acknowledgements) were added to the blots and incubated for 16 h at 4°C . Blots were washed three times in TBS to remove unbound antibodies and then incubated with IRDye secondary antibodies (1:4000, LiCor Biosciences, Lincoln, NE) for 1 h at room temperature. Immunoreactive bands were visualized by enhanced fluorescence and the relative optical densities of DAT-reactive bands were determined by imaging with an Odyssey CLx Infrared Image System (LiCor Biosciences) and quantified using ImageJ software (NIH, Bethesda, MD).

Statistical Analyses

All data were analyzed and graphed in Prism 6 (Graphpad). For the day 1–5 locomotor activity data, an analysis of variance was performed with drug and day as main factors and posthoc analysis followed using repeated-measures (multiple) two-tailed *t*-test. For the day 8

locomotor activity data, an independent two-tailed *t*-test was used for analysis. For the analysis of baseline locomotor activity on days 5 (without Mn²⁺) and 8 (with Mn²⁺), an independent two-tailed paired *t*-test was used. MEMRI data were analyzed by independent two-tailed *t*-tests. Separate Spearman correlation (two-tailed) and linear regression analyses were conducted using data from both groups to analyze relationships between behavior (day 8) and MEMRI (NAC, AST, or hippocampus). Immunoblotting data on striatal DAT concentration were standardized as percentage of control and analyzed by a two-tailed *t*-test. Confidence intervals were set at 95% and $\alpha=0.05$; therefore, significance was set at $p<0.05$.

Results

Behavior

As shown in Figure 2A, cocaine significantly increased locomotor activity compared to saline-control on days 1–5 (interaction: $F_{4,65}=2.843$; drug: $F_{4,65}=54.61$; day: $F_{4,65}=1.898$), and posthoc analysis revealed that each day of binge-pattern administration of cocaine was higher than that of control: day 1 ($t_{13}=2.90$, $p=0.012$), day 2 ($t_{13}=3.44$, $p=0.004$), day 3 ($t_{13}=5.08$, $p=0.002$), day 4 ($t_{13}=4.15$, $p=0.001$), and day 5 ($t_{13}=2.78$, $p=0.015$). On day 8, which followed a 2-day drug-free period and was the day of MEMRI testing, cocaine significantly increased the locomotor activity of rats compared to saline-control injections (Figure 2B; $t_4=4.172$, $p=0.001$) as hypothesized.

In order to evaluate the potential confounding effects of Mn²⁺ on locomotor activity, we determined the effects of Mn²⁺ on baseline locomotor activity measured on day 5 (no Mn²⁺) and day 8 (with Mn²⁺). Since the 15 min baseline period was prior to cocaine or saline injection, the only difference between data collected on days 5 and 8 was the absence or presence of Mn²⁺; therefore, we combined between-group data and focused on within-group data to evaluate the effects of Mn²⁺ on locomotor activity. We found that locomotor activity (mean beam breaks $\pm$ standard error of the mean) on day 5 in the absence of Mn²⁺ was 365 ± 39 and on day 8 in the presence of Mn²⁺ was 289 ± 34 and that there was no statistical difference between locomotor activity on these days ($t_{14}=1.18$, $p=0.257$; data not shown).

MEMRI

Following repeated cocaine administration, supranormal signal intensity was found in the NAC ($t_{13}=2.27$, $p=0.041$; Figure 3A) consistent with our hypothesis. A non-significant increase in signal intensity in the AST ($t_{13}=1.94$, $p=0.074$; Figure 3B) was observed. The NAC, AST, and muscle ROIs are shown in Figure 1B and representative images from a saline- (Figure 3C) and a cocaine- (Figure 3D) treated rat is shown. Also, we found that the average signal intensity in the hippocampus was not significantly affected by cocaine ($t_{13}=0.18$, $p=0.859$; Figure 4A). Furthermore, the mean muscle signal intensity, which served as a control region used to normalize among rats, was not significantly affected by cocaine ($t_{13}=0.48$, $p=0.627$; Figure 4B). The hippocampus and muscle ROIs are shown in Figure 1C and representative images from a saline- (Figure 4C) and a cocaine- (Figure 4D) treated rat is shown.

Comparing Behavior and MEMRI

The locomotor activity on day 8 and signal intensity in the NAC were correlated ($p=0.039$, $r=0.541$, $r^2=0.293$; Figure 5A). On the other hand, no correlation was found between behavior on day 8 and signal intensity in the AST ($p=0.063$, $r=0.495$, $r^2=0.245$; Figure 5B) or hippocampus ($p=0.863$, $r=-0.050$, $r^2=0.003$; Figure 5C).

Immunoblotting

No significant difference was found in striatal synaptosomal membrane DAT protein content between Mn^{2+} treated and control rats ($t_{14}=2.03$, $p=0.237$, Figure 6). A blot of the cytosolic fraction from these striatal tissues showed no detectable DAT bands (data not shown).

Discussion

In this study we found that repeated cocaine administration produced robust behavioral sensitization that was positively correlated with increased MEMRI signal intensity in the NAC, but not in AST or hippocampus. The NAC underlies the reinforcing and rewarding effects of cocaine and other drugs of abuse in self-administration and conditioned place preference rodent models of addiction (32–34). The role of the NAC in the development and expression of behavioral sensitization to cocaine and other drugs suggests that the neurocircuitry of sensitization mirrors that of the reward neurocircuitry (5, 34). We were somewhat surprised that the AST did not show a significant increase in signal intensity in the cocaine-treated rats. It is well established that NAC and AST have differential roles in cocaine-mediated reward, reinforcement, and habit behaviors. For example, evidence suggests that as cocaine dependence develops a transition occurs from reward- and goal-mediated behaviors, which are regulated by ventral striatum including NAC, to habit and compulsive behaviors, which are governed by the dorso-lateral striatum, including AST (32). Given that the cocaine behavioral sensitization paradigm used here does not engage habit behavior in the animal, the AST may not have been as robustly activated as the NAC by cocaine exposure, and thus not detected by MEMRI analysis. Alternatively, low statistical power may have limited our ability to detect a statistically-significant difference in the AST because justifiable use of 1-tail t -test analysis yields significant differences. The hippocampus plays a contextual role in drug-taking, -seeking, and -administration (4, 5, 33, 35). Since cocaine was administered in the same context daily for several days in our study, causing habituation to the environment and experimental procedures, hippocampal activity was not expected to be activated significantly using MEMRI. Based on these considerations, our data suggests that on the day of MEMRI testing (day 8), cocaine selectively activated the NAC, a key brain region involved in drug-induced locomotor behavior (5); AST and hippocampus neuroactivity were not significantly affected because by the time of MEMRI testing the environmental context was not novel to the animal and habit behavior was not part of the model (32, 36).

MEMRI is an emerging technology in neuroscience with applications in, for example, models of visual stimulation (6, 27), Parkinson's disease (37), epilepsy (38), posttraumatic stress disorder (39) and drug action (10, 12). One study combined cocaine-provocation with MEMRI. In that study, anesthetized rats, whose blood brain barrier had been compromised,

received acute injection of cocaine while inside the magnet and were studied using MEMRI (12). A dose-dependent increase in MEMRI signal intensity in the NAC was reported although no behavioral testing was performed (12). Similarly, the neurotoxic effects of methamphetamine have been studied using MEMRI (10). Rats were treated with an established neurotoxic regimen (40) and 6 days later a track-tracing variation of MEMRI was employed. Rats that received injection of Mn^{2+} into the habenular nucleus, and then treated with methamphetamine, had significantly greater Mn^{2+} accumulation in the ventral tegmental area, the dorso-medial striatum, and the medial prefrontal cortex (10). MEMRI has recently been used to show that repeated amphetamine treatment increases neuroactivity in the dorsal horn of the spinal cord in association with enhanced pain sensitivity in mice (11). An advantage and innovation of our study is that we administered Mn^{2+} to awake and freely moving rats without having to compromise the blood brain barrier during cocaine-induced locomotor activity. Our findings demonstrate that MEMRI can be used to correlate psychostimulant-related behaviors and changes in localized neuronal activity in animals exposed to chronic cocaine, providing the framework for further investigation into the functional neuroactivity caused by drugs of abuse and integration of neuroimaging with more complex rodent behavioral tasks associated with the study of addiction.

As discussed, the signal intensity changes observed using MEMRI are dependent on Ca^{2+} channel activity (13–16). Therefore, it is reasonable to assume that the increases in cocaine-induced signal activity in the NAC observed in this study are Ca^{2+} -dependent and reflect enhanced neuronal activity. Aberrant Ca^{2+} channel activity in neurons of the reward circuitry is well-established as a mechanism of cocaine action in the animal literature (41–44), and genes involved in Ca^{2+} regulation play a role in the pathophysiology of cocaine addiction (19). For example, in animals, Ca^{2+} channel blockers decrease behavioral sensitization to cocaine and reduce cocaine-mediated synaptosomal Ca^{2+} transport in the NAC (45). Cocaine-induced reward and psychological dependence behavior, as measured in the conditioned place preference paradigm, is blocked by intracerebroventricular administration of the LTCC blocker, nifedipine, with this effect also reported for morphine- and methamphetamine-induced reward behavior (46). The role of LTCCs in cocaine-induced behavior and neuronal plasticity in the NAC is complex and findings on post-mortem examination suggest that subunit-specific signaling is important. Specifically, a molecular switch in the NAC occurs with $Ca_v1.3$ channels, which is posited to be critical to the development of sensitization, while $Ca_v1.2$ channels appear to contribute to the expression of sensitization to cocaine (47, 48). $Ca_v1.2$ LTCCs also mediate cocaine-induced AMPA receptor trafficking in the NAC that accompanies cocaine-induced behavioral sensitization (49). Furthermore, Ca^{2+} -permeable AMPA receptors in the NAC and ventral tegmental area appear to play a direct role in the transition to cue-induced cocaine craving (44). However, the Ca^{2+} mechanism is complex because repeated cocaine administration suppresses, not enhances, high-voltage Ca^{2+} potentials in the rat NAC (48). Given that it is possible to distinguish the separate contributions of $Ca_v1.2$ and $Ca_v1.3$ LTCCs with appropriate pharmacological agents, we anticipate future studies that use MEMRI can investigate and aid in our understanding of the temporal evolution and contribution of LTCC subtypes in the actions of cocaine.

Despite best efforts to minimize confounds, a few limitations of our study may exist. One possible limitation of the present study is that Mn^{2+} may directly regulate the expression or cellular distribution of the dopamine transporter (DAT), which is important because DAT is the molecular target of cocaine (50). For example, Roth and colleagues used an *in vitro* preparation to show that Mn^{2+} causes DAT internalization and thus inactivation (21). On the other hand, an increase in DAT binding sites using positron emission topography was observed in 2 monkeys that received Mn^{2+} injection days earlier (20). The applicability of these studies to the present work is unclear since the Mn^{2+} administration paradigms differ greatly, so we determined whether or not our specific Mn^{2+} administration paradigm altered DAT in striatal synaptosomal membranes and found that it did not. Furthermore, we found that Mn^{2+} did not significantly affect baseline locomotor activity and that it did not have a substantially negative effect on the magnitude of cocaine's behavioral response, which is an outcome metric that is innately dependent on DAT expression and function. It is now established that similar doses of Mn^{2+} to those used in the present study have been shown to be non-toxic over a range of endpoints (15, 51–53). Our neurochemical and behavioral data and MEMRI neuronal activity maps clearly demonstrate the expected cocaine-induced increased in locomotor activity (behavioral sensitization) and enhanced neuronal activity in the NAC. These considerations suggest that Mn^{2+} is not exerting a substantial confounding (neurotoxic) influence in our study. Methodological issues of our study that may have been limiting are the use of T_1 -weighted imaging, lack of baseline (pretreatment) MEMRI scans, lack of spatial normalization, and a potentially confounding influence of cocaine on vasculature that might influence MEMRI observations. Use of voxel-wise T_1 -mapping and incorporation of a longitudinal (repeated design) approach may have lessened a concern that B_1/B_0 -inhomogeneities and surface-coil sensitivity profile, for example, confound our result. However, our use of the ratio (MPRAGE/PDGE) imaging and normalization to muscle procedures served to minimize these confounds. Spatial normalization, rather than manual segmentation of ROIs, is thought to reduce error and variance across measures; however, manipulating the brain images to fit them into a defined stereotaxic coordinate system may lead to ROI warping and alterations in signal intensities. Thus for this pilot study, we choose to focus our measurements to hypothesis-driven testing of key brain regions. Finally, we have considered the cardiovascular effects of cocaine on MEMRI and believe this not to be an issue. Cocaine is a vasoconstrictor, which would limit Mn^{2+} availability, if anything, so this does not readily explain our observed increase in signal intensity. Also, whereas cocaine induces an immediate rise in neural Ca^{2+} , it causes an initial decrease in hemoglobin oxygen content and cerebral blood flow followed by an overshoot in these measures that lasts over 20 min (Yuan et al., 2011), which collectively suggests that the vasoconstrictor effects of cocaine in the brain do not directly influence the Ca^{2+} -mediated (neuronal) activity. Future studies should consider employing alternative methodological strategies to optimize design and data analysis.

In summary, the present results demonstrate the potential of MEMRI for application in the field of addiction. MEMRI will likely be useful in clarifying underlying Ca^{2+} channel-based mechanisms of action of psychostimulants and has potential in further defining the mechanistic effects of drugs of abuse on the reward neurocircuitry in relationship to their behavioral effects.

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Abbreviations

NAC	nucleus accumbens
AST	anterior striatum
MEMRI	manganese-enhanced magnetic resonance imaging
Mn²⁺	manganese
Ca²⁺	calcium
LTCC	L-type calcium channels
MPRAGE	magnetization prepared rapid acquisition gradient echo
PBS	phosphate-buffered saline
PDGE	proton density weighted
ROIs	regions of interest
SDS	sodium dodecyl sulfate
SI	signal intensities
TBS	TRIS-buffered saline

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Figure 1. The MEMRI data analysis process and brain regions of interests are shown
 (A) For the image analysis process the MPRAGE (top) and PDGE (middle) images were registered on a voxel-by-voxel basis and then the signal intensities from MPRAGE by PDGE were divided to produce the resulting ratio MPRAGE/PDGE (bottom) image. The scale bar represents mean values for that image and the units are arbitrary. (B) A sample ratio image depicting the anterior striatum (AST), nucleus accumbens (NAC), and muscle (M) regions of interest outlined in yellow is shown. (C) A sample ratio image identifying the hippocampus (Hip) and muscle (M) regions of interest outlined in yellow is shown.

Figure 2. Cocaine increases locomotor activity and induces behavioral sensitization in rats
 Behavioral (locomotor) activity was measured for 30 min followed by measurement over a 3 hour period after saline or cocaine injections daily for 5 days (only 3 h of activity after injection is shown). This was followed by a 2-day drug free period, and then a challenge day given on day 8, where Mn^{2+} was injected in all animals for later MEMRI analysis and locomotor activity was measured after saline or cocaine injections over a 6 hour period. (A) Cocaine (10 mg/kg, 3 injections daily) increased locomotor activity and induced robust behavioral sensitization over the first 5 days. (B) On the challenge day (day 8) after a 2-day

drug free period, cocaine (3 mg/kg, 10 injections) induced robust locomotor activity compared to saline control. N=7 rats in the cocaine group and N=8 rats in the saline group; * $p<0.05$, ** $p<0.01$, or *** $p<0.001$ for cocaine versus saline.

Figure 3. Neurofunctional activity only in the NAC is increased upon a cocaine challenge

Cocaine (3 mg/kg, repeated) or saline was given after a 2 day drug free period that followed a 5-day binge-pattern saline or cocaine injection paradigm, and following 6 h of Mn²⁺ uptake and measurement of locomotor activity on day 8, MEMRI was conducted. Group means show that on day 8 cocaine increases signal intensity relative to controls in the (A) NAC but not (B) AST. Sample ratio (MPRAGE/PDGE) images of a (C) saline-treated and (D) cocaine-treated rat illustrate the effect of cocaine on signal intensity and Mn²⁺ uptake (i.e. MEMRI) in the NAC and AST. A pseudo-color palette was used such that more signal intensity and Mn²⁺ uptake is represented by brighter colors (and higher values on the scale to the right). N=7 rats in the cocaine group and N=8 rats in the saline group; *p<0.05 for cocaine versus saline.

Figure 4. Neurofunctional activity in the hippocampus or average temporalis muscle is not affected by a cocaine challenge

Cocaine (3 mg/kg, repeated) or saline was given after a 2 day drug free period that followed a 5-day binge-pattern saline or cocaine injection paradigm, and following 6 h of Mn^{2+} uptake and measurement of locomotor activity on day 8, MEMRI was conducted. Group means show that cocaine challenge on day 8 does not affect signal intensity in the (A) hippocampus normalized to average muscle or (B) average temporalis muscle alone. Sample ratio (MPRAGE/PDGE) images of a (C) saline-treated and (D) cocaine-treated rat illustrate the effect of cocaine on signal intensity and Mn^{2+} uptake (i.e. MEMRI) in the hippocampus and average muscle. A pseudo-color palette was used such that more signal intensity and Mn^{2+} uptake is represented by brighter colors (and higher values on the scale to the right). N=7 rats in the cocaine group and N=8 rats in the saline group.

Figure 5. A significant correlation between locomotor activity on day 8 and signal intensity exists in the NAC, but not in the AST or hippocampus, of rats administered saline or cocaine
Spearman correlation and linear regression analyses revealed that a significant relationship between locomotor activity on day 8 and MEMRI signal intensity in (A) NAC but not (B) AST or (C) hippocampus. N=15 for each brain region analysis.

Figure 6. Administration of Mn^{2+} (88 mg/kg) does not alter DAT content of striatal synaptosomal membranes

A two-tailed t-test revealed no significant difference between Mn^{2+} administration and control on DAT protein in synaptosomal membranes isolated from whole striatum. Blots of the cytosolic fraction had no detectable DAT protein bands (data not shown). N=8 per group.