Published in final edited form as:

Brain Imaging Behav. 2018 February ; 12(1): 201–216. doi:10.1007/s11682-017-9691-1.

Cocaine Differentially Affects Synaptic Activity in Memory And Midbrain Areas Of Female And Male Rats: An In Vivo MEMRI Study

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Abstract

Manganese enhanced magnetic resonance imaging (MEMRI) has been previously used to determine the effect of acute cocaine on calcium-dependent synaptic activity in male rats. However, there have been no MEMRI studies examining sex differences in the functional neural circuits affected by repeated cocaine. In the present study, we used MEMRI to investigate the effects of repeated cocaine on brain activation in female and male rats. Adult female and male rats were scanned at 4.7 Tesla three days after final treatment with saline, a single cocaine injection (15 mg kg⁻¹, i.p. × 1 day) or repeated cocaine injections (15 mg kg⁻¹, i.p. × 10 days). A day before imaging rats were provided with an i.p. injection of manganese chloride (70 mg kg⁻¹). Cocaine produced effects on MEMRI activity that were dependent on sex. In females, we observed that a single cocaine injection reduced MEMRI activity in hippocampal CA3, ventral tegmental area (VTA), and median Raphé, whereas repeated cocaine increased MEMRI activity in dentate gyrus and interpeduncular nucleus. In males, repeated cocaine reduced MEMRI activity in VTA. Overall, it appeared that female rats showed a general trend towards increase MEMRI activity with single cocaine and reduced activity with repeated exposure, while male rats showed a trend towards opposite effects. Our results provide evidence for sex differences in the *in vivo* neural response to cocaine, which involves primarily hippocampal, amygdala and midbrain areas.

Keywords

Addiction; Cocaine; Manganese; MEMRI; Sex differences; Synaptic activity

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Ethical approval of the use of animals in research: The University of Florida Institutional Animal Care and Use Committee approved the experimental protocols. All procedures adhered to the Guide for the Care and Use of Laboratory Animals (8th Edition, 2011), National Institutes of Health and the American Association for Laboratory Animal Science.

Ethical approval for human subjects: Does not apply

Informed consent: Does not apply

Disclosure: The authors have no commercial, financial, or other conflict of interests that influenced the present work.

Conflict of interest: There are no conflict of interests

Introduction

Over 24 million individuals aged 12 years or older reported illicit drug use in the U.S. in 2013 (SAMHSA, 2014). Among these, 1.5 million reported using cocaine and about 855,000 had a cocaine use disorder (SAMHSA, 2014). Individuals with a cocaine use disorder experience repeated episodes of relapse despite prolonged periods of abstinence. Cue-triggered drug craving, exaggerated neurobehavioral responses to stress, co-morbid anxiety/panic during withdrawal, cognitive impairment, and the development of other psychiatric conditions (e.g., psychosis, paranoia) can all contribute to multiple chronic relapse episodes. Importantly, the physiological, psychological and neurobiological factors that influence cocaine seeking and repeated intake can differ between women and men (Kennedy et al., 2013; Lejuez et al., 2007; Lynch et al., 2008; Najavits and Lester, 2008; Wagner and Anthony, 2007). Thus, the study of cocaine-induced alterations in neuronal and synaptic circuitry is significantly improved by including sex comparisons (Clayton and Collins, 2014; Sanchis-Segura and Becker, 2016).

Identifying the brain wide *in vivo* effect of cocaine administration on neuronal and synaptic activity, particularly during drug-free abstinence periods, may improve our understanding of the neural circuit mechanisms underlying cocaine addiction and relapse in female and male users. Human neuroimaging studies (and to a lesser extent animal neuroimaging studies) have shed light on the functional neural circuit adaptations following chronic cocaine administration and abstinence. Abstinent male and female users showed functional connectivity reductions between amygdala and prefrontal cortex and between hippocampus and prefrontal cortex (Gu et al., 2010). Cocaine addicted subjects that relapse within 30 days of treatment also displayed reduced connectivity between amygdala subregions and ventromedial and rostral anterior cingulate cortices (McHugh et al., 2014). Increased functional connectivity between prefrontal cortex and striatum has also been reported in recently abstinent cocaine users (Hu et al., 2015), which have also been shown to have increased connectivity between structures involved in memory, visuospatial processing, and motivation (Wang et al., 2015b). Functional neural circuit adaptations may differ between women and men, particularly in hormone sensitive areas mediating cognitive and emotion-related neural activity during drug free abstinence periods (Li et al., 2005; Potenza et al., 2012; Sinha et al., 2007; Volkow et al., 2011).

Animal fMRI studies have partially supported the above human imaging results, and may provide a valuable means to investigate sex differences in the acute and chronic neural response to cocaine. Male rats undergoing abstinence for 20 days following 10 days of intravenous cocaine administration showed both increases in functional connectivity between nucleus accumbens and its target areas, and decreased functional connectivity involving the prefrontal cortex, accumbens and dorsal striatum (Lu et al., 2014). Cerebral blood volume responses to cocaine were blunted in the prefrontal cortex, anterior cingulate, medial dorsal thalamus, and hippocampus (Lu et al., 2012). This blunted hemodynamic response to chronic cocaine exposure is consistent with previous work using noncontingent cocaine administration in male rats (Febo et al., 2005b), but is inconsistent with results obtained in female rats (Febo et al., 2005a).

Because of the neurovascular and brain endothelial actions of cocaine and of other drugs of abuse (Sajja et al., 2016), interpreting the functional neural circuitry changes observed in fMRI studies is limited by 'off-target' non-neuronal hemodynamic effects (Kaufman et al., 2001; Tucker et al., 2004). Animal imaging studies can help overcome this limitation using contrast agent enhanced imaging that offers a closer indication of neuronal and synaptic activity (Duong et al., 2000; Inoue et al., 2011; London et al., 1989; Silva et al., 2004). Manganese enhanced MRI (MEMRI) has been used to track calcium-dependent synaptic activity under several experimental paradigms, including experimental conditions involving acute and chronic drug exposure (Chiu et al., 2015; Dudek et al., 2015; Hsu et al., 2008; Lu et al., 2007; Perrine et al., 2015). The manganese ion (Mn^{2+}) is a calcium analog that enters active synapses through voltage-gated calcium channels (Fukuda and Kawa, 1977; Narita et al., 1990) and is sequestered and transynaptically transported antero- and retrogradely across active neural circuits (Murayama et al., 2006; Pautler et al., 1998; Saleem et al., 2002; Sloot and Gramsbergen, 1994; Takeda et al., 1998a; Takeda et al., 1998b). The presence of the paramagnetic Mn^{2+} ion in the brain increases longitudinal relaxation rates and enhances signal intensity in T_1 weighted scans, and is utilized for functional mapping of synaptic activity (Duong et al., 2000).

The MEMRI technique has proven useful in mapping synaptic activity by acutely driving brain neural responses with sensory (Aoki et al., 2002; Duong et al., 2000; Yu et al., 2005) or drug stimulation (Lu et al., 2007; Perrine et al., 2015). However, in the absence of neuronal-driving stimuli, and without the aid of drugs to increase its entry to the CNS (central nervous system), Mn^{2+} shows a robust distribution in the brain (Lee et al., 2005) through both activity independent and dependent mechanisms (Wang et al., 2015a), and has been used to map calcium-dependent synaptic activity under chronic disease conditions (Gallagher et al., 2012; Kim et al., 2011; Perez et al., 2013; Smith et al., 2007). This has opened an opportunity for mapping synaptic activity during abstinence periods following acute or chronic cocaine exposure. The present study used MEMRI in male and female rats given single or repeated non-contingent cocaine and imaged after an abstinence period. Previously, cocaine-induced alterations in synaptic plasticity, voltage sensitive calcium currents and neuronal excitability in areas receiving dopaminergic inputs such as the prefrontal cortex and nucleus accumbens have been described (Hu et al., 2004b; Kamii et al., 2015; Nasif et al., 2005; Zhang et al., 2002). Such drug-induced changes in the firing of neurons were expected to lead to Mn^{2+} accumulations that differed between control and cocaine-exposed animals. Given the well-documented sex differences in the psychomotor stimulant (Hu and Becker, 2003) and reinforcing effects of cocaine (Lynch and Taylor, 2004), as well as the synaptic and neuronal changes produced by the drug (Wissman et al., 2011), we predicted that female and male rats would vary in the intensity of MEMRI signal changes due to chronic cocaine administration. Our results indicate that females have a much greater baseline Mn^{2+} signal in memory and midbrain areas and show very distinct neuroadaptations in comparison to males when given acute or chronic cocaine.

Materials and Methods

Subjects

Male (n = 20) and female (n = 21) Long Evans rats (200–350g; Charles River Labs, Wilmington, MA) were housed in same-sex pairs in a temperature and humidity-controlled vivarium (12 h light-dark cycle, lights off at 19:00 h; food and water were provided *ad libitum*). The University of Florida Institutional Animal Care and Use Committee approved the experimental protocols. All procedures adhered to the Guide for the Care and Use of Laboratory Animals (8th Edition, 2011), National Institutes of Health and the American Association for Laboratory Animal Science.

Cocaine-induced locomotor and stereotyped activity

The locomotor effects of cocaine or saline administration were first measured. All rats were habituated for 60 minutes inside automated locomotor activity testing cages a day before starting experiments. These cages consisted of clear and smooth plastic acrylic walls and floor (40 cm along height × length × width) surrounded by an aluminum frame containing 3 pairs of infrared beam emitters and receivers. The 1st two sets of beams were located along the sides of the floor at 2 cm distance from the floor forming an x-y grid (horizontal beams), and the 3rd set of beams along the sides of the walls at a distance of 5 cm from the floor (z axis vertical beams). Interruption of a single beam (out of 16 beams along each of the x, y and z coordinates, each beam 2.5 cm apart) by rats moving inside test boxes registered a count on a personal computer running Versamax® software (Accuscan Instruments Inc., Columbus, OH). Consecutive counts along the x-y direction were defined as horizontal activity, repeated interruptions of the same series of beams as stereotyped activity, and interruption of vertical z axis beams as vertical activity (standing on hind limbs). Rats received an intraperitoneal (i.p.) saline (0.9% sterile saline, 1ml/kg) or cocaine injection (15 mg/kg) and were then placed into an activity test cage for 1 h. Activity was recorded on the computer during this post-injection period (60 minutes total in six 10 minute bins). The procedure was repeated for 10 consecutive days. Male and female rats were all tested under the same conditions but in separate daily sessions. Cages were cleansed with 3% hydrogen peroxide solution to remove odors from previous sessions. Following the final injection day, rats were given a 3-day washout period before Mn²⁺ treatment and imaging.

A subgroup of male and female rats that were administered saline for 4 days received an i.p. 15 mg/kg cocaine injection on a 5th and final day (single cocaine treatment). No behavior was carried in these animals, but injections were made in the same environment as animals assessed for their locomotor response to repeated cocaine. This group was added to distinguish between the MEMRI activity produced by single and repeated cocaine administration. Imaging was carried out 3 days after the final cocaine injection. The following experimental groups are included in this study: male/saline (n = 5), male/single cocaine (n = 5), male/repeated cocaine (n = 5), female/saline (n = 6), female/single cocaine (n = 9), female/repeated cocaine (n = 6). An additional group of 5 male rats that were not given any injection served as controls for Mn²⁺ treatment. A repeated measure two-way analysis of variance (ANOVA) was used with drug treatment and sex as independent factors,

and post-injection time as a repeated measure ($p < 0.05$; Fisher's least significant difference test used in posthoc analyses).

Manganese enhanced magnetic resonance imaging (MEMRI) following systemic Mn^{2+} injection

Manganese (II) chloride tetrahydrate ($MnCl_2$; Sigma-Aldrich, St. Louis, MO) was dissolved in sterile filtered ddH₂O prior to use. Twenty-two to 24 h prior to imaging, all rats were behaviorally tested and were given an i.p. injection of $MnCl_2$ (70 mg/kg) in their home cage. A group of 5 naïve male rats were used as controls for Mn^{2+} treatment and were scanned under the same conditions as the rest of the animals. These Mn^{2+} controls served to validate and assess the quality of the processing procedure used here for analyzing the MEMRI data. Previous studies have shown T_1 signal enhancement at 4 hours following systemic administration with doses between 44–88 mg/kg and significant brain distribution to various cortical layers and subcortical structures by 14–24 h (Kimura et al., 2007; Lee et al., 2005). In addition, we conducted pilot studies to determine the optimum dose of $MnCl_2$ solution. In these initial studies, the selected dose was observed to provide significant T_1 signal enhancement with no signs of motor, cardiac, or respiratory impairment.

Images were collected on a 4.7 Tesla Magnex Scientific MR scanner controlled by Agilent Technologies VnmrJ 3.1 console software. A 38-mm quadrature transmit/receive radiofrequency coil tuned to 200.6 MHz was used (airmri, LLC, Holden, MA). Anesthesia was initially induced under 3–4% isoflurane delivered in 100% medical grade oxygen for 30–60 seconds (flow rate 1L/min), and the levels of isoflurane were then maintained between 1.5–2.0% throughout the entire setup and imaging. Rats were placed prone on a plastic cradle with a respiratory pillow connected to a force transducer placed underneath the abdomen. Body temperatures were maintained using a warm air recirculation system that received feedback from a fiber optic thermocouple probe (SA Instruments, Inc., New York). Respiratory rates were monitored continuously and average breaths per minute across subjects were 50–70. Images were acquired using a T_1 -weighted multi-slice multi-echo sequence with the following parameters: repetition time (TR) = 460 ms, echo time (TE) = 16 ms, data matrix 256×256 , field of view 25.6×25.6 mm, 20 slices at 0.8 mm thickness. Slices were in axial view (coronal in the rat) with the first slice starting at the rostral-most extension of the prefrontal/motor cortex and excluding the olfactory bulb. Scan time was 60 minutes per rat for 30 averages.

Data processing and statistical analysis

MEMRI data were processed and analyzed using previously published methods (Perez et al., 2013). Brain masks were manually drawn over T_1 anatomical scans using the drawing tool in itkSNAP (www.itksnap.org). The masks were then used to remove non-brain signal from each scan (including the *pituitary*, which showed highest signal intensity that could skew the distribution of voxel signal intensities on histograms). The resulting cropped images were aligned with a rat brain template (Ekamsolutions, LLC, Holden, MA) using the FMRIB Software Library's automated linear registration tool *flirt* (Jenkinson et al., 2002). We used 12-parameter affine registration with a correlation ratio search cost, full angular search along x y z, a mutual information cost function, and trilinear interpolation, and other default

parameters in *flirt*. The quality of registration was judged adequate based on the precision of the alignment of important landmarks inside the rat brain, such as the corpus callosum, lateral ventricles and hippocampus, which were qualitatively evaluated for each subject. The subject-to-atlas transformation vectors were saved as a numerical matrix. The FSL command '*convert_xfm*' was used to calculate an inverse transformation matrix (atlas-to-subject) that was subsequently applied to the rat brain template to generate a subject-specific region of interest (ROI) mask using *flirt* (nearest neighbor interpolation used in this step).

Cropped T₁ scans were converted to z score maps through a voxel-wise signal-to-noise processing step. We first calculated the global mean signal and standard deviation. For each individual voxel, we subtracted the global mean signal and then divided the result by the standard deviation of the mean global brain signal. This produced images with $-z$ to $+z$ values (with voxel values generally between -4 to $+4$) centered slightly above a mean z value of 0 (center z value ~ 0.4). Voxels exceeding a threshold value of $z = 0.8$ are considered in our statistical analysis as having high signal intensities primarily associated with MnCl₂ accumulation (in most cases, $z \geq 1$ indicates highest putative Mn²⁺ accumulation). In a preliminary analysis of rats without Mn²⁺ treatment, we tested various threshold values ($z = 0.8, 1.0$ and 1.2) and determined that applying a threshold of $z = 0.8$ was sufficient for eliminating most brain signal in this group. We applied the same threshold to brain images of rats treated with Mn²⁺ and this consistently showed signal intensity above the threshold in several brain regions (see results). We also analyzed the intensity distribution histograms for all groups as a way to verify the accuracy of this image processing and thresholding procedure. All voxels with z score values below this threshold were set to zero. Composite maps were produced for each group by averaging atlas-registered and thresholded z score maps. ROI masks for each subject were used to export nonzero signal intensity values for 56 brain areas. Unless otherwise noted, a two-way ANOVA was used to analyze these data for each ROI independently, with drug treatment and sex as independent factors ($p < 0.05$; Fisher's least significant difference [LSD] test was used for individual posthoc analyses). Statistical analyses were carried out in Graphpad Prism Software (Version 7.02).

Results

Repeated cocaine produced behavioral sensitization in both male and female rats

Results for locomotor activity are shown in Figure 1. Cocaine produced greater levels of locomotor (main effect drug treatment in females $F_{3,44} = 22$ and $p < 0.0001$ and in males $F_{3,16} = 35$ and $p < 0.0001$), stereotyped (main effect drug treatment in females $F_{3,44} = 21$ and $p < 0.0001$ and in males $F_{3,16} = 33$ and $p < 0.0001$) and vertical activity (main effect drug treatment in females $F_{3,44} = 5$ and $p = 0.0045$ and in males $F_{3,16} = 7.8$ and $p = 0.009$) than saline. Both male and female rats showed greater levels of locomotor and stereotyped activity on day 10 than on day 1 (locomotor activity females, $p < 0.05$ at 20–50 minute post-injection and males, $p < 0.05$ for the entire 60 minutes; stereotyped activity females, $p < 0.05$ at 20–60 minute post-injection and males, $p < 0.05$ at 30–60 minutes). Males also showed a lower vertical activity on day 10 compared to day 1 ($p < 0.05$ at 20–40 minutes; Figure 1). Our results indicate that both male and female rats showed sensitized locomotor response to cocaine 5 days before imaging.

Regions showing T_1 weighted magnetic resonance signal enhancement following Mn^{2+} administration

The regional distribution of brain signal enhancement following systemic Mn^{2+} was consistent with previous research (Lee et al., 2005). Particularly consistent was the enhanced signal intensity in the dorsal hippocampal CA3/dentate gyrus (Online Supplemental Figure 1). This was observed in all 6 saline treated females and all 5 saline male rats, and not in any of the 5 Mn^{2+} control rats tested (Online Supplemental Figure 1). Strong signal enhancement is also observed in the highly vascularized pituitary gland, pituitary stalk and median eminence, and this was used to confirm effective treatment with Mn^{2+} (Online Supplemental Figure 1). Signal enhancement in the brain was observed across several regions, mostly localized near ventricular areas. These regions included the dorsal and ventral CA3 and dentate, the habenula, interpeduncular nucleus, paraventricular nucleus and bed nucleus of the stria terminalis, among other regions (e.g., septal area, anterior hypothalamus) (Figure 2A). Composite maps also showed distribution of the Mn^{2+} related signal enhancement, which was highly concentrated in forebrain areas (Figure 2B and C).

We first compared overall brain signal intensity between male and female control animals with rats not treated with manganese but scanned using the same T_1 weighted MRI sequence (Figure 3). Whole brain signal intensity among the three groups was within a similar range, which supports the notion that regional MEMRI differences are selective to certain CNS regions and not merely the result of general and non-specific signal intensity increases across the entire brain (Figure 3A). Indeed, a close examination of whole brain signal intensity distribution in Figure 3A shows effects consistent with greater signal variation with Mn^{2+} exposure than in non-exposed controls. This is observed as broader tails of the distribution and lower peak height in Mn^{2+} rats (Figure 3A). The overall differences in whole brain are, however, less overt than when looking at smaller ROI's (primarily due to the washing out of differences because most voxels in the distribution overlap significantly in signal intensity). When analyzing signal intensity values in various regions, we noted that in areas such as the hippocampus and midbrain the signal intensity is higher in animals with manganese treatment (Figure 3B). Interestingly, these regions also showed greater signal intensity values in females compared to males (Figure 3C).

Effects of single and repeated cocaine injections on brain MEMRI signal intensity

Composite maps in Figure 4 summarize the spatial extent of Mn^{2+} signal intensity in females rats administered saline, single and repeated cocaine injections and Figure 5 summarizes data for male rats. Analyses of signal intensity in each of the 56 ROIs revealed sex differences with repeated cocaine administration in subregions of the hippocampus, amygdala and midbrain (Figures 6–7).

Hippocampus

In the dorsal CA3 subregion of the hippocampus there was a sex x treatment interaction ($F_{2,30} = 6.8$, $p = 0.0037$). Female rats given repeated cocaine showed a significant increase in dorsal CA3 MEMRI signal compared to acutely treated females (Fisher's posthoc test: $t_{30} = 3.2$, $p = 0.003$) and to males given repeated cocaine (Fisher's posthoc test: $t_{30} = 3.5$, $p = 0.002$). A similar effect was observed in male rats, but in the opposite direction, with

repeated cocaine decreasing signal intensity compared to acutely exposed males (Fisher's posthoc test: $t_{30} = 2.1$, $p = 0.04$) (Figure 6). In the ventral CA3, there was a significant sex x treatment interaction ($F_{2,30} = 12$, $p = 0.0002$) and a main effect of sex ($F_{1,30} = 16$, $p = 0.0004$). Females given saline showed a greater baseline MEMRI signal than males administered saline (Fisher's posthoc test: $t_{30} = 2.1$, $p = 0.04$) (Figure 6). A single cocaine injection reduced ventral CA3 MEMRI signal in female rats (Fisher's posthoc test: $t_{30} = 2.9$, $p = 0.007$) and repeated cocaine increased signal intensity (Fisher's posthoc test: $t_{30} = 2.1$, $p = 0.04$). Females administered repeated cocaine also showed a greater signal intensity in ventral CA3 than males given the same cocaine treatment (Fisher's posthoc test: $t_{30} = 5.7$, $p < 0.0001$). Male rats given repeated cocaine showed a lower signal intensity in this region than acutely exposed animals (Fisher's posthoc test: $t_{30} = 2.05$, $p = 0.048$). In the CA2 hippocampal subregion, there was a significant main effect of sex ($F_{1,30} = 7.8$, $p = 0.008$), with the main difference between saline treated females and males (Fisher's posthoc test: $t_{30} = 2.6$, $p = 0.01$) (Online Supplementary Figure 2). Similar to the CA3 region, a two-way ANOVA revealed a significant sex x treatment interaction in the dorsal dentate gyrus (DG) ($F_{2,30} = 7.5$, $p = 0.002$) and a sex x treatment interaction ($F_{2,30} = 12.1$, $p = 0.0001$) and main effect of sex in the ventral dentate gyrus ($F_{1,30} = 16.4$, $p = 0.003$). In the dorsal DG, repeated cocaine increased MEMRI signal intensity in females compared to saline treated (Fisher's posthoc test: $t_{30} = 2.6$, $p = 0.01$) and single cocaine treated female rats (Fisher's posthoc test: $t_{30} = 3.6$, $p = 0.001$), and males given repeated cocaine (Fisher's posthoc test: $t_{30} = 3.6$, $p = 0.001$) (Figure 6). We observed similar effects in ventral DG, with repeated cocaine increasing MEMRI signal intensity in female rats compared to those given saline (Fisher's posthoc test: $t_{30} = 2.3$, $p = 0.026$), acute cocaine (Fisher's posthoc test: $t_{30} = 4.3$, $p = 0.0002$), and males given repeated cocaine (Fisher's posthoc test: $t_{30} = 5.4$, $p < 0.0001$) (Figure 6). This subregion of the DG also showed a significantly greater signal intensity in saline treated female rats compared to saline treated males (Fisher's posthoc test: $t_{30} = 2.7$, $p = 0.01$) (Online Supplementary Figure 2). In males, repeated cocaine reduced signal intensity in ventral CA3 compared to saline controls (Fisher's posthoc test: $t_{30} = 2.3$, $p = 0.026$) and acutely treated rats (Fisher's posthoc test: $t_{30} = 2.7$, $p = 0.01$) (Figure 6).

Midbrain

We observed significant effects of sex and treatment in several midbrain regions, which included the median and dorsal Raphé nuclei, central grey (CG), ventral tegmental area (VTA), and interpeduncular nucleus (IPN). In the median Raphé there was a significant sex x treatment interaction ($F_{2,30} = 5.2$, $p = 0.01$) and main effects for sex ($F_{1,30} = 27.7$, $p < 0.0001$) and treatment ($F_{2,30} = 5.2$, $p = 0.01$). A single cocaine injection reduced signal intensity in this region in female rats (Fisher's posthoc test: $t_{30} = 4.5$, $p = 0.0001$). Female rats administered repeated cocaine showed a greater signal intensity than males given a similar treatment (Fisher's posthoc test: $t_{30} = 2.1$, $p = 0.04$) (Figure 6). This brain area also showed a significantly greater signal intensity in saline treated females than males (Fisher's LSD test, $p < 0.0001$) (Online Supplementary Figure 2). In the dorsal Raphé there was a significant main effect for sex ($F_{1,30} = 25.6$, $p < 0.0001$) and treatment ($F_{2,30} = 4.1$, $p = 0.03$). In this region, a single cocaine injection reduced signal intensity in female rats (Fisher's posthoc test: $t_{30} = 3.0$, $p = 0.005$). Female rats administered repeated cocaine showed a greater signal intensity than males given a similar treatment (Fisher's posthoc test:

$t_{30} = 3.8$, $p = 0.0006$) (Figure 6). This brain area also showed a significantly greater signal intensity in saline treated females than males (Fisher's posthoc test: $t_{30} = 3.0$, $p = 0.005$). In the CG there was a significant sex x treatment interaction ($F_{2,30} = 4.3$, $p = 0.02$) and main effects for sex ($F_{1,30} = 5.2$, $p = 0.03$) and treatment ($F_{2,30} = 6.2$, $p = 0.006$). An acute cocaine injection reduced MEMRI signal intensity in the CG of female rats (Fisher's posthoc test: $t_{30} = 3.8$, $p = 0.0006$). Female rats given repeated cocaine showed a greater MEMRI signal than their male counterparts (Fisher's posthoc test: $t_{30} = 3.3$, $p = 0.002$). In males, repeated cocaine administration reduced MEMRI signal intensity in the CG (Fisher's posthoc test: $t_{30} = 2.3$, $p = 0.03$) (Figure 6). In the VTA there was a significant sex x treatment interaction ($F_{2,30} = 3.8$, $p = 0.03$) and main effects for sex ($F_{1,30} = 4.4$, $p = 0.04$) and treatment ($F_{2,30} = 3.8$, $p = 0.03$). An acute cocaine injection decreased MEMRI signal intensity in the VTA of female rats (Fisher's posthoc test: $t_{30} = 2.9$, $p = 0.006$). Females given repeated cocaine showed greater MEMRI signal intensity than their male counterparts (Fisher's posthoc test: $t_{30} = 3.0$, $p = 0.005$). In males, repeated cocaine significantly reduced signal intensity in the VTA compared to saline control males (Fisher's posthoc test: $t_{30} = 2.5$, $p = 0.02$) (Figure 6). In the IPN there was a significant sex x treatment interaction ($F_{2,30} = 3.8$, $p = 0.03$) and main effect for sex ($F_{1,30} = 10.1$, $p = 0.003$). Repeated cocaine administration increased signal intensity in the IPN of female rats (Fisher's posthoc test: $t_{30} = 2.6$, $p = 0.01$) but not males. Females given repeated cocaine had a greater signal intensity in IPN than males (Fisher's posthoc test: $t_{30} = 4.0$, $p = 0.0004$).

Amygdala

In the medial amygdala, there was a significant sex x treatment interaction ($F_{2,30} = 4.4$, $p = 0.02$) and main effect of treatment ($F_{2,30} = 4.6$, $p = 0.02$). A single cocaine injection in female rats reduced signal intensity in this region (Fisher's posthoc test: $t_{30} = 3.2$, $p = 0.003$) (Figure 7). Females given repeated cocaine had greater signal intensity in the medial amygdala than male rats given repeated cocaine (Fisher's posthoc test: $t_{30} = 2.6$, $p = 0.01$) (Figure 7). In the central amygdala, there was a significant sex x treatment interaction ($F_{2,30} = 5.2$, $p = 0.01$) and main effect of sex ($F_{1,30} = 5.8$, $p = 0.02$). A single cocaine injection in female rats reduced signal intensity in this region (Fisher's posthoc test: $t_{30} = 2.7$, $p = 0.01$). Females given repeated cocaine had greater signal intensity in the medial amygdala than male rats given repeated cocaine (Fisher's posthoc test: $t_{30} = 3.5$, $p = 0.001$) (Figure 7). Finally, a two-way ANOVA showed a significant sex x treatment interaction ($F_{2,30} = 4.6$, $p = 0.02$) and main effect of sex ($F_{1,30} = 6.8$, $p = 0.01$) in the lateral amygdala. Repeated cocaine in female rats increased signal intensity in this amygdala region relative to acutely treated females (Fisher's posthoc test: $t_{30} = 2.8$, $p = 0.008$) and male rats given repeated cocaine (Fisher's posthoc test: $t_{30} = 3.5$, $p = 0.002$).

Other regions

We analyzed a number of hypothalamic, cortical, striatal and thalamic regions and none showed effects despite showing evidence of Mn^{2+} accumulation (see next section). Among the regions outside the hippocampus, midbrain and amygdala, only the anterior hypothalamic area showed a significant sex x treatment interaction ($F_{2,30} = 3.5$, $p = 0.04$) and main effect of sex ($F_{1,30} = 6.1$, $p = 0.02$). Acute cocaine reduced signal intensity in the AHA of females (Fisher's posthoc test: $t_{30} = 2.5$, $p = 0.02$). Females given repeated cocaine

showed a greater signal intensity than repeatedly exposed males (Fisher's posthoc test: $t_{30} = 3.1$, $p = 0.004$).

Sex differences in basal MEMRI signal

Finally, several regions showed sex differences in MEMRI signal regardless of cocaine treatment. We used a non-parametric Mann-Whitney median test to compare these two groups (two-tailed, $p < 0.05$). These included the median (p = 0.004) and dorsal nuclei of Raphé (p = 0.017), and the hippocampal CA2 (p = 0.017), ventral dentate (p = 0.004), and lateral geniculate (p = 0.027), all of which showed lower signal intensity in males compared with females (Online Supplementary Figure 2).

Discussion

There are two principal findings in the present study. First, female rats showed greater levels of MEMRI signal intensity than males in several brain areas. This was not a generalized effect across the entire brain since the regions that showed greater Mn^{2+} signal in females were limited to hippocampal areas (CA2, ventral dentate), Raphé nuclei and the lateral geniculate nucleus. The implication of this first result is that these areas of the female rat brain might sustain higher levels of ongoing synaptic activity than males, which would lead to greater synaptic accumulation of Mn^{2+} in these regions during a 24 h period. This in turn suggests the existence of sex differences in the basal activity of neurons involved in learning and memory, emotion modulation (perhaps involving serotonergic neurons in the Raphé), and visual processing. It is important to note, however, that Mn^{2+} can accumulate in some regions through activity-dependent (Aoki et al., 2002) and independent mechanisms (Wang et al., 2015a). It is interesting that the regions showing greater MEMRI activity in females in the present work (namely, hippocampal CA2, dentate, Raphé and lateral geniculate) do not show sex differences in local cerebral glucose utilization under basal conditions (Nehlig et al., 1985). Thus, the greater MEMRI signal in these areas of females might also be associated with activity-independent mechanisms of Mn^{2+} accumulation (Wang et al., 2015a). Interestingly, all of these areas express estrogen receptors (α and β subtypes) in female rats (Horvath et al., 1999; Leranth et al., 1999; Shughrue and Merchenthaler, 2000; Suzuki et al., 2013). The presence of estrogen receptors implies that neurons in these regions can respond to estrogenic stimulation. However, it remains to be determined whether estrogen modulates synaptic activity in these regions, and this in turn is the cause for greater Mn^{2+} signal.

The second finding of importance is that acute and repeated cocaine exerts diametrically distinct actions on MEMRI signal in the male and female brain. In female rats, a single cocaine injection reduced MEMRI signal intensity in several regions (compared to saline controls) and repeated cocaine administration either had no effect or increased MEMRI activity. In male rats, a single cocaine injection increased MEMRI signal in a few brain areas (compared to saline controls) and repeated cocaine either reduced or had no effect on MEMRI activity. Areas of the female and male rat brain showing this pattern of MEMRI activity with acute and repeated cocaine included areas of the hippocampus, amygdala and midbrain. One implication of this second set of findings is that acute and repeated cocaine

exert distinct effects via learning and emotional memory centers in male and female rats. This is consistent with previous work (Sato et al., 2011; Wissman et al., 2011; Zhou et al., 2014). Another implication of the results is that the mechanism of Mn^{2+} accumulation in the brain is affected differently by cocaine in males and females. One possibility is that in female rats a single cocaine injection may cause a lasting suppression of spontaneous firing of neurons in these regions, and may exert an opposite effect in males (increased excitability). In male rats, there is evidence of cocaine-induced synaptic potentiation of VTA neurons (Dong et al., 2004; Ungless et al., 2001) and cocaine itself can alter neuronal excitability by affecting dopamine transporter mediated conductance (Ingram et al., 2002). Also, cocaine can alter dendritic and spine morphology in accumbens and prefrontal cortex in male rats (Robinson and Kolb, 1999) and this could alter Mn^{2+} accumulation in the brain. However, these effects of acute and repeated cocaine have been studied in male rats and it is unclear if differential outcomes in females may underlie the distinct patterns of MEMRI activity observed in the present study. Overall, our results provide evidence for sex differences in the *in vivo* neural response to cocaine, which involves primarily hippocampal, amygdala and midbrain areas.

There have been a number of studies applying MEMRI to the study of the functional neural circuit activation by cocaine and other drugs of abuse. The approach used in applying MEMRI varies across several of these studies, and the choice of technique in turn is associated with the specific questions being addressed in using this functional imaging strategy. Mn^{2+} ions can traverse the BBB (blood-brain barrier) within seconds of intra-carotid injection, but its penetration into CNS shows saturable transport kinetics (Aschner and Gannon, 1994). Thus, examining the acute effects of drugs using MEMRI is limited by this slow entry of Mn^{2+} in sufficient amounts to brain areas that are activated during a limited period. To overcome this issue, Lu and colleagues (Lu et al., 2007) utilized methods originally used in studies by Aoki et al in which BBB permeability is increased by mannitol to facilitate rapid Mn^{2+} penetration (Aoki et al., 2002). Using this approach, Lu et al showed the areas of the brain that are dose-dependently activated by intravenously administered cocaine in male rats (Lu et al., 2007). The areas included well-established brain reward regions such as the striatum and prefrontal cortex, which confirms the utility of the MEMRI technique to study the neural circuits activated by this drug. In addition, they also showed that the calcium channel blocker diltiazem prevents the increases in MEMRI signal in response to electrical forepaw stimulation; confirming that signal enhancement in neural circuits is mediated in part through sequestration in active synapses. Mn^{2+} appears to accumulate largely inside neurons and dendrites and not in the extracellular medium (Watanabe et al., 2013), and may be sequestered in intracellular compartments and bound to macromolecules (Watanabe et al., 2015). Its neuronal distribution can provide a clear architectural depiction of cortical layers *in vivo* (Silva et al., 2008). Our present results showed very little Mn^{2+} signal enhancement in cortical and striatal regions (except in regions near ventricles; Figure 2), although layered distribution is somewhat apparent in comparison with T1 scans without Mn^{2+} treatment (Online Supplemental Figure 1). This may be largely because at the single acute systemic dose of manganese used in the present study these areas show very little Mn^{2+} penetration under control or non-drug exposed conditions. Perrine et al (Perrine et al., 2015) studied the relationship between MEMRI

signal in ventral and striatal regions and the development of behavioral sensitization in male rats. Rats were administered a binge pattern of experimenter-delivered cocaine for 5 days and on a final day imaged 6 h after Mn^{2+} administration. They observed greater signal increases in nucleus accumbens of cocaine vs. saline treated animals (Perrine et al., 2015). Although they found a linear relationship between levels of locomotor activity and signal intensity in the nucleus accumbens and anterior (dorsal) striatum, they found no relationship between hippocampal MEMRI activity and cocaine-induced locomotion. The goal of the present study was to measure MEMRI activity in a drug free state following chronic cocaine exposure, but we did not consider the relationship with locomotor sensitization more directly (hours after drug exposure). One reason for this objective was to assess whether MEMRI detects changes in synaptic activity not linked to the transient or acute pharmacological actions of cocaine (e.g., increased extracellular levels of monoamines). MEMRI has also been utilized to investigate functional neural circuitry changes in response to other drugs, such as methamphetamine (Hsu et al., 2008), morphine (Sun et al., 2006), and methylenedioxymethamphetamine (MDMA) (Chiu et al., 2015). It should be noted that the above-cited studies were carried out in male rats only.

In one interesting application of MEMRI designed to track functional pathways more specifically, Mn^{2+} was injected directly to the habenula (which is one of the regions showing most Mn^{2+} accumulated signal with systemic administration) to measure activity over time along the fasciculus retroflexus with repeated administration of a neurotoxic dose of methamphetamine. Activity along the fasciculus retroflexus connecting the habenula to the VTA/interpeduncular nucleus was clearly visible (Hsu et al., 2008). We observed very high signal intensity in these three brain regions with systemic exposure. Females showed increased signal intensity in the interpeduncular nucleus with repeated cocaine, and males showed a reduction in MEMRI signal in the VTA with the same amount of cocaine injections (Figure 6). This differential outcome between males and females suggests that different synaptic circuits are active in the drug free condition tested here. In the case of males, the reduction in VTA activity suggests blunted activity of dopamine neurons in this region. There is some evidence of this in the literature, although a majority indicates increased plasticity of VTA synapses following acute or chronic cocaine (Dong et al., 2004; Ungless et al., 2001). In just one supporting example, withdrawal from chronic opiate exposure affected the ability of GABAergic interneurons in the VTA to disinhibit DA neurons (Kaufling and Aston-Jones, 2015). If a similar mechanism were present with chronic cocaine exposure, this would result in greater suppression of activity in cocaine exposed vs. saline treated rats. Unfortunately, in the present study we were unable to discern tail of the VTA from other VTA subareas to make a direct comparison of MEMRI activity (Barrot, 2015). Interestingly, a single cocaine injection results in insertion of calcium permeable AMPA receptors in VTA neurons, an effect that is short-lived with experimenter-delivered cocaine (as carried out in the present study) (Wolf and Tseng, 2012). Such a mechanism should have led to greater Mn^{2+} accumulation in the VTA with single cocaine exposure. However, the VTA was not among the brain regions showing increased MEMRI activity with single cocaine in males. Female rats, on the other hand, showed increased MEMRI activity in the interpeduncular nucleus with repeated cocaine. This increased activity could be related to the existence of an aversive state, and suggests the involvement

of cholinergic transmission in females given repeated cocaine (Zhao-Shea et al., 2015). One potentially interesting possibility is that females showing greater interpeduncular activity with repeated cocaine might also sustain greater levels of anxiety (not measured here). Indeed, optogenetic silencing of the medial habenula-interpeduncular nucleus pathway in mice exposed to chronic nicotine suppresses activity in the latter region and reduces anxiety (Zhao-Shea et al., 2013).

Sex differences in the locomotor stimulating effects of cocaine have been reported. Studies have reported a greater reactivity to acute cocaine in female rats compared to males (Festa et al., 2004; Glick et al., 1983), higher sensitivity to the conditioned rewarding effects (Russo et al., 2003), and a faster onset of behavioral sensitization than males (Hu and Becker, 2003). We observed that both female and male rats show behavioral sensitization to cocaine, although a sex difference in locomotor and stereotyped behavior was not apparent in the present work. The lack of sex differences in locomotor behavior in this study is perhaps due to grouping of female rat data regardless of their stage of the hormonal cycle. Estrous variations in circulating ovarian hormones influence the acute behavioral response to cocaine (Sell et al., 2005; Sell et al., 2000), and it seems that this is mediated through central dopaminergic actions of estrogen (Febo et al., 2003; Peris et al., 1991; Zhang et al., 2008). Estrogen also modulates other key neurotransmitters in areas of the reward system such as glutamate (Oberlander and Woolley, 2016), GABA (Febo and Segarra, 2004; Huang and Woolley, 2012), and serotonin (Zhou et al., 2002), which may also underlie differences in the behavioral response to cocaine. Importantly, functional neural circuit adaptations in response to cocaine may be contingent upon these hormonal effects, particularly estrogen, in female rats. Moving forward, this will be an important question to address using MEMRI and other *in vivo* functional and structural imaging modalities. Circulating estrogen may act through central α and β receptor subtypes, which are widely distributed across several important regions affected by drugs of abuse (Horvath et al., 1999; Leranath et al., 1999; Shughrue and Merchenthaler, 2000; Suzuki et al., 2013). Central actions of estrogen may mediate greater propensity to self-administer cocaine (Hu et al., 2004a), reinstatement of self-administration following extinction (Larson and Carroll, 2007), and greater motivation to seek cocaine (Lynch and Taylor, 2005). Female rats trained to self-administer cocaine showed greater levels of reinstatement in the presence of cue and cue-stressor than males (Feltenstein et al., 2011). Female rats also show a greater preference for cocaine over food reward than male rats, and this is mediated by estrogen in the female (Kerstetter et al., 2012). While our behavioral paradigm is circumscribed to experimenter-delivered cocaine, limiting comparisons with the cited work, it is interesting to note that in females the primary effects of acute and repeated cocaine on MEMRI activity involved memory and emotion regions. The CA2 of the hippocampus, Raphé and amygdala have been reported to express estrogen receptors and could therefore represent areas responsive to cocaine and estrogen in female rats. It will be important in future studies to: (1) apply MEMRI in male and female rats using intravenous cocaine self-administration using an extended access paradigm, (2) determine the effects of estrogen in modulating MEMRI activity during baseline and drug intake conditions, (3) and compare results using systemic Mn^{2+} and dynamic MEMRI as previously used by Lu et al (Lu et al., 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

This work was supported by NIH grant DA038009, DA019946 and the University of Florida McKnight Brain Foundation. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the funding agencies. Authors acknowledge the support from the National High Magnetic Field Laboratory's Advanced Magnetic Resonance Imaging & Spectroscopy (AMRIS) Facility (National Science Foundation Cooperative Agreement No. DMR-1157490 and the State of Florida). PDP is currently in the Department of Biomedical Engineering at Penn State University.

Funding: This study was funded by NIH grants DA019946 and DA038009 to Dr. Marcelo Febo.

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Figure 1. Repeated cocaine administration produced behavioral sensitization in female and male rats.

Injections (i.p.) were administered at time = 0 minutes. A significant main effect of drug and day is observed for locomotor activity (ANOVA $p < 0.05$). Data are shown as mean $\pm$ standard error. *Significant difference between cocaine day 10 and day 1 (Fisher's LSD posthoc test, $p < 0.05$).

Figure 2. Systemic administration of manganese (Mn^{2+} ; 70 mg/kg, i.p.) enhanced brain signal intensity 22–24 h later.

A) Signal enhancement in T_1 weighted images was observed in hippocampus, habenula, bed nucleus of stria terminalis, septum, interpeduncular nucleus, hypothalamus, and hippocampus, among other regions of saline-treated rats. Arrows highlight these regions in a representative unprocessed MRI scan and atlas maps to the right show the same region in orange color. B) Composite map highlighting subcortical areas showing increased signal following Mn^{2+} administration ($n = 6$ saline treated female rats). Middle panel shows the same composite map with a lower threshold value of 0.9 and overlaid onto an MRI atlas of the rat brain. Bottom panel in B shows specific brain areas located within the signal-enhanced region. C) Areas of the brain showing signal enhancement with Mn^{2+}

administration are primarily located near ventricular regions and in regions previously shown to have higher permeability to trypan blue dye because of disrupted blood brain barrier sites (Saunders et al., 2014). Abbreviations: dHPC, dorsal hippocampus; Hb, habenula; Spt, septum; Hyp, hypothalamus; VTA, ventral tegmental area; IP, interpeduncular.

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Figure 3. The female rat brain showed greater signal enhancement with Mn^{2+} than males.

A) Shown is a histogram of average signal intensity across all brain voxels of male and female rats treated with Mn^{2+} , and in rats without Mn^{2+} . Sex differences in Mn^{2+} signal are not due to general increases in brain signal intensity in MRI scans. B) Regional differences in Mn^{2+} signal. C) Representative processed brain maps for Mn^{2+} treated female, male, and Mn^{2+} rats. Images are thresholded between $z = -3$ and $z = +3$ as a comparison for the histograms in A-B (see Materials and Methods for details on the image processing procedure).

Figure 4. Composite MEMRI maps for female rats.

Shown are maps for saline ($n = 6$), single cocaine ($n = 9$) and repeated cocaine ($n = 6$) treated animals. Male rats not treated with Mn^{2+} ($n = 5$) are shown in the far left as a comparison. Scale bar on right indicates signal intensity. Abbreviations: SSC, somatosensory cortex; PVN, paraventricular nucleus; CeA, central amygdala; CA3d(v), dorsal and ventral hippocampal CA3; DGd, dorsal dentate; Hab, habenula; 3Vd, dorsal 3rd ventricle; LDT, laterodorsal thalamus; PAG, periaqueductal grey; Sub, subiculum; IPN, interpeduncular nucleus; DRN/MRN, dorsal and medial nucleus of Raphé.

Figure 5. Composite MEMRI maps for male rats.

Shown are maps for saline ($n = 5$), single cocaine ($n = 5$) and repeated cocaine ($n = 5$) treated animals. Male rats not treated with Mn^{2+} ($n = 5$) are shown in the far left as a comparison. Scale bar on right indicates signal intensity. Abbreviations: SSC, somatosensory cortex; PVN, paraventricular nucleus; CeA, central amygdala; CA3d(v), dorsal and ventral hippocampal CA3; DGd, dorsal dentate; Hab, habenula; 3Vd, dorsal 3rd ventricle; LDT, laterodorsal thalamus; PAG, periaqueductal grey; Sub, subiculum; IPN, interpeduncular nucleus; DRN/MRN, dorsal and medial nucleus of Raphé.

Figure 6. Cocaine differentially affects MEMRI signal in hippocampal and midbrain regions of female and male rats.

A) Hippocampal areas. B) Midbrain areas. Data presented as Tukey box and whisker plots (median, 25–75th percentile and 1.5 interquartile range). *Significant effect of single cocaine; **significant effect of repeated cocaine; ***significant difference between male and female. Specific differences are highlighted by dashed lines.

Figure 7. Cocaine differentially affects MEMRI signal in amygdala and hypothalamic areas of female and male rat brain.

Top) Data for amygdala and anterior hypothalamus presented as Tukey box and whisker plots (median, 25–75th percentile and 1.5 interquartile range). *Significant effect of single cocaine; **significant effect of repeated cocaine. Specific differences are highlighted by dashed lines. Bottom) Composite MEMRI maps for female and male rats, which highlight amygdala and hypothalamic areas.