

RESEARCH ARTICLE

Manganese-Enhanced Magnetic Resonance Imaging Reveals Differential Long-Term Neuroadaptation After Methamphetamine and the Substituted Cathinone 4-Methylmethcathinone (Mephedrone)

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Abstract

Background: In recent years there has been a large increase in the use of substituted cathinones such as mephedrone (4-methylmethcathinone, 4-MMC), a psychostimulant drug that shows a strong resemblance to methamphetamine (METH). Unlike METH, which can produce clear long-term effects, the effects of 4-MMC have so far remained elusive. We employ manganese-enhanced magnetic resonance imaging (MEMRI), a highly sensitive method for detecting changes in neuronal activation, to investigate the effects of METH and 4-MMC on the brain.

Methods: In Wistar rats we performed a MEMRI scan two weeks after binge treatments (twice daily for 4 consecutive days) of METH (5 mg/kg) or 4-MMC (30 mg/kg). Furthermore, locomotor activity measurements and novel object recognition tests were performed.

Results: METH produced a widespread pattern of decreased bilateral activity in several regions, including the nucleus accumbens, caudate putamen, globus pallidus, thalamus, and hippocampus, as well as several other cortical and subcortical areas. Conversely, 4-MMC produced increased bilateral activity, anatomically limited to the hypothalamus and hippocampus. Drug treatments did not affect the development of locomotor sensitization or novel object recognition performance.

Conclusions: The pattern of decreased brain activity seen after METH corresponds closely to regions known to be affected by this drug and confirms the validity of MEMRI for detecting neuroadaptation two weeks after amphetamine binge treatment. 4-MMC, unlike METH, produced increased activity in a limited number of different brain regions. This highlights an important difference in the long-term effects of these drugs on neural function and shows precisely the anatomical localization of 4-MMC-induced neuroadaptation.

Keywords: 4-methylmethcathinone, magnetic resonance imaging, mephedrone, methamphetamine, psychostimulants

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Introduction

Amphetamine-type psychostimulants are amongst the most widely used illicit drugs in the world today. One of the most commonly used amphetamine-type stimulants, methamphetamine (METH), has an annual and lifetime non-medical use prevalence of 2.8% and 8.6%, respectively (Iritani et al., 2007; Durell et al., 2008). However, in recent years, there has also been a particularly strong increase in the use of novel substituted cathinones. One of the most widely used substituted cathinones is 4-methylmethcathinone (4-MMC), also known as mephedrone, a close structural analogue of METH (UNODC, 2011; Winstock et al., 2011; Deluca et al., 2012). The importance of brain imaging techniques, such as magnetic resonance imaging (MRI), for understanding the effects of psychostimulants on the brain has been noted repeatedly (Martin and Sibson, 2008; Lukas, 2014). Extending on this, we here describe the use of manganese-enhanced MRI (MEMRI), a novel preclinical method for investigating the long-term effects of central nervous system stimulants such as METH and 4-MMC on the brain.

In rodents, METH produces long-term deficits in markers of the dopamine (DA) and serotonin (5-HT) systems, including decreases in levels of DA and 5-HT and their respective transporters and synthesizing enzymes. These decreases may persist for weeks or even months after the final drug administration (Seiden et al., 1988; Cass and Manning, 1999; Haughey et al., 2000).

Recent studies which attempted to assess the effect of 4-MMC on markers of the monoamine systems have failed to find any evidence of alterations in levels of DA or 5-HT or their respective transporters and synthesizing enzymes (Angoa-Perez et al., 2012, 2013, 2014; Motbey et al., 2012). Moreover, other gauges of neurotoxicity, such as microglial activation and glial fibrillary acidic protein levels, likewise remain unaffected by 4-MMC, even following repeated high-dose binge treatments at normal ambient temperatures (Angoa-Perez et al., 2012, 2014). On the other hand, some reports indicate that 4-MMC may have a long-term effect on memory function (Motbey et al., 2012; den Hollander et al., 2013), suggesting that there may be changes occurring in the brain that are not detectable with conventional neurochemical and immunological methods.

Here we employ MEMRI as a novel method for detecting long-term (two weeks after the final drug treatment) alterations in brain regional activity in rats following treatment with METH or 4-MMC. The MEMRI method used here employs manganese (Mn^{2+}) delivery via osmotic minipumps. Changes in signal intensity occur as a result of activity-dependent translocation of Mn^{2+} into neurons via a voltage-gated Ca^{2+} channel (Silva and Bock, 2008) and signal alterations are hypothesized to reflect altered neural activity preceding the actual scan. This method has been employed previously to detect brain activity changes in rodents following various behavioral paradigms (Bangasser et al., 2013; Hoch et al., 2013), suggesting it can be capable of detecting alterations in brain function that are not visible using other neurochemical or immunological methods. We also assessed the development of sensitization during drug treatments and measured recognition memory performance one week after the drug treatments.

In line with our hypothesis, the MEMRI method detected a pattern of decreased activity in the striatum and other regions known to be affected by METH. However, in contrast to what was seen with METH, 4-MMC produced a pattern of increased activity in a limited number of brain regions that showed little overlap with those affected by METH. Moreover, the drug treatments did not produce any significant behavioral effects. These

findings are important as they show, for the first time, clear long-term neurophysiological effects of 4-MMC in specific neuroanatomical locations, and because they highlight the MEMRI method as a valuable tool for studying the long-term effects of psychostimulants on neural function.

Materials and Methods

Animals

A total of 26 Wistar rats were used in the experiments. Of these, 24 were randomly assigned to two different experimental groups (METH or 4-MMC, $n = 8$ each) or one control group ($n = 8$), while two rats were used for initial tests of drug doses and the behavioral apparatus. Animals were 6 weeks old at arrival and single-housed in internally-ventilated cages containing aspen chip bedding and nesting material, with food pellets and water available *ad libitum*. The experiments started after a 14-day habituation period. The environmental conditions were a 12h light-dark cycle, lights on at 06:00h, temperature 20–22°C, relative humidity 50–60%. All animal tests were approved by the Laboratory Animal Committee of the Southern Finland Provincial Government.

Drug Treatments

Racemic 4-MMC was acquired from the Department of Forensic Medicine, Hjelt Institute, University of Helsinki. The compound was analyzed for purity by gas chromatography and Fourier transform infrared spectroscopy and found to be pure (>95%) HCl salt. METH HCl was acquired from Sigma-Aldrich (St. Louis). The drugs were dissolved in saline and administered intraperitoneally at a volume of 1 ml/kg while a control group was given a corresponding injection of saline. All other chemicals and reagents used in the experiments were analytical grade and also acquired from Sigma-Aldrich, unless otherwise specified.

Dose Selection

Drug doses were chosen based on previous literature and on an estimation of the relative difference in potency between 4-MMC and METH. METH and 4-MMC are self-administered by humans and animals at a ratio of between 1:4 and 1:10 (Vardakou et al., 2011; Motbey et al., 2013). In line with previous studies, we employed a neurotoxic METH dose of 5 mg/kg (Abekawa et al., 2001; Clemens et al., 2004; Angoa-Perez et al., 2013) and a 4-MMC dose of 30 mg/kg, which is also similar to previous studies on 4-MMC neurotoxicity (Motbey et al., 2012; den Hollander et al., 2013) and, furthermore, corresponds to a METH to 4-MMC ratio of 1:6 and thus corresponds to observed differences in self-administered doses.

Experimental Design

The experiment started with the drug treatments. 4-MMC (30 mg/kg) or METH (5 mg/kg) was administered twice daily, 6h apart, for 4 consecutive days. Drug administration using this schedule aims to mimic a multi-day drug binge and reliably produces neurotoxicity following treatment with psychostimulants such as METH, 3,4-methylenedioxymethamphetamine (MDMA), and D-amphetamine (Bittner et al., 1981; Battaglia et al., 1987; Reneman et al., 2002; den Hollander et al., 2013).

To assess the development of sensitization, locomotor activity of the rats was measured during treatment days 1 and 4, after the morning drug injections. On treatment day 2, the core body temperature of the animals was measured at regular intervals following the morning injections. On treatment day 3, animals were returned to the home cage after the drug injections without any further interventions. Body weights were also monitored during the experiment. One week after the last drug treatments, the recognition memory performance of the animals was assessed using the novel object recognition test. After this test the animals were implanted with an osmotic pump for delivery of $MnCl_2$. The MEMRI scan took place two weeks after the last drug treatments.

Behavioral Experiments

Locomotor Activity and Sensitization

The locomotor activity of animals was measured on the first and last days of the drug treatments. For two days prior to the first measurement, the animals had been habituated to the cages used for this test for 2 h each day. On the day of the locomotor activity measurement, the animals were placed in the transparent polycarbonate cages (26 x 43 x 18 cm) similar to their home cages 30 min before the drug injections in order to further habituate the animals to the novel cage environment. The locomotor activity of the animals was measured continuously for 6 h following injection using Ethovision software (Noldus Information Technology) connected to a closed-circuit television camera with an overview of all 8 cages in the test arena. Sensitization was assessed by comparing the locomotor activity until 1 h after dosing between the first and last measurement (Motbey et al., 2012).

Novel Object Recognition

The novel object recognition test was performed one week following the last drug treatment. The parameters used were chosen to mimic closely a previous 4-MMC novel object recognition experiment (Motbey et al., 2012). Rats were habituated to the testing arena, a rectangular opaque blue box (50 x 80 x 30 cm), for two days prior to the day of the test. The test consisted of one 3-min sample trial, in which the rat was exposed to two identical objects, followed 15 min later by a 3-min test trial, in which the rat was exposed to one novel object and one object which was previously used in the sample trial. The objects used were a brown coffee mug (8 x 8 x 12 cm) and two white, translucent, rectangular plastic bottles taped together at the lids (3 x 3 x 18 cm). All objects were securely mounted to the bottom of the box using poster putty in order to prevent the animals from moving the objects. The test trials were recorded using a digital video camera and subsequently analyzed using Ethograph v. 2.06 by an operator blind to the experimental conditions. Active exploration of the object, such as touching and sniffing, was counted as investigation, while being in the vicinity of, or perching over the object, was not.

MRI imaging

MnCl₂ administration

Manganese chloride was dissolved into Tris-buffered saline (pH 7.4) and administered with osmotic minipumps (Alzet, model 2001) that delivered 200 μ l of $MnCl_2$ (1 μ l/h) during a 7-day infusion, corresponding to a total $MnCl_2$ dose of 120 mg/kg/week. The concentration of $MnCl_2$ in the pumps was adjusted according to the body weight of the animals. Before the surgery, the

pumps were primed overnight in saline at 37°C. Animals were anesthetized with isoflurane and the pumps were implanted subcutaneously on the dorsum, slightly caudal to the scapulae. For post-surgical analgesia, animals received a subcutaneous injection of carprofen (5 mg/kg) immediately after implantation.

MRI data acquisition

Magnetic resonance imaging was performed on a 4.7 T scanner (Bruker, PharmaScan 47/16 US) using a 38 mm linear volume coil for transmitting and receiving. Rats were anesthetized with 5% isoflurane in oxygen (1 L/min) and secured on a custom-made holding apparatus with a stabilizing tooth bar and a nose cone. During scanning, the isoflurane concentration was maintained at 2–3%, and the body temperature was kept constant with a heating pad. T1-weighted images were acquired using a three-dimensional rapid acquisition-relaxation enhanced pulse sequence (repetition time = 300 ms; echo time = 12.5 ms; number of averages = 7; number of slices: coronal = 127, sagittal = 57, axial = 57; flip angle = 180°; field of view = 26 x 17 x 17 mm; and matrix size = 128/54/54, resulting in 0.2 x 0.31 x 0.31 mm voxel resolution).

Statistics and MRI Data Processing

Bodyweights, body temperatures, novel object recognition performance, and individual region of interest (ROI) comparisons were analyzed using one-way or two-way analyses of variance (ANOVAs) followed by Fisher's least significant difference (LSD) post hoc tests. Sensitization was analyzed using a repeated-measures ANOVA of average locomotor activity during the first two hours following drug administration. Global effect of treatment on ROI intensity was reported using a two-way ANOVA with brain region as the within-subjects factor. All analyses were performed in IBM SPSS v. 21 and GraphPad Prism v. 5.01.

The MRI images were converted to Analyze format and scaled up by a factor of 10. The MRI images were spatially pre-processed with custom-developed MatLab functions (version R2010a). In brief, brain-extracted T1-weighted images were spatially normalized using a rat brain template co-registered to a rat brain atlas (Schwarz et al., 2006) by a 12-parameter affine transformation using the FSL/FLIRT tool (Jenkinson et al., 2002). Co-registration of the template to the digitized rat brain atlas (Paxinos and Watson, 1998) enabled atlas-based generation of ROI masks for further anatomical analysis. The resulting images were smoothed with a 4 x 4 x 4 mm at full width at half-maximum Gaussian kernel to improve signal-to-noise ratio. For identifying the brain regions in which activation in the drug-treated groups differed from the saline control group, voxel-wise independent *t*-tests were performed in SPM8 (www.fil.ion.ucl.ac.uk/spm/). Systemic Mn^{2+} infusion can lead to inter-individual differences in Mn^{2+} accumulation that are reflected by inter-individual differences in global signal intensity. Mean global intensity was therefore treated as a nuisance factor that was removed on a voxel-by-voxel basis using analysis of covariance normalization in SPM (Friston et al., 1990). The resulting statistical parametric maps were thresholded voxel-wise at the arbitrary significance of $p < 0.0001$ and cluster-size thresholded with a threshold of $K = 70$ voxels obtained from iterative Monte Carlo simulations, which resulted in an overall significance level of $p < 0.01$ corrected for multiple comparisons across the whole brain.

As METH is known to affect specific brain regions, we also performed anatomical ROI analyses on brain regions known to be affected by psychostimulants as well as regions with hypothesized involvement in circuits mediating goal-directed behavior

(Sesack and Grace, 2010; Klomp et al., 2012; Noori et al., 2012). These regions included both individual nuclei and larger composite structures. Three-dimensional ROI masks were created with a WFU_PickAtlas toolbox (Maldjian et al., 2003), and the selected ROI masks were applied to previously-generated SPM contrast files. Thus, ROI analysis was performed using the SPM design from the global analysis, but without thresholding, yielding all voxels within the anatomical regions for analysis of differences between the drug exposures. Normalized mean signal intensity values from individual ROIs were extracted using the REX tool (Duff et al., 2007).

Results

Body Weights

The mean body weight across all groups prior to the drug treatments was 310 ± 3 g ($n = 24$), and no group differences in body weight existed prior to the drug treatments ($F_{2,23} = 0.48, p > 0.05$). Furthermore, no differences in body weights were observed directly after ($F_{2,23} = 0.77, p > 0.05$) or one week after ($F_{2,23} = 2.8, p > 0.05$) drug treatments, indicating the treatments had no effect on body weights and were well-tolerated.

Body Temperature

The effect of the drug treatments on body temperatures is shown in Figure 1. A two-way repeated-measures ANOVA revealed a significant effect of treatment (4-MMC, METH or saline drug treatments produced a differing effect on body temperature, $F_{2,21} = 13.24, p < 0.0005$), time (differences in body temperatures were observed between 0 and 360 min after the injections, $F_{8,168} = 23.91, p < 0.0005$), and a time \times treatment interaction (a different pattern of body temperatures was observed over time depending on the drug treatment given, $F_{16,168} = 4.27, p < 0.0005$). Treatment-induced elevations in body temperature compared to saline were observed between 0.5–4 h in the drug-treated groups at various time points (see Figure 1).

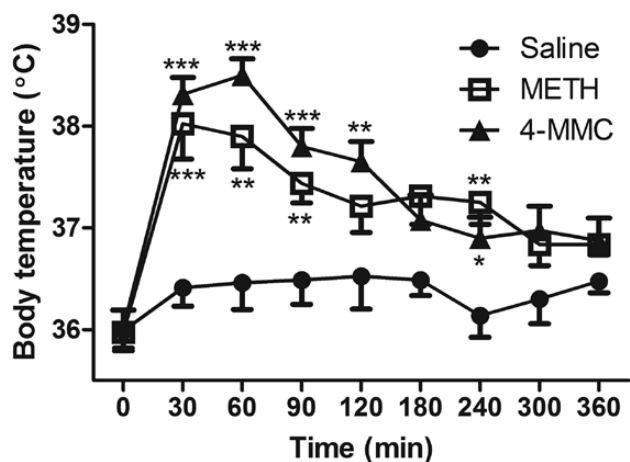


Figure 1. Core body temperature was measured for up to 6 h after administration of either methamphetamine (METH; 5 mg/kg), 4-methylmethcathinone (4-MMC; 30 mg/kg), or saline. T = 0 indicates the baseline temperature measured when the animals were handled for the first time that day. The drug injections were performed directly after this measurement, after which the animals were returned the home cage until the next measurement. All treatments produced significant elevations in body temperatures, although there were differences in the rate of onset and duration. Data are expressed as mean \pm standard error of the mean. $N = 8$ per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to saline.

Locomotor Activity and Sensitization

The effects of all drug treatments on locomotor activity between 0 and 6 h after the morning drug treatments on days 1 and 4 are shown in Figure 2A and B, respectively. A two-way ANOVA revealed a significant main effect of time ($F_{5,105} = 5.24, p < 0.0005$) and a time \times treatment interaction ($F_{10,105} = 2.45, p < 0.05$) but no main effect of treatment ($F_{2,21} = 3.29, p > 0.05$) during the first day of drug treatments. Subsequent testing revealed that only METH produced a significant increase in locomotor activity, and only between 2–3 h following treatment (see Figure 2A).

During the locomotor activity measurement on the last treatment day, significant main effects were observed for time ($F_{5,75} = 8.79, p < 0.0005$) and treatment ($F_{2,15} = 12.42, p < 0.01$). The treatment effect was due to an overall increase in locomotor activity by the METH-treated rats, compared to other groups. Furthermore, there was a time \times treatment interaction ($F_{10,75} = 3.83, p < 0.0005$) which was due to increases in locomotor activity in both drug-treated groups at various time points (see Figure 2B).

In order to assess whether sensitization took place during the drug treatments, the difference in locomotor activity between day 1 and 4 was calculated. The difference was calculated using the average activity between 0–1 h following drug injections. A repeated-measures ANOVA of locomotor activity between day 1 and 4 showed a significant main effect of treatment day ($F_{1,21} = 4.36, p < 0.05$), which was due to an overall decrease in locomotor activity at day 4. No significant treatment ($F_{2,21} = 1.96, p > 0.05$) or time \times treatment interaction ($F_{3,27} = 0.90, p > 0.05$) was observed, indicating that sensitization did not take place.

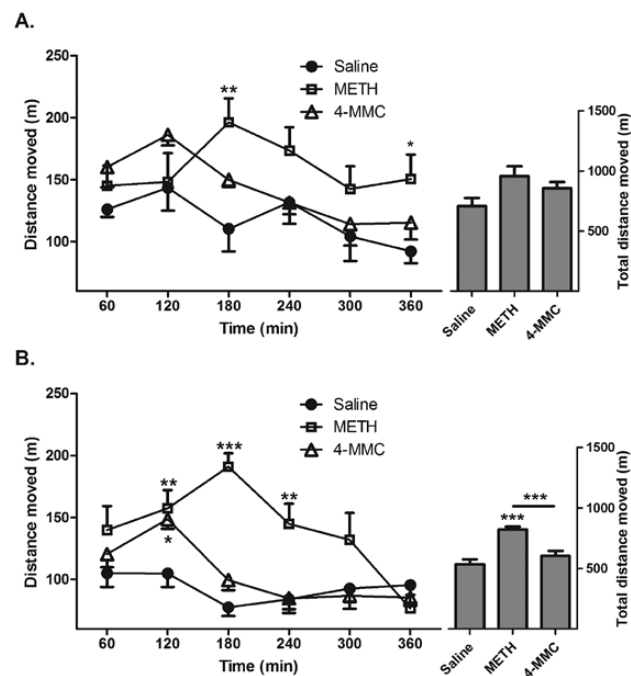


Figure 2. The effects of methamphetamine (METH; 5 mg/kg), 4-methylmethcathinone (4-MMC; 30 mg/kg), or saline on locomotor activity measured between 0 and 6 h after the drug treatments are shown for (A) the first day and (B) the last day of the binge treatment. The graph on the left shows locomotor activity over time while the bars represent cumulative locomotor activity during the entire session. Data are expressed as mean \pm standard error of the mean. $N = 8$ per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to saline.

Novel Object Recognition

The effect of treatments on the main novel object recognition test variable, measured as the percentage of time spent exploring the novel object, is shown in Figure 3. Data acquired from the first out of 4 days of testing was discarded due to a noisy environment in the test room from construction work in adjacent rooms. No effect of treatment was found on any measures of test performance, such as the percentage of time spent exploring the novel object ($F_{2,17} = 0.63, p > 0.05$), the number of instances exploring the novel object ($F_{2,17} = 0.39, p > 0.05$), or the total time spent exploring the novel object ($F_{2,17} = 1.15, p > 0.05$), demonstrating treatment had no effect on recognition memory performance.

Functional Mapping of Brain Activity

Statistical parametric maps showing statistically significant ($p < 0.01$, corrected) alterations in voxel-wise T1-signal intensity suggestive of altered brain activity in the drug-treated groups, compared to the saline control group, are shown in Figure 4. These maps revealed reduction of the signal by METH in many cortical regions, including the primary motor cortex, cingulate, and insular cortex. In the forebrain, the most prominent reduction was seen in the dorsal and ventral striata. More caudally, deactivated areas were observed in thalamic areas, dorsal hippocampus, and amygdala. In the hindbrain, the superior colliculus and raphe nuclei showed conspicuous reduction of signal. In a sharp contrast, rats treated with 4-MMC exhibited increased signal intensities, with most prominent clusters confined to cortical areas. For exploring differences in brain activity directly between METH and 4-MMC, we also created an additional contrast (4-MMC > METH). Because this comparison revealed wide-spread differences encompassing large areas at the uncorrected p value of 0.0001, we chose to explore these differences using a higher uncorrected p value ($p < 0.0000001$) in order to see the most important differentially-affected regions. Thus, Figure 5 shows that compared to 4-MMC, METH treatment resulted in lower signal intensity in many cortical regions, including the motor, somatosensory, and insular cortices, striatum, thalamic regions, periaqueductal grey, and raphe nucleus.

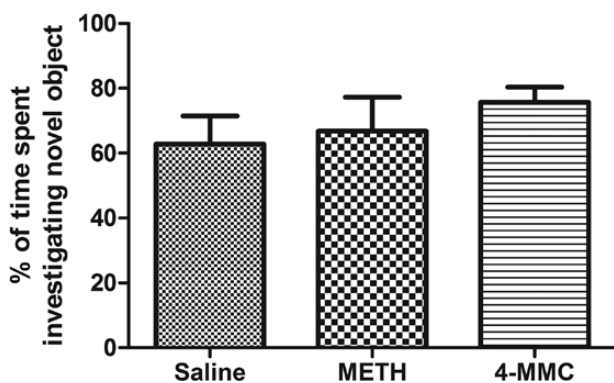


Figure 3. The effect of binge treatments with methamphetamine (METH) or 4-methylmethcathinone (4-MMC) on the novel object recognition test parameter “percentage of time spent investigating the novel object” during the 3-min trial, at one week after the last drug treatment. None of the drug treatments produced a significant change in novel object recognition test performance compared to saline-treated animals. Data are expressed as mean \pm standard error of the mean. $N = 6$ per group.

ROI Signal Intensity

Signal intensities from 22 ROIs expressed as intensities relative to the saline-treated control group are shown in Table 1. A two-way ANOVA with treatment as the between-subjects factor and brain region as the within-subjects factor revealed significant main effects of treatment ($F_{2,21} = 5.49, p < 0.05$) and region ($F_{21,441} = 440.28, p < 0.0005$) as well as a significant treatment \times region interaction ($F_{42,441} = 2.10, p < 0.0005$). See Table 1 for details.

Discussion

Here we show that high-dose, binge treatments with METH that produce acute elevations in body temperature and locomotor activity also result in a long-lasting effect on brain activity in the nucleus accumbens, caudate putamen, globus pallidus, cingulate cortex, thalamus, hippocampus, and raphe nucleus, as well as a number of other cortical and subcortical regions. Conversely, 4-MMC produced an increase in brain activity, but only in the hypothalamus, hippocampus, and parietal cortex. Furthermore, no evidence was found of sensitization or decreased memory performance as a result of the drug treatments.

In the METH-treated animals we observed decreased activity in 5-HT-rich and DA-rich areas such as the striatum and raphe nuclei. The decreases in nuclei heavily dependent on monoaminergic neurotransmission are not surprising, considering that the monoamine systems are the primary target of METH (Khoshbouei et al., 2004; Daberkow et al., 2013), and that METH at high doses such as those employed here produces long-term decreases in markers of monoamine system integrity, such as reductions in levels of DA and 5-HT and their transporters and synthesizing enzymes (Seiden et al., 1988; Cass and Manning, 1999; Haughey et al., 2000; Schroder et al., 2003). The fact that the MEMRI method is capable of detecting neurophysiological alterations in regions known to be affected by METH-treatment speaks for the validity of this method in terms of detecting brain regions affected by psychostimulants. Furthermore, the regional pattern of altered activity observed here overlaps to a large extent with the pattern of metabolic alterations seen in abstinent human METH users (Volkow et al., 2001; London et al., 2004; Wang et al., 2004) and suggests that the MEMRI method has potential as a translational tool that offers many benefits to preclinical research, such as excellent spatial resolution and the possibility to perform multiple measurements.

Looking at the effects of 4-MMC on brain activity, there appear to be two important distinctions. First, 4-MMC produced an increase, rather than a decrease, in neural activity, and second, the number of brain regions affected was much lower than what was seen after METH, and also showed little overlap with regions affected by METH.

The data presented here imply primarily the parietal cortex, hippocampus, and the hypothalamus as the targets of long-lasting effects of 4-MMC. Whether this is a direct drug effect or mediated by stress due to the high-dose drug treatments is an important question, particularly considering the pivotal role of the hypothalamus in responding to stress (Herman and Cullinan, 1997), and remains to be answered. Nonetheless, the regions shown to be affected by 4-MMC in this study provide specific guidance for future studies needed to outline the exact nature and cause of the alterations reported here.

The fact that so few brain regions appear to be affected by 4-MMC compared to METH highlights an important distinction which is somewhat surprising, considering that METH and 4-MMC work in a similar fashion by increasing synaptic

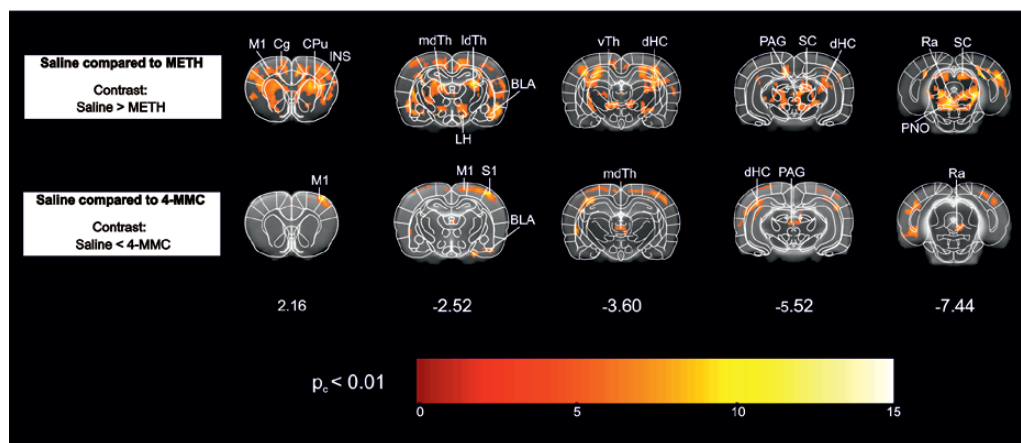


Figure 4. Functional brain activity revealed by decreased intensity (SAL > METH contrast) or increased intensity (SAL < 4-MMC contrast) of a T1-weighted magnetic resonance imaging signal in rats treated with METH or 4-MMC compared to saline-treated rats, at two weeks after the last dose. $N = 8$ per group. Statistical t -maps (thresholded at $p < 0.01$, corrected) are superimposed on T2-weighted sections from the brain template (Schwarz et al., 2006), with the corresponding atlas sections (Paxinos and Watson, 2007) manually overlaid (see Methods for full details regarding image preprocessing). Numbers between the columns indicate the positions of the sections from bregma in millimeters. 4-MMC, 4-methylmethcathinone; BLA, basolateral amygdala; Cg, cingulate cortex; CPu, caudate putamen; dHC, dorsal hippocampus; INS, insular cortex; ldTh, laterodorsal thalamus; LH, lateral hypothalamus; M1, primary motor cortex; mdTh, mediodorsal thalamus; METH, methamphetamine; PAG, periaqueductal grey; PNO, pontine reticular nucleus; Ra, raphe nucleus; SAL, saline; SC, superior colliculus; vTh, ventral thalamus.

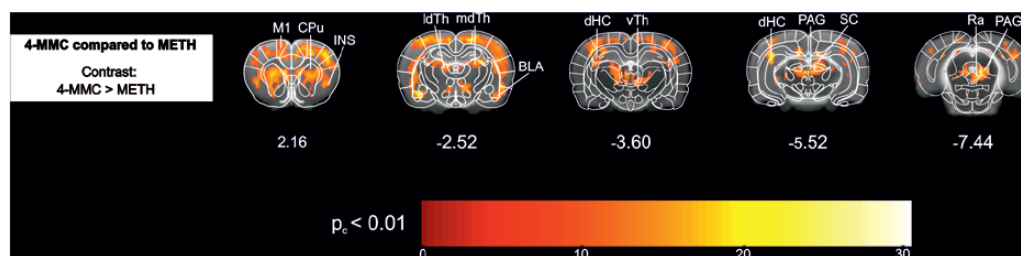


Figure 5. Functional brain activity revealed by increased intensity of a T1-weighted magnetic resonance imaging signal in rats treated with 4-methylmethcathinone (4-MMC)-treated rats compared to methamphetamine (METH)-treated rats, at two weeks after the last dose. $N = 8$ per group. Statistical t -maps (thresholded at $p < 0.01$, corrected) are superimposed on T2-weighted sections from the brain template (Schwarz et al., 2006), with the corresponding atlas sections (Paxinos and Watson, 2007) manually overlaid (see Methods for full details regarding image preprocessing). Numbers between the columns indicate the positions of the sections from bregma in millimeters. BLA, basolateral amygdala; CPu, caudate putamen; dHC, dorsal hippocampus; INS, insular cortex; ldTh, laterodorsal thalamus; M1, primary motor cortex; mdTh, mediodorsal thalamus; PAG, periaqueductal grey; Ra, raphe nucleus; SC, superior colliculus; vTh, ventral thalamus.

monoamine levels by inhibiting their reuptake and enhancing their release (Kehr et al., 2011; Baumann et al., 2012), and thus are very similar in terms of their pharmacological action. On the other hand, this appears to support and confirm recent studies demonstrating that 4-MMC does not affect various neurochemical and immunological measurements of neurotoxicity (Angoa-Perez et al., 2012; Baumann et al., 2012; den Hollander et al., 2013), and confirms that the long-term effects of 4-MMC indeed appear to be very limited compared to METH. These results are particularly compelling in light of the recently renewed interest in the possible clinical benefits of empathogen/entactogen-class drugs (Mithoefer et al., 2011, 2013).

With regards to the results on behavioral measures, our results are generally in line with previous literature. We found no effect of 4-MMC on memory performance. Previous studies on the effects of cathinones on memory have yielded ambiguous results, with both decreases and increases being reported (Motbey et al., 2012; den Hollander et al., 2013). We employed a novel object recognition protocol that has previously demonstrated decreased memory performance in 4-MMC-treated rats at a higher, but not at the lower, cumulative doses than used in this study (Motbey et al., 2012). On the other hand, we measured novel object recognition performance 7 days after the final treatment, while Motbey et al.

(2012) measured 47 days after the final treatment. Future studies will have to investigate the possibility that 4-MMC-induced memory deficits take longer than a week to develop. METH also did not produce decreased novel object recognition performance in this study, which is in line with previous studies, as not all studies report decreased novel object recognition performance after METH (Clemens et al., 2007; Herring et al., 2008). The lack of effect of 4-MMC and METH on memory does not support the notion of a clear detrimental effect of these drugs on memory performance. It is possible that variation in test performance between studies is due to secondary factors, such as stress associated with non-contingent, high-dose stimulant treatments. A recent review also concluded that the effects of METH on memory and cognitive function in human users may have been exaggerated (Hart et al., 2012), which suggests the observed brain activity changes may not be related to changes in cognitive function. Further, we found no evidence of sensitization. This is in accord with a previous study (Motbey et al., 2012) and in agreement with evidence that sensitization takes place primarily following treatments with lower (1 mg/kg) doses, but not higher doses of psychostimulants (5 mg/kg; Frey et al., 1997).

One limitation of this study pertains to the fact that acute high-dose Mn^{2+} challenges are known to be neurotoxic and to

Table 1. Effects of Methamphetamine (METH) and 4-Methylmethcathinone (4-MMC) on Magnetic Resonance Imaging Signal Intensity in Pre-selected Regions of Interest Two Weeks Following the Last Dose of the Binge Treatment. Data are normalized per each region (saline = 100) and expressed as mean \pm standard error of the mean. N = 8 per group. * $p < 0.05$; ** $p < 0.01$ compared to saline.

Region of interest	F	p	Mean			Δ
			Saline	METH	4-MMC	4-MMC - METH
Ventral tegmental area	2.054	> 0.05	100.0 \pm 2.1	93.7 \pm 2.1	99.2 \pm 2.1	5.5 \pm 4.0
Nucleus accumbens, core	7.988	0.003	100.0 \pm 1.9	93.1 \pm 1.9*	103.0 \pm 1.9	9.9 \pm 2.7**
Nucleus accumbens, shell	6.623	0.006	100.0 \pm 2.0	94.7 \pm 2.0	104.8 \pm 2.0	10.1 \pm 2.6**
Caudate putamen	7.228	0.004	100.0 \pm 1.9	93.5 \pm 1.9*	103.3 \pm 1.9	9.8 \pm 3.0**
Lateral globus pallidus	6.639	0.006	100.0 \pm 2.0	93.0 \pm 2.0*	102.4 \pm 2.0	9.4 \pm 2.9**
Insular cortex	3.070	> 0.05	100.0 \pm 2.4	95.3 \pm 2.4	103.9 \pm 2.4	8.6 \pm 4.1
Cingulate cortex	7.802	0.003	100.0 \pm 2.0	93.8 \pm 2.0*	104.5 \pm 2.0	10.7 \pm 3.2**
Frontal association cortex	4.258	0.028	100.0 \pm 2.2	94.6 \pm 2.2	103.6 \pm 2.2	9.0 \pm 3.8**
Primary motor cortex	6.957	0.005	100.0 \pm 2.0	94.4 \pm 2.0	104.5 \pm 2.0	10.1 \pm 3.2**
Infralimbic cortex	5.176	0.015	100.0 \pm 2.4	93.0 \pm 2.4	104.5 \pm 2.4	11.5 \pm 4.4**
Orbital cortex	4.726	0.020	100.0 \pm 2.3	94.3 \pm 2.3	104.2 \pm 2.3	9.9 \pm 3.8**
Parietal association cortex	5.256	0.014	100.0 \pm 2.4	95.6 \pm 2.4	105.7 \pm 2.4	10.1 \pm 3.5**
Prelimbic cortex	5.347	0.015	100.0 \pm 2.3	93.9 \pm 2.3	104.5 \pm 2.3	10.6 \pm 3.9**
Dorsal hippocampus	4.293	0.027	100.0 \pm 2.3	95.0 \pm 2.3	104.5 \pm 2.3	9.5 \pm 3.9**
Ventral hippocampus	1.927	> 0.05	100.0 \pm 3.6	97.6 \pm 3.6	107.1 \pm 3.6	9.5 \pm 5.4
Amygdala	1.879	> 0.05	100.0 \pm 4.2	99.0 \pm 4.2	108.8 \pm 4.2	9.8 \pm 5.5
Dorsal thalamus	5.333	0.013	100.0 \pm 2.2	93.8 \pm 2.2	104.0 \pm 2.2	10.2 \pm 3.9**
Ventral thalamus	5.965	0.009	100.0 \pm 2.1	94.0 \pm 2.1	103.9 \pm 2.1	9.9 \pm 3.5**
Hypothalamus	7.635	0.003	100.0 \pm 2.1	100.1 \pm 2.1	109.6 \pm 2.1**	9.5 \pm 2.1**
Raphe nucleus	8.500	0.002	100.0 \pm 1.7	93.5 \pm 1.7**	102.3 \pm 1.7	8.8 \pm 2.8**
Substantia nigra	1.819	> 0.05	100.0 \pm 2.2	95.4 \pm 2.2	100.9 \pm 2.2	5.5 \pm 3.1
Superior colliculus	6.335	0.007	100.0 \pm 2.0	93.9 \pm 2.0*	103.2 \pm 2.0	9.3 \pm 3.1**

affect locomotor activity in rodents. This was the main reason for employing a continuous infusion paradigm which, unlike acute high-dose injections, has been shown to be devoid of any effects on locomotor activity or feeding behavior (Malecki, 2001; Eschenko et al., 2010b), suggesting this method clearly reduces the negative effects of Mn²⁺ compared to acute high-dose manganese injection. Importantly, in this study the Mn²⁺ infusions were not started until after the behavioral experiments were completed. The week-long continuous infusion paradigm leads to a slow, gradual build-up of Mn²⁺ and it is likely that trans-synaptic transport of Mn²⁺ takes place during this period (Inoue et al., 2011). Therefore, the observed activation pattern may reflect not only brain regions directly affected by treatments, but could also reveal functional and anatomical connectivity across synaptically-connected regions. This should be taken into consideration when interpreting the imaging data. Although we observed clear decreases in signal intensity in the striatum and other regions known to be vulnerable to METH-induced toxicity, caution should be exercised in interpreting MEMRI signal intensity changes as evidence of toxicity. This applies particularly to 4-MMC which, unlike METH, increased rather than decreased signal intensity in certain regions. Finally, the employed binge-dosing regimen, where large doses are administered over several days without any preconditioning, may not correctly model a human pattern of drug use which is normally characterized by intake of relatively low doses over a longer period of time. Even if drug use escalates to repeated intake of large doses, there is evidence that preconditioning with lower METH doses reduces the toxicity of subsequent toxic high doses (Graham et al., 2008; Hodges et al., 2011), suggesting that studies employing binge-treatment regimens, including this one, may produce more severe effects than what occurs in most humans during recreational use.

Additional research is needed to determine the exact nature of the physiological adaptations responsible for the changes in

MEMRI signal intensity due to psychostimulant drugs. This research may be challenging, and also require animals not exposed to manganese, as Mn²⁺ itself is known to produce certain neurophysiological changes, such as decreases in excitatory postsynaptic potentials and spike amplitudes in the hippocampus (Eschenko et al., 2010a). A recent microarray study examining striatal gene expression after a METH treatment regimen comparable to the one employed in this study found 55 up-regulated and 17 down-regulated genes belonging to multiple functional categories, including those involved in transcription, angiogenesis, cell adhesion, apoptosis, and neuronal development (Dela Pena et al., 2014), while epigenetic changes have also been reported (Mychasiuk et al., 2013). Furthermore, Golgi-Cox staining has revealed specific amphetamine-induced synaptic changes, such as increases in the density and branching of dendritic spines (Robinson and Kolb, 1999). Similar examinations of regions implicated in 4-MMC neuroadaptation, such as the hypothalamus, hippocampus, and parietal cortex, may reveal interesting correlates to MEMRI signal intensity changes and begin to explain in more detail the exact synaptic and structural plasticity which takes place during and after exposure to 4-MMC or other psychostimulants.

In conclusion, using MEMRI, we show that METH produces long-term widespread decreases in brain activity in regions including the nucleus accumbens, caudate nucleus, and thalamus, as well as a number of other cortical and subcortical brain regions. Compared to METH, the substituted cathinone 4-MMC produces a long-term pattern of neural activation which is detectable, but much more limited in terms of the number of affected regions. Alterations were observed in the hypothalamus, hippocampus, and parietal cortex, and future work is warranted to determine the exact nature and cause of the physiological alterations reported here in order to gain a better understanding of the neuropsychopharmacological differences between cathinones and non-keto amphetamines.

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Statement of Interest

The authors declare no conflicts of interest.

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