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SHORT COMMUNICATION

Chronic methamphetamine exposure prior to middle cerebral artery occlusion increases infarct volume and worsens cognitive injury in Male mice

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Abstract Emerging evidence indicates that methamphetamine (MA) abuse can impact cardiovascular disease. In humans, MA abuse is associated with an increased risk of stroke as well as an earlier age at which the stroke occurs. However, little is known about how chronic daily MA exposure can impact ischemic outcome in either humans or animal models. In the present study, mice were injected with MA (10 mg/kg, i.p.) or saline once daily for 10 consecutive days. Twenty-four hours after the final injection, mice were subjected to transient middle cerebral artery occlusion (tMCAO) for one hour followed by reperfusion. Mice were tested for novel object memory at 96 h post-reperfusion, just prior to removal of brains for quantification of infarct volume using 2,3,5-Triphenyltetrazolium Chloride (TTC) staining. Mice treated with MA prior to tMCAO showed decreased object memory recognition and increased infarct volume compared to saline-treated mice. These findings indicate that chronic MA exposure can worsen both cognitive and morphological outcomes following cerebral ischemia.

Keywords Methamphetamine · middle cerebral artery occlusion · memory · cognition

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Introduction

In the United States, methamphetamine (MA) is abused by approximately 1.2 million individuals over the age of 12 and MA-associated health issues contribute to a large healthcare burden (SAMHSA 2010). MA use increases risk for stroke, dramatically reduces the average age of onset (43 versus 73 years), and is associated with both hemorrhagic and ischemic stroke (Ho et al. 2009). MA-associated stroke has been reported to occur both immediately following MA use as well as up to months following chronic MA abuse (Ho et al. 2009; Ohta et al. 2005). Potential factors that may influence elevated stroke risk following MA include rupturing and occlusion of blood vessels (Polesskaya et al. 2011; Anderson and Sung 2003) and elevated oxidative stress (Wang et al. 2001).

Consistent with the human studies, rodent models indicate that short-term MA can worsen ischemic outcome. Male mice repeatedly injected with MA over a few hours just prior to transient middle cerebral artery occlusion (tMCAO) show increased infarct volume (Wang et al. 2001; Shen et al. 2008). These studies involved short-term effects of MA and a tMCAO which occurs while MA is at high levels in the brain and plasma. However, while clinically more relevant, effects of *chronic* MA exposure over more than one day on ischemic outcome and cognitive performance have not been investigated in rodent models. Based on our recent study, which indicated significant alterations in neural activation with chronic exposure, we anticipate profound differences between acute

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and chronic MA exposure on the brain (Zuloaga et al. 2016). Therefore in the present study we tested the hypothesis that treating mice with MA once daily for 10 days prior to induction of tMCAO would worsen cognitive injury as well as histological outcomes. Since MA is a drug that is chronically abused in the human population, the development of an animal model for understanding chronic effects of MA on stroke outcome is critical. Although human findings indicate that MA can affect stroke, these studies are confounded by multi-drug use, differences in individual exposure to MA, and genetic risk factors. As a result it remains unclear as to whether MA worsens stroke outcome.

Materials and methods

Animals Male C57BL/6 J mice (105 days old) were purchased from the Jackson Laboratory (Sacramento, CA) and maintained on a 12/12 light/dark cycle with lights on at 6:00 am. Mice were singly housed for one week prior to experiments and were between 120 and 140 days old at the time of testing. Rodent chow (PicoLab Rodent Diet 20, #5053; PMI Nutrition International, St. Louis, MO) and tap water were available ad libitum. All procedures complied with the Guide for the Care and Use of Laboratory Animals and were approved by the Oregon Health & Science University animal care and use committee.

MA treatment and tMCAO Mice were randomly assigned to treatment groups and received daily injections with (*d*)-MA hydrochloride dissolved in saline (10 mg/kg, i.p.) or saline alone at 10:00 am PST for 10 consecutive days. (*d*)-MA hydrochloride was obtained from the Research Triangle Institute (Research Triangle Park, NC) through the National Institute of Drug Abuse drug supply program. The MA dose was chosen based on previous studies in which there was short-term MA administration in the context of tMCAO (Wang et al. 2001; Shen et al. 2008). Twenty-four hours after the final injection, mice were subjected to tMCAO. tMCAO was induced in male C57BL/6 J mice for 1 h by reversible right MCAO under isoflurane anesthesia followed by 96 h of reperfusion as described previously (Banerjee et al. 2013). The 96 h time point was selected based on our previous work (Zhu et al. 2015), indicating that it is a valid time point for assessment of infarct volume by 2,*3*,*5*-*Triphenyltetrazolium Chloride* (*TTC*) staining post-ischemia. The investigator performing the tMCAO surgery was blinded to treatment group. No mice in either treatment group were observed to be hyperthermic at the time body and head temperature monitoring was initiated for tMCAO surgery 24 h following administration of the final dose of MA or saline. Head and body temperatures were controlled at 37.0 ± 1.0 °C throughout MCAO surgery with warm water pads and a heating lamp. Occlusion and reperfusion were verified in each animal by laser Doppler flowmetry (LDF) (Model DRT4, Moor Instruments Ltd., Wilmington, DE). The common carotid artery was exposed and the external carotid artery was ligated and cauterized. Unilateral MCAO was accomplished by inserting a 6–0 nylon monofilament surgical suture (Ethicon, Inc., Somerville, NJ, USA) with a heat-rounded and silicone-coated (Xantopren Comfort Light, Heraeus, Hanau, Germany) tip into the internal carotid artery via the external carotid artery stump. Adequacy of MCAO was confirmed by monitoring cortical blood flow at the onset of the occlusion with an LDF probe affixed to the skull. Criteria for exclusion involved the mean intra-ischemic LDF being greater than 30 % pre-ischemic baseline. No animals in this study were excluded based on this LDF exclusion criterion. After 1 h of occlusion, the occluding filament was withdrawn to allow for reperfusion. Mice were then allowed to recover from anesthesia and survived for 96 h following initiation of reperfusion. Mice were euthanized if their post-tMCAO body weight decreased by greater than 20 % compared to body weight recorded just prior to initiation of tMCAO surgery (pre-tMCAO). No animals in this study were excluded based on this post tMCAO body weight exclusion criterion.

Neurological deficit score Neurological function was evaluated at baseline (before MCAO surgery), just after reperfusion, and 24, 48, 72, and 96 h after reperfusion using a 0- to 4-point neurological deficit score as follows: 0, no neurological dysfunction; 1, failure to extend left forelimb fully when lifted by tail; 2, circling to the contralateral side; 3, falling to the left; 4, no spontaneous movement or in a comatose state (Banerjee et al. 2013). The investigator performing these assessments was blinded to treatment group (Saline, $N = 12$; MA, $N = 11$).

Cognitive assessment Forty-eight hours after tMCAO, mice (Saline, $N = 12$; MA, $N = 11$) were tested for novel object recognition by an investigator who was blinded to experimental condition. The novel object recognition test is used to measure cortex- and hippocampus-dependent recognition memory (Broadbent et al. 2009). The particular novel object recognition paradigm used in this study involved a 24-h period between training and testing and relies primarily on the hippocampus and entorhinal, perirhinal, and parahippocampal cortices (Baxter 2010). Previous studies in our laboratory have indicated that this test is particularly sensitive in detecting cognitive injury following environmental challenges (Haley et al. 2013). On day 1 (habituation), 48 h after tMCAO, mice were placed into an open field (16″ × 16″ Kinder Scientific, Poway, CA) for 15 min. On day 2, the mice were placed in the open field containing two identical objects (hexagonal orange blocks) placed near the center of the arena (approximately 6-cm apart) and were allowed to freely explore for 15 min. On

day 3, the mice were again placed in the open field, but one familiar object was replaced with a novel object (green triangular block) of similar dimensions. Mice were allowed to explore for 15 min. Movement and time spent exploring each object was recorded and analyzed using EthoVision XT video tracking system (Noldus Information Technology, Sterling, VA). Percentage of time exploring the novel object versus the familiar object on day 3 was used to assess novel object recognition. The open field arena and objects were cleaned with 5 % acetic acid between all trials. To assess potential group differences in locomotor function, the total distance moved during the three trials of the Novel Object Recognition Test was also analyzed.

TTC Staining and brain infarct volume analysis Immediately following the final Novel Object Recognition trial (96 h following tMCAO), mice were euthanized by cervical dislocation followed by rapid decapitation. Brains were removed and cut into five 2 mm-thick coronal sections for staining with 1.2 % TTC (Sigma Aldrich, St. Louis, MO, USA) in saline, as described previously (Banerjee et al. 2013). The 2 mm-thick brain sections were incubated in 1.2 % TTC for 15 min at 37 °C, and then fixed in 10 % formalin for 24 h. Infarct volume was measured using digital imaging and image analysis software (Systat, Inc., Point Richmond, CA, USA). To control for edema, regional infarct volume (cortex, striatum, and hemisphere) was determined by subtraction of the ipsilateral non-infarcted regional volume from the contralateral regional volume. This value was then divided by the contralateral regional volume and multiplied by 100 to yield regional infarct volume as a percentage of the contralateral region. The analysis was performed by an investigator who was blinded to the experimental condition (Saline, $N = 12$; MA, $N = 11$).

Fluoro-Jade Staining and analysis To analyze potential changes in degenerating cells, Fluoro-Jade staining was assessed within discrete regions involved in regulating cognitive and motor function (CA1, CA3, dentate gyrus, and somatosensory cortex). A subset of brains (Saline $N = 8$, MA $N = 7$) was used for this analysis. These were brains from a second cohort of mice that were tested. Brains from the first cohort were discarded after TTC analysis, so they were therefore unavailable to include in the analysis. One 2-mm thick coronal brain section corresponding to the 3rd section shown in Fig. 2c as one moves from anterior to posterior aspect of the brain was sectioned into 50 μm sections and processed for Fluoro-Jade staining. Briefly, sections were mounted onto slides and were dried on a slide warmer at 50 °C. Slides were immersed in 80 % ethanol solution with 1 % NaOH, followed by 70 % ethanol solution, then washed in distilled H_2O (dH_2O). Slides were then incubated in 0.06 % potassium permanganate for 15 min, and washed in dH_2O again before incubation in 0.0001 % Fluoro-Jade C (16 A, Histo-Chem, Inc., AR, USA) for 15 min. Slides were washed in dH_2O, dried overnight, and cover-slipped with Permount™ mounting medium (Fisher Scientific, MA, USA) the following day. Fluoro-Jade staining was quantified bilaterally within fixed area frames; CA1 (box, 275 × 860 μm), CA3 (box, 625 × 850 μm), dentate gyrus (box, 77 × 93 μm), and sensorimotor cortex (860 × 500 um). Background threshold levels were set and applied to all images for comparison. Pixel intensities above this threshold were used for quantification (area occupied by pixels).

Statistical analysis

All data are reported as means ± standard error of the mean (SEM). Data were analyzed using GraphPad Prism v.4. The Gehan-Breslow-Wilcoxon test was used to compare group survival rates following tMCAO. Median neurological scores were compared using Mann Whitney T-tests. For infarct and Fluoro-Jade analyses, data were analyzed separately by region using Student's T-tests and 2-way ANOVA, respectively. Total distance moved in the three trials of the novel object test was analyzed by 2-Way ANOVA. Student's T-tests were also used to compare novel object recognition in trial 3 of the novel object recognition test. For this test, a percentage was calculated for each mouse that indicates the amount of time it spent investigating the novel versus the familiar object. These individual mouse percentages were averaged by treatment group to obtain a group mean percentage.

Results and discussion

Survival of mice at 96 h post reperfusion was 66.6 % (12/18) in the saline group compared to 45.8 % (11/24) in the MA-treated group. Comparison of survival curves using a Gehan-Breslow-Wilcoxon test revealed a trend toward a greater mortality rate in MA pre-treated mice ($X^2(1) = 2.85, p = 0.091$) but it did not reach statistical significance. Body weights significantly decreased following tMCAO (($F(1,42) = 25.36, p < 0.001$) although change in body weight did not differ by prior treatment with saline or MA (Saline pre-tMCAO = 28.1 ± 0.6 g; saline 96 h post-tMCAO = 24.7 ± 0.6 g (11.9 ± 1.7 % decrease); MA pre-tMCAO = 29.2 ± 0.6 g; MA 96 h post-tMCAO = 25.1 ± 1.0 g (14.3 ± 1.9 % decrease)). There were no significant differences in median neurological scores at any time point (Supplemental Table 1). At 48 and 72 h post reperfusion, the distribution of neurological deficit scores would suggest that MA-treated mice tended to have higher neurological deficit scores than saline-treated mice (Supplemental Table 1).

Table 1 Neurological deficit score distribution and median scores at various reperfusion time points following 1 h of middle cerebral artery occlusion in saline and methamphetamine (MA) treated male C57BL/6 J mice

	Distribution Of Neurological Deficit Scores																								
Experimental Groups	1 Hour					24 Hours					48 Hours					72 Hours					96 Hours				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Saline (n = 12)	0	0	11	1	0	0	7	5	0	0	7	3	1	1	0	9	1	2	0	0	9	2	0	1	0
MA (n = 11)	0	0	10	1	0	0	6	5	0	0	2	9	0	0	0	3	8	0	0	0	8	2	1	0	0

	Median Neurological Deficit Scores				
Experimental Groups	1 Hour (P = 1.000)	24 Hours (P = 0.899)	48 Hours (P = 0.288)	72 Hours (P = 0.153)	96 Hours (P = 0.974)
Saline (n = 12)	2	1	0	0	0
MA (n = 11)	2	1	1	1	0

Distance traveled was not significantly different between MA and saline-treated mice in any of the three trials of the Novel Object (NO) Recognition Test. There was an effect of testing day with all mice showing a greater amount of activity in the day 2 trial in which they were first introduced to objects in their environment 48 h following tMCAO ((F(2, 63) = 22.09, $p < 0.001$; Fig. 1c). In the testing trial, (NO Day 3, See Fig. 1a), saline-treated mice explored the novel object significantly more than the familiar object (t(22) = 3.33; p = .003; Fig. 1b), indicating novel object recognition, but MA-treated mice did not (t(20) = 0.61; p = .548; Fig. 1b).

Figure 2a shows that relative cerebral perfusion, measured by laser Doppler probe, of the MCA territory was similar between MA and saline pre-treated mice at all time points evaluated during 1 h of tMCAO and the first 5 min of reperfusion.

Infarct volume was increased in the striatum (t(21) = 2.61; p = .016; Fig. 2b) and total hemisphere (t(21) = 2.24; p = .032; Fig. 2b). In addition, there was a trend toward increased infarct volume in the cortex (t(21) = 1.76; p = .092; Fig. 2b). Representative images of TTC-stained coronal brain sections from MA and saline pre-treated mice are shown in Fig. 2c.

Neuronal degeneration, analyzed using Fluoro-Jade staining, was significantly elevated in the tMCAO occluded (right) hemisphere in the sensorimotor cortex (p = 0.0005, F(1, 26) = 15.52; Fig. 2d), dentate gyrus (p = 0.0052, F(1, 26) = 9.314; Fig. 2d), CA1 (p = 0.0007, F(1,26) = 14.76; Fig. 2d), and CA3 (p = 0.0005, F(1,26) = 15.09; Fig. 2d, 2e) compared to the non-occluded (left) hemisphere. However, no significant effects of prior treatment with MA on this measure were found in any brain region examined compared to saline-treated mice.

The results of this study indicate that chronic MA exposure prior to tMCAO increases brain infarct volume and causes short-term cognitive deficits. MA-induced increase in infarct volume was greatest in the striatum, an area that is densely populated by dopamine-containing cells. Previous studies have shown that bolus MA administration induces apoptosis within dopaminergic neurons (Thiriet et al. 2005). There is currently no evidence to indicate that the lower daily dose of MA repeatedly administered in the current study can induce apoptotic effects on its own; however, the combination of chronic MA exposure and tMCAO may have contributed to specific damage to the striatum due to the high percentage of dopaminergic neurons found in this region. Although MA-treated mice showed greater striatal damage, this did not translate into a functional deficit in locomotor activity. On the contrary, cortical regions which contribute to object memory showed relatively lesser infarction compared to the striatum in MA pre-exposed mice, but were associated with greater functional impairments. Although hemisphere infarct volume as determined by TTC staining (indicator of dead cells) was elevated following prior exposure to MA, the number of surviving neurons undergoing degeneration (Fluoro-Jade) in hippocampal regions and somatosensory cortex were unaffected by MA exposure. This indicates that, within these brain regions, ongoing neurodegeneration at 96 h post-tMCAO was unaffected by prior chronic MA treatment. Interestingly, in contrast to higher doses of MA such as those commonly abused by addicts, low dose MA administered around the time of cerebral ischemia can *reduce* neurodegeneration (Rau et al. 2016).

Although not assessed in the current study, it remains possible that MA pre-treatment may have increased infarct volume in the hippocampus which could be a key factor that contributes to the deficits in novel object recognition reported in the current study. In future studies, it will be important to determine whether these deficits in cognitive function persist for weeks or months following tMCAO. In the present study, the focus was solely on short-term deficits in cognitive functions; however there are limitations to interpretation of these findings. During recovery from tMCAO, mice tend to be stressed, anxious, and show contralateral rotations particularly during early recovery (Balkaya et al. 2013). These factors may play a role in behavioral performance in the novel object task and should be considered in interpreting cognitive function. Furthermore, since sham-operated mice were not included in

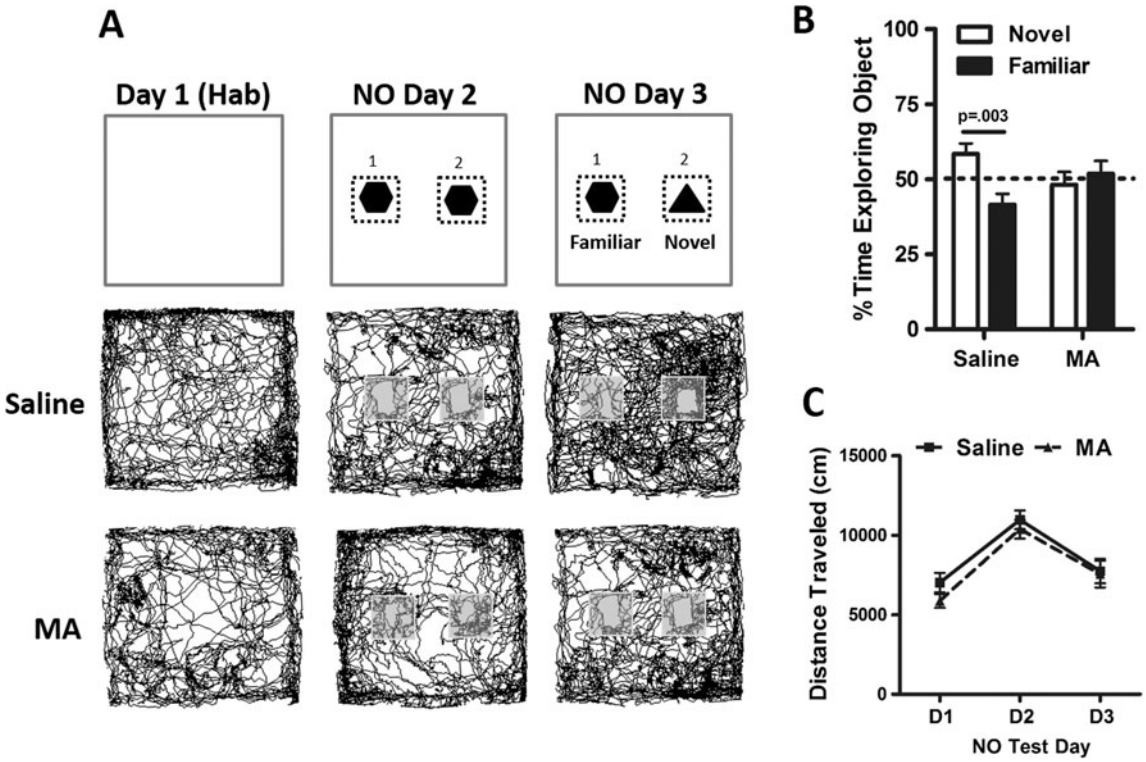

Fig. 1 Male mice treated with MA prior to tMCAO show impaired novel object (NO) recognition. **a** Description of the Novel Object Recognition Test and tracking patterns from representative saline- and MA-treated male C57BL/6J mice; Day 1 (habituation, 48 hours post MCAO), Day 2 (exposure to 2 identical objects, 72 hours post MCAO), and Day 3 (exposure to familiar and novel object, 96 hours post MCAO). **b** Saline-treated mice ($n = 12$) show a greater preference for the novel vs. familiar object indicating novel object recognition in these mice. However, MA-treated mice ($n = 11$) show no preference toward either object indicating a lack of object recognition. **c** Locomotor activity did not significantly differ between saline- and MA-treated mice during the three days of testing

this study we cannot dismiss the possibility that MA exposure per se, not MA followed by tMCAO, contributed to cognitive deficits. Lastly, significant limitations in preclinical stroke models in the absence of MA exposure have also been identified (Dirnagl 2006). They include low statistical power and hence reproducibility, problems in statistical analysis, lack of blinding and randomization, lack of quality-control mechanisms, deficiencies in reporting, negative publication bias, and quality-related sources of bias relevant to mechanism-driven basic stroke research.

These findings complement previous studies, which demonstrated that 4 injections of MA resulted in elevated infarct volume compared to saline controls (Wang et al. 2001; Shen et al. 2008). However, blood MA levels mice in this study were likely near undetectable at the time of tMCAO, given that the half-life is between 60 and 70 min (Yamanaka et al. 1983). This indicates that group differences reflect effects of prior/chronic exposure to MA rather than an acute effect. MA has been implicated as a factor that contributes to stroke risk in humans (Ho et al. 2009). However, what remains unclear is whether chronic MA-use in humans can worsen stroke outcome compared to non-MA users. Our current findings demonstrate that indeed chronic MA does worsen ischemic outcome in mice. This indicates that chronic MA may also worsen stroke outcome in humans. Controlled clinical studies are warranted to test this possibility.

Short-term MA dosing may worsen stroke outcome by increasing oxidative stress (Wang et al. 2001), elevating levels of caspase-3/7 activity which contributes to apoptotic cell death (Shen et al. 2008), decreasing the neuroprotective factor bone morphogenic protein 7 (BMP7; Shen et al. 2008), and affecting the hypothalamic-pituitary-adrenal (HPA) axis (DeVries et al. 2001). Other potential contributing mechanisms include chronic MA-induced rupturing, narrowing, or occlusion of large blood vessels which can exacerbate ischemic events produced by tMCAO (Polesskaya et al. 2011; Anderson and Sung 2003). Repeated MA administration can deplete striatal dopamine levels (Wagner et al. 1980), thereby reducing potential neuroprotective effects of dopamine in this region. Furthermore, repeated MA exposure can alter dopamine receptor (D1 and D2) sensitivity, as well as dopamine transporter function (Bennett et al. 1998; Shishido et al. 1997), factors which may also contribute to differences in ischemic cell death. MA has also been associated with vascular damage which may be a factor that contributed to larger infarct volume following cerebral ischemia (Sharma and Ali 2006). However, in the current study we found no differences between MA- and saline-treated mice in laser Doppler probe assessed cerebral

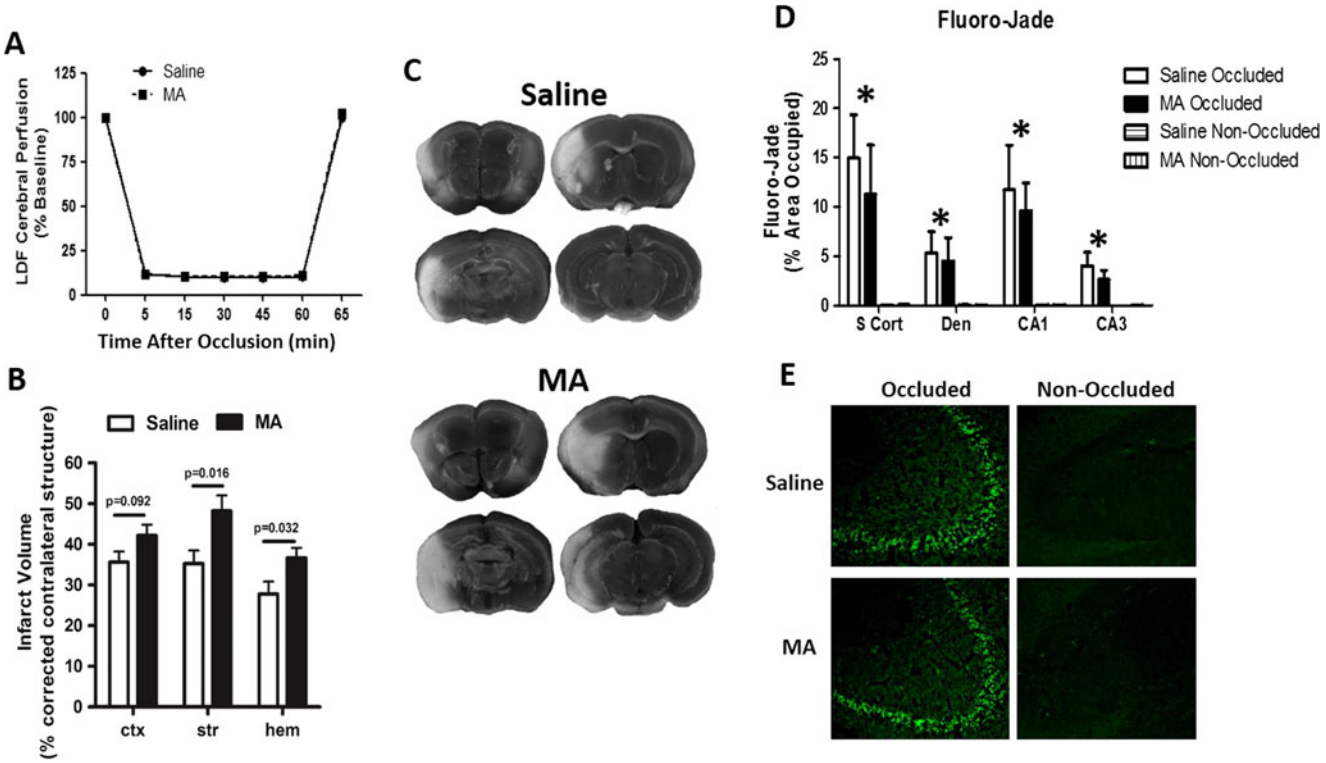

Fig. 2 Infarct volume and Fluoro-Jade labeling in male mice chronically treated with MA or saline. **a** Cerebral perfusion during tMCAO (60 min) and early reperfusion is similar in MA- and saline-pretreated male C57BL/6 J mice. Relative cerebral perfusion of the MCA territory measured continuously by laser Doppler probe (averages of 5–15 min intervals) is shown at baseline (0 min), during the occlusion (5–60 min), and during 5 min post-reperfusion (65 min). N = 11–12 per group. **b** Infarct volumes were significantly greater in MA- (n = 11) than saline-treated mice (n = 12) in the striatum (str, p = .016) and total hemisphere (hem, p = .032) after 1 h of tMCAO and 96 h of reperfusion. There was a trend toward increased infarct volume in the cortex (ctx) of MA-treated mice (p = .092). **c** Representative TTC-stained coronal brain sections from saline- and MA-treated mice. **d** Neuronal degeneration was significantly greater in somatosensory cortex (S Cort; p = 0.0005), dentate gyrus (p = 0.0052), CA1 (p = 0.0007), and CA3 (p = 0.0005) within the occluded (ischemic) vs. non-occluded (non-ischemic) hemisphere regardless of treatment group. There was no difference observed in MA- (n = 7) compared to saline-treated mice (n = 8) in neuronal degeneration following MCAO in either occluded or non-occluded hemisphere. **e** Representative images of Fluoro-Jade in CA3. * indicates increase in occluded compared to non-occluded hemisphere

perfusion change at 5 min of reperfusion, which suggests that altered cerebral perfusion may not have been a major contributing factor. A recent study utilizing the same tMCAO model indicates that vascular damage can be detected by laser Doppler probe assessed cerebral perfusion change at 5 min of reperfusion (Zuloaga et al. 2014). Since no change was detected in laser Doppler probe assessed cerebral perfusion change between MA and saline pretreated mice, this suggests that vascular damage was potentially not severe enough to be detected using this method although it is quite possible that some vascular damage was incurred due to MA treatment.

MA has been repeatedly shown to induce hyperthermia and as a result could contribute to vascular damage and neurodegeneration (Bowyer et al. 2008). In male mice, MA at a dose of 10 mg/kg does has been shown to induce an increase in body temperature that peaks at around 38 °C (Yamanaka et al. 1983) and this sustained hyperthermia may lead to neuroinflammation, neurotoxicity, and vascular rupture. In this study, we did not find evidence of neurodegeneration (assessed by Fluoro-Jade) or significant alterations in vascular perfusion (assessed by laser Doppler flowmetry) resulting from prior exposure to MA. Furthermore, at the time body and head temperature monitoring was initiated for tMCAO surgery 24 h following administration of the final dose of MA, no mice were observed to be hyperthermic. However, it remains likely that MA-induced hyperthermia compromised neurons and vasculature although more subtle changes may not have been detectable using procedures employed in this study. Hyperthermia also increases reactive oxygen species (ROS) and it is possible they remained elevated at the time of tMCAO (24 h after final MA injection). Elevated ROS may therefore be associated with elevated infarct volume resulting from ROS induction of apoptotic pathways (Wang et al. 2001). Further studies are needed to determine these mechanisms.

In summary, the present findings provide the first direct evidence that chronic exposure to MA exacerbates ischemic cell death and worsens cognitive function following tMCAO. This model of chronic MA exposure prior to stroke can be utilized in future studies to investigate the mechanisms

through which MA abuse worsens ischemic outcome and facilitate the development of therapeutic interventions for stroke patients with a history of MA use.

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Compliance with ethical standards

Conflict of interest None.

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