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4 **The danger-associated molecular pattern HMGB1 mediates the**
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7 **neuroinflammatory effects of methamphetamine**
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Abstract

Methamphetamine (METH) induces neuroinflammatory effects, which may contribute to the neurotoxicity of METH. However, the mechanism by which METH induces neuroinflammation has yet to be clarified. A considerable body of evidence suggests that METH induces cellular damage and distress, particularly in dopaminergic neurons. Damaged neurons release danger-associated molecular patterns (DAMPs) such as high mobility group box-1 (HMGB1), which induces pro-inflammatory effects. Therefore, we explored the notion here that METH induces neuroinflammation indirectly through the release of HMGB1 from damaged neurons. Adult male Sprague-Dawley rats were injected IP with METH (10 mg/kg) or vehicle (0.9% saline). Neuroinflammatory effects of METH were measured in nucleus accumbens (NAcc), ventral tegmental area (VTA) and prefrontal cortex (PFC) at 2h, 4h and 6h after injection. To assess whether METH directly induces pro-inflammatory effects in microglia, whole brain or striatal microglia were isolated using a Percoll density gradient and exposed to METH (0, 0.1, 1, 10, 100, or 1000 uM) for 24h and pro-inflammatory cytokines measured. The effect of METH on HMGB1 and IL-1 β in striatal tissue was then measured. To determine the role of HMGB1 in the neuroinflammatory effects of METH, animals were injected intra-cisterna magna with the HMGB1 antagonist box A (10 ug) or vehicle (sterile water). 24h post-injection, animals were injected IP with METH (10 mg/kg) or vehicle (0.9% saline) and 4h later neuroinflammatory effects measured in NAcc, VTA, and PFC. METH induced robust pro-inflammatory effects in NAcc, VTA, and PFC as a function of time and pro-

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4 inflammatory analyte measured. In particular, METH induced profound effects on
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6 IL-1 β in NAcc (2h) and PFC (2h and 4h). Exposure of microglia to METH *in vitro*
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8 failed to induce a pro-inflammatory response, but rather induced significant cell
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10 death as well as a decrease in IL-1 β . METH treatment increased HMGB1 in
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12 parallel with IL-1 β in striatum. Pre-treatment with the HMGB1 antagonist box A
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14 blocked the neuroinflammatory effects (IL-1 β) of METH in NAcc, VTA and PFC.
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17 The present results suggest that HMGB1 mediates, in part, the
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19 neuroinflammatory effects of METH and thus may alert CNS innate immune cells
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24 to the toxic effects of METH.
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53 **Key words:** methamphetamine; neuroinflammation; microglia; HMGB1; DAMP;
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56 neurotoxicity.
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1. Introduction

A number of studies have found that methamphetamine (METH) activates microglia, which are considered the predominant innate immune effector in the CNS (Bowyer and Ali, 2006; Buchanan et al., 2010; Escubedo et al., 1998; Fantegrossi et al., 2008; Goncalves et al., 2010; Guilarte et al., 2003; Kelly et al., 2012; Ladenheim et al., 2000; LaVoie et al., 2004; Pubill et al., 2003; Sekine et al., 2008; Sharma and Kiyatkin, 2009; Sriram et al., 2006; Theodore et al., 2006; Thomas et al., 2004; Thomas et al., 2008; Thomas and Kuhn, 2005). Moreover, METH induces an array of neuroinflammatory effects including pro-inflammatory cytokines (PICs), chemokines, and oxidative stress (Cadet and Krasnova, 2009; Krasnova and Cadet, 2009). The initial reports demonstrated that acute METH treatment induces hypothalamic interleukin (IL)-1 β (Yamaguchi et al., 1991a; Yamaguchi et al., 1991b). Several groups have extended this finding to show that METH induces PICs across several brain regions including brain reward pathways. For example, acute METH treatment increased striatal and frontal cortex expression of tumor necrosis factor (TNF) α and IL-1 β (Flora et al., 2003; Flora et al., 2002). Likewise, Kelly and colleagues found that acute METH up-regulated the expression of pro-inflammatory cytokines and chemokines, including TNF α , IL-6, IL-1 β , leukemia inhibitory factor, and CCL-2 in whole striatum (Kelly et al., 2012). Similarly, chronic METH increased interferon α/β in frontal lobe (Coutinho et al., 2008), striatal expression of TNF α and NF- κ B (Lai et al., 2009), as well as TNF α throughout several brain regions (Nakajima et al., 2004). Consistent with a key role for microglia in the neuroinflammatory effects of

METH, acute METH induced expression of $\text{TNF}\alpha$, $\text{TNF}\alpha_1$, $\text{TNF}\alpha_2$, $\text{IL-1}\alpha$ and IL-6 , effects that were blocked by pretreatment with the microglia inhibitor minocycline (Sriram et al., 2006). Similarly, the non-steroidal anti-inflammatory indomethacin prevented the neuroinflammatory effects of METH (Goncalves et al., 2010).

While the preponderance of evidence suggests that METH induces neuroinflammation, in part mediated by microglia, the mechanisms by which METH does so have yet to be clarified. It should be noted that there is little evidence that METH acts directly on microglia to induce a neuroinflammatory response. Therefore, we explore here the notion that the neuroinflammatory effects of METH may be secondary to the effects of METH on the neuronal release of danger-associated molecular patterns (DAMPs), which are capable of inducing neuroinflammatory processes. This notion extends from studies showing that under a variety of neuroinflammatory conditions, neuronal release of DAMPs, in particular high mobility group box-1 (HMGB1), plays a mediating role in neuroinflammation (reviewed in (Frank et al., 2015). For example, ethanol exposure induces the neuronal secretion of HMGB1, which then functions to elicit a neuroinflammatory response via activation of microglia and the toll-like receptor (TLR)4 pathway (Zou and Crews, 2014). Here, we explore the idea that METH induces the neuronal release of HMGB1, which may then signal through its cognate receptors (e.g. TLR4; receptor for advanced glycation endproducts, RAGE) on microglia or other immunocompetent cells, resulting in the induction of neuroinflammatory processes.

2. Methods

2.1. Animals

Male Sprague Dawley rats (60–90 d-old; Harlan) were pair housed with food and water available ad libitum. The colony was maintained at 25°C on a 12 h light/dark cycle (lights on at 7:00 A.M.). Rats were allowed 1 week of acclimatization to the colony rooms before experimentation. All experimental procedures occurred between 9:00 A.M. and 12 P.M. and were conducted in accord with the University of Colorado Institutional Animal Care and Use Committee. A total of 64 animals were used in the following experiments.

2.2. Experimental design

2.2.1. Experiment I: Effects of METH on neuroinflammatory mediators.

Rats were injected IP with vehicle (0.9% saline; N = 4) or METH (d-methamphetamine hydrochloride; 10 mg/kg; Sigma, St. Louis, MO; N = 4 per timepoint post-METH). Prior studies suggest that this dose and route of METH administration is capable of inducing neuronal damage (Imam and Ali, 2001), therefore this dosing regimen was selected to observe HMGB1-mediated neuroinflammatory effects. METH-induced neuronal damage was observed 4h after treatment in striatal tissue (Imam and Ali, 2001). Therefore, we conducted an initial timecourse experiment (2, 4 and 6h post-treatment) in several brain reward-related structures including the nucleus accumbens (NAcc), prefrontal cortex (PFC), and ventral tegmental area (VTA) to determine the timepoint at which the maximal neuroinflammatory effect of METH (mRNA changes) occurs across brain regions. This experiment was repeated at the 4h timepoint post-

METH (N = 5) and vehicle (N = 5) treatment to determine whether METH-induced mRNA changes in pro-inflammatory mediators extended to the protein level.

2.2.2. Experiment II: In vitro effects of METH on microglia

Whole brain (N = 4) or striatal microglia (N = 3) were isolated from naïve animals and exposed to varying concentrations of METH (0, 0.1, 1, 10, 100 and 1000 uM) for 24h, and pro-inflammatory mediators (mRNA and protein) measured. A 24h incubation period was chosen to maximize the chances of detecting a pro-inflammatory effect of METH.

2.2.3. Experiment III: Effect of METH on striatal HMGB1 and IL-1 β expression.

Rats were injected IP with vehicle (0.9% pyrogen-free saline; N = 4) or METH (10 mg/kg; N = 4). 4h post-treatment, protein levels were measured in whole striatum.

2.2.4. Experiment IV: Effect of the HMGB1 antagonist box A on the neuroinflammatory effects of METH.

Rats were injected intra-cisterna magna (ICM) with vehicle (5 μ l pyrogen-free sterile water) or box A (10 μ g; HMGBiotech, Milan, IT). Box A is a competitive antagonist of HMGB1 at TLR4 (Yang et al., 2004). This dose of box A was selected because of its efficacy in blocking stress-induced neuroinflammatory priming (Weber et al., 2015) and as well as HMGB1 mediated seizure (Maroso et al., 2010). 24h post-ICM injection, animals were injected IP

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4 with vehicle (0.9% pyrogen-free saline) or METH (10 mg/kg). 4h post-IP injection,
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6 neuroinflammatory mediators were measured (mRNA) in NAcc, PFC, and VTA.
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9 Group sample sizes: ICM veh/IP veh (N = 6), ICM veh/IP METH (N = 5), ICM
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11 boxA/IP veh (N = 6) and ICM boxA/IP METH (N = 6).
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14 15 2.3. General procedures 16 17

18 2.3.1. Brain Tissue Collection 19 20

21 All rats were administered a lethal dose of sodium pentobarbital and
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23 transcardial perfusion performed for 3 min with ice-cold pyrogen-free 0.9%
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25 saline. In experiment I and IV, brain was dissected and flash frozen in isopentane
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27 (−40°C). Bilateral micropunches (1 mm³) were collected while the brain was
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29 mounted on a freezing cryostat from PFC, NAcc and VTA and stored at −80°C.
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31 In experiment II, whole brain or striatum was dissected and microglia rapidly
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33 isolated. In experiment III, whole striatum was dissected, flash frozen in liquid
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35 nitrogen and stored at -80°C.
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41 2.3.2. ICM injection 42 43 44

45 ICM injection was selected as an intra-cerebral route of drug
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47 administration because this procedure obviates the potential neuroninflammatory
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49 effects caused by cannulation, minimizes anesthesia exposure and effectively
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51 delivers compounds throughout the CNS (Proescholdt et al., 2000). Rats were
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53 briefly anesthetized with isoflurane (~3 min). The dorsal aspect of the skull was
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55 shaved and swabbed with 70% EtOH. A 27-gauge needle, attached via
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57 polyethylene-50 tubing to a 25 µl Hamilton syringe, was inserted into the cisterna
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magna. To verify entry into the cisterna magna, CSF (~2 µl) was withdrawn and gently pushed back. Injection proceeded only if CSF appeared clear of red blood cells.

2.3.3. Microglia Isolation

Whole brain or striatal microglia were isolated using a Percoll density gradient as described previously (Frank et al., 2006), in which we have shown that this microglia isolation procedure yields highly pure (> 95%) microglia. Rapidly isolated microglia are typically positive for ionized calcium-binding adapter molecule 1 (Iba-1; microglia/macrophage marker) and major histocompatibility complex II (MHCII; microglia/macrophage marker) and negative for cluster of differentiation 163 (CD163; perivascular macrophage marker) and glial fibrillary acidic protein (GFAP; astrocyte marker). Immunophenotype and purity of microglia was assessed and verified using real-time RT-PCR of Iba-1, MHCII, CD163, and GFAP. Microglia were routinely found to be +Iba-1/+MHCII/-CD163/-GFAP (data not shown) indicating that microglia were devoid of perivascular macrophages and astrocytes. Microglia were cultured in 100 µl of DMEM plus 10% FBS, and microglia concentration was determined by trypan blue exclusion. Microglia were plated in individual wells of a 96-well v-bottom plate and incubated at 37° C, 5% CO₂ under the experimental conditions described above. Supernatants were collected for protein assay and cells were washed in ice-cold 1x PBS. Cells were lysed/homogenized and cDNA synthesis was performed according to the protocol of the manufacturer using SuperScript III CellsDirect cDNA Synthesis System (Life Technologies, Grand

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4 Island, NY). Gene expression of proinflammatory cytokines was measured using
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7 real-time RT-PCR.

8 9 *2.3.4. MTT assay of microglia viability.*

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11 Microglia were incubated with 12 mM MTT (3-(4,5-dimethylthiazol-2-yl)-
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13 2,5-diphenyltetrazolium bromide; Life Technologies) at 37° C for 4h. Dimethyl
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15 sulfoxide (50 µl) was added to each well and incubated at 37° C for 10 min.
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17 Absorbance was measured at 540 nm, and cell viability was determined
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19 according to the protocol of the manufacturer.
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22 23 *2.3.5. RNA extraction, cDNA synthesis and real time RT-PCR*

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27 RNA was extracted using a standard Trizol protocol (Chomczynski and
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29 Sacchi, 1987). cDNA was synthesized using Superscript II reverse transcriptase
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31 according to the manufacturer's protocol (Life Technologies). A detailed
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33 description of the PCR amplification protocol has been published previously
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35 (Frank et al., 2006). cDNA sequences were obtained from GenBank at the
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37 National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov).
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39 Primer sequences were designed using the Eurofins MWG Operon Oligo
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41 Analysis and Plotting Tool (<http://www.operon.com/technical/toolkit.aspx>) and
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43 tested for sequence specificity using the Basic Local Alignment Search Tool at
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45 the NCBI (Altschul et al., 1997). Primers were obtained from Invitrogen. Primer
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47 specificity was verified by melt curve analysis. All primers were designed to span
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49 exon/exon boundaries and thus exclude amplification of genomic DNA (for primer
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51 description and sequences, see Table 1). PCR amplification of cDNA was
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53 performed using the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA).
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4 Formation of PCR product was monitored in real time using the MyiQ Single-
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6 Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Relative gene
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8 expression was determined by taking the expression ratio of the gene of interest
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10 to the housekeeping gene β -actin.
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16 Striatum was sonicated in a mixture containing extraction buffer (Life
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18 Technologies) and protease inhibitors (Sigma). Ice-cold tissue samples were
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20 centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was removed, and
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22 the protein concentration for each sample was quantified using the Bradford
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24 method. Samples were heated to 75° C for 10 min and loaded into a standard
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26 polyacrylamide Bis-Tris gel (Life Technologies). SDS-PAGE was performed in 3-
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28 (N-morpholino)-propanesulfonic acid running buffer (Life Technologies) at 175 V
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30 for 1.25 h. Protein was transferred onto a nitrocellulose membrane using the iblot
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32 dry transfer system (Life Technologies). The membrane was blocked with
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34 Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h and incubated
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36 with a primary antibody in blocking buffer overnight at 4°C. The following day, the
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38 membrane was washed in 1x PBS containing Tween 20 (0.1%) and then
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40 incubated in blocking buffer containing either goat anti-rabbit or goat anti-mouse
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42 (LI-COR) IRDye 800CW secondary antibody at a concentration of 1:10,000 (LI-
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44 COR) for 1 h at room temperature. Protein expression was quantified using an
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46 Odyssey Infrared Imager (LI-COR) and expressed as a ratio to the housekeeping
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48 protein. Primary antibodies included rabbit anti-rat HMGB-1 (1:4000 dilution;
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50 Abcam) and mouse anti-rat β -actin (1:200,000 dilution; Sigma-Aldrich), which
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served as a housekeeping protein.

2.3.7. Enzyme-linked immunosorbent assay (ELISA)

A standard sandwich ELISA was used to measure IL-1 β protein according to the manufacturer's protocol (R & D Systems, Minneapolis, MN). For cell culture supernatants, protein concentration is expressed as pg/ml and for tissue homogenates, concentration is expressed as pg/mg total protein. Total protein was quantified using a Bradford assay.

2.3.8. Statistical Analysis

Data is presented as the mean $\pm$ SEM. Statistical analysis consisted of T-test or ANOVA followed by Dunnett's (experiment I and II) or Tukey's (experiment IV) post-hoc tests using Prism 5.0. Threshold for statistical significance was set at $\alpha = 0.05$.

3. Results

3.1. Effects of METH on neuroinflammatory mediators (**Fig. 1**)

Initially, a time-course experiment was conducted to determine the maximal neuroinflammatory effect of METH in several brain nuclei involved in brain reward. In NAcc, METH induced a significant change in IL-1 β (df = 3, 12, F = 71.65, p < 0.0001), TNF α (df = 3, 12, F = 7.86, p = 0.003) and NF- κ B α (df = 3, 12, F = 18.18, p < 0.0001) gene expression. Post-hoc comparisons show that METH increased IL-1 β (p < 0.001), TNF α (p < 0.01) and NF- κ B α (p < 0.001) at 2h post-treatment compared to vehicle control.

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4 In PFC, METH induced a significant change in IL-6 (df = 3, 12, F = 13.68,
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6 p = 0.004) and NF- κ B α (df = 3, 12, F = 5.61, p = 0.012) gene expression. Post-
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8 hoc comparisons show that METH increased IL-6 at 2h (p < 0.05) and 4h (p <
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10 0.001) and NF- κ B α at 2h (p < 0.05) and 4h (p < 0.05) post-treatment compared
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12 to vehicle control.
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17 In VTA, METH induced a significant change in IL-1 β (df = 3, 12, F = 5.87,
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19 p = 0.01), TNF α (df = 3, 12, F = 5.07, p = 0.017), IL-6 (df = 3, 12, F = 9.85, p =
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21 0.001) and NF- κ B α (df = 3, 12, F = 11.54, p = 0.0008) gene expression. IL-1 β (p
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23 < 0.01), TNF α (p < 0.01), IL-6 (p < 0.01) and NF- κ B α (p < 0.001) were
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25 significantly increased at 4h post-treatment compared to vehicle control.
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31 To determine whether the neuroinflammatory effects of METH extended to
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33 the level of protein expression, the above experiment was repeated at the 4h
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35 time-point post-METH treatment and IL-1 β protein levels measured in NAcc, PFC
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37 and VTA (**Fig. 2**). METH significantly increased IL-1 β protein levels in NAcc (df =
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39 8, t = 3.21, p = 0.012), PFC (df = 8, t = 6.39, p = 0.0002) and VTA (df = 8, t =
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41 3.58, p = 0.0071), compared to vehicle control.
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45 46 3.2. *In vitro* effects of METH on microglia

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49 In light of these observed neuroinflammatory effects of METH, we sought
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51 to test whether METH directly induces proinflammatory responses in isolated
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53 microglia. Initially, primary whole brain microglia were utilized to conduct *in vitro*
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55 experiments because sufficient numbers of microglia could not be isolated from
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57 CNS micropunches (**Fig. 3**). METH failed to significantly modulate the gene
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expression of IL-1 β (df = 5, 18, F = 1.76, p = 0.17), IL-6 (df = 5, 18, F = 0.99, p = 0.45), TNF α (df = 5, 18, F = 1.91, p = 0.14) and NF- κ B α (df = 5, 18, F = 0.84, p = 0.54). Interestingly, METH significantly modulated IL-1 β protein levels in cell culture supernatants (df = 5, 18, F = 63.64, p < 0.0001). Post-hoc comparisons show that METH significantly reduced IL-1 β protein at 100 μ M (p < 0.05) and 1000 μ M (p < 0.001) compared to media control. To assess whether this effect of METH on IL-1 β protein was due to effects on cell viability, an MTT assay was conducted and showed that METH significantly affected cell viability (df = 5, 12, F = 101.6, p = 0.0002). Post-hoc comparisons showed that METH induced a significant decrease in microglia viability at 1000 μ M (p < 0.001) compared to media control.

A concern regarding these *in vitro* experiments is that use of whole brain microglia may have obscured neuroinflammatory effects of METH in discrete brain regions. Therefore, these *in vitro* experiments were repeated using microglia isolated from striatum, which yielded sufficient numbers of microglia and encompasses the NAcc. Consistent with the effects of METH on whole brain microglia, METH failed to induce a pro-inflammatory response in primary striatal microglia (data not shown).

3.3. Effect of METH on striatal HMGB1 and IL-1 β expression

The results of experiment II suggest that METH may not directly exert pro-inflammatory effects on microglia. These results prompted us to explore the possibility that METH induces the release of DAMPs, which then target microglia

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4 to induce a pro-inflammatory response. Towards exploring this possible
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6 mechanism, we examined the effect of METH on striatal HMGB1 levels at 4h
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8 post-treatment. Whole striatum was used here because of technical challenges
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10 of reliably measuring HMGB1 protein levels in brain tissue micropunches. METH
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12 treatment resulted in a significant upregulation of HMGB1 (**Fig. 4A**) protein
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14 compared to vehicle control (df = 6, t = 5.00, p = 0.0025). Similarly, METH also
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16 induced a significant increase in IL-1 β protein (**Fig. 4B**) compared to vehicle
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18 control (df = 6, t = 6.43, p = 0.0007).
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24 *3.4. Effect of the HMGB1 antagonist box A on the neuroinflammatory effects of* 25 26 *METH* 27 28 29

30 The results of experiment III demonstrated that METH induces HMGB1 in
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32 parallel with increases in IL-1 β protein, therefore, we tested whether METH-
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34 induced HMGB1 mediates the neuroinflammatory effects of METH (**Fig. 5**).
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38 In NAcc, box A treatment significantly modulated the effect of METH on IL-
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40 1 β (interaction df = 1, 19, F = 16.28, p = 0.0007), but not TNF α (interaction df =
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42 1, 19, F = 0.0473, p = 0.83), IL-6 (interaction df = 1, 19, F = 2.73, p = 0.115) or
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44 NF- κ B α (interaction df = 1, 19, F = 2.32, p = 0.14) gene expression. Post-hoc
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46 comparisons show that vehicle/METH treatment induced a significant increase in
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48 IL-1 β compared to vehicle/vehicle control (p < 0.001), while box A treatment
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50 significantly reduced METH-induced IL-1 β expression compared to
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52 vehicle/METH treatment (p < 0.001). The main effect of METH was significant for
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54 IL-6 (df = 1, 19, F = 5.27, p = 0.03) and NF- κ B α (df = 1, 19, F = 6.32, p = 0.02)
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demonstrating that METH upregulated expression of these proinflammatory genes independent of box A treatment (data not shown).

In PFC, box A treatment significantly modulated the effect of METH on IL-1 β (interaction df = 1, 19, F = 6.89, p = 0.0039), but not TNF α (interaction df = 1, 19, F = 1.73, p = 0.2), IL-6 (interaction df = 1, 19, F = 0.28, p = 0.6) or NF- κ B α (interaction df = 1, 19, F = 2.32, p = 0.14) gene expression. Post-hoc comparisons show that vehicle/METH treatment induced a significant increase in IL-1 β compared to vehicle/vehicle control (p < 0.01), while box A/METH treatment significantly reduced IL-1 beta expression compared to vehicle/METH treatment (p < 0.05). The main effect of METH was significant for IL-6 (df = 1, 19, F = 5.75, p = 0.027) and NF- κ B α (df = 1, 19, F = 7.13, p = 0.0064), which showed that METH upregulated expression of these proinflammatory genes (data not shown).

In VTA, box A treatment significantly modulated the effect of METH on IL-1 β (interaction df = 1, 19, F = 6.64, p = 0.0185), but not TNF α (interaction df = 1, 19, F = 1.73, p = 0.2), IL-6 (interaction df = 1, 19, F = 0.28, p = 0.6) or NF- κ B α (interaction df = 1, 19, F = 2.32, p = 0.14) gene expression. Post-hoc comparisons show that vehicle/METH treatment induced a significant increase in IL-1 β compared to vehicle/vehicle control (p < 0.01), while box A/METH treatment significantly reduced IL-1 β expression compared to vehicle/METH treatment (p < 0.01). The main effect of METH was significant for NF- κ B α (df = 1, 19, F = 9.197, p = 0.0068) showing that METH increased expression of this gene (data not shown).

4. Discussion

Consistent with prior findings (Coutinho et al., 2008; Flora et al., 2003; Flora et al., 2002; Kelly et al., 2012; Lai et al., 2009; Nakajima et al., 2004; Yamaguchi et al., 1991a; Yamaguchi et al., 1991b), the present study found that acute METH treatment induced a transient, but robust neuroinflammatory response in several brain reward-related structures. Notably, METH had pronounced effects on IL-1 β mRNA and protein as well as NF- κ B α gene expression, which is induced by the transcription factor NF- κ B (Sun et al., 1993). This finding suggests that METH activates the NF- κ B pathway, resulting in the induction of pro-inflammatory cytokines, most notably IL-1 β .

A key question arising from this finding was whether METH would directly induce a pro-inflammatory response in microglia, which mediate, in large part, neuroinflammatory responses (Ransohoff and Perry, 2009). Although, a priori, a mechanistic basis for a direct effect of METH on microglia is lacking, we nevertheless examined this possibility given several reports of METH inducing pro-inflammatory cytokines in microglia cell lines (Coelho-Santos et al., 2012; Tocharus et al., 2010). Here, METH failed to induce a pro-inflammatory cytokine response in whole brain primary microglia even at millimolar concentrations. Rather, we found that METH significantly reduced the level of IL-1 β protein in cell culture supernatants below levels observed in the media control. METH also reduced microglia cell viability at high concentrations, which most likely is the basis for the observed effect of METH on IL-1 β protein. This effect on cell viability is consistent with the findings in microglia cell lines (Coelho-Santos et al., 2012;

Tocharus et al., 2010). A concern here is that use of whole brain microglia may have obscured differential pro-inflammatory effects of METH as a function of brain regional sensitivity of microglia to METH. To address this possibility, we examined the *in vitro* effects of METH on microglia isolated from whole striatum. Likewise, we found that METH failed to induce a pro-inflammatory response in striatal microglia. It should be noted that primary microglia were highly responsive to the TLR4 agonist lipopolysaccharide, which excludes the possibility that these null effects of METH were simply due to microglia anergy. Our inability to replicate prior *in vitro* effects of METH on microglia pro-inflammatory cytokines may be due to our use of primary microglia rather than microglia cell lines. Nonetheless, a concern regarding these prior studies as well as the findings reported here is the effect of METH on microglial cell viability, which is a potential confound of the observed effects of METH on cytokine expression.

In light of our observations that METH does not directly exert pro-inflammatory effects on microglia, we explored the possibility that METH-induced neuroinflammation may be a consequence of neuronal release of DAMPs, in particular HMGB1, which then target innate immune cells to induce a neuroinflammatory cascade. The basis for this notion extends from several studies demonstrating that neuronal release of HMGB1 mediates the neuroinflammatory response in several conditions (reviewed in (Frank et al., 2015)) including seizure and ethanol-induced neuroinflammation. Therefore, towards exploring this possible role of HMGB1, we initially examined whether METH exposure results in the induction of HMGB1. We examined the effects of

METH on striatal HMGB1 given the large body of evidence that the striatum is key target of the neurotoxic/neuroinflammatory effects of METH (Krasnova and Cadet, 2009). Indeed, we found that METH increased striatal expression of HMGB1 in parallel with increased IL-1 β protein.

HMGB1 exerts its pro-inflammatory effects through the pattern recognition receptors TLR2 and TLR4, as well as RAGE (Yang and Tracey, 2005). The primary structure of HMGB1 consists of an A box domain and a B box domain (Yang and Tracey, 2005). While the B box domain mediates the pro-inflammatory effects of HMGB1, the A box domain functions as a competitive receptor antagonist of HMGB1 at TLR4 (Yang et al., 2004). Here, we utilized the box A fragment to test the mediating role of HMGB1 in the neuroinflammatory effects of METH. METH treatment induced a robust increase in IL-1 β gene expression in NAcc (3.34 fold increase), PFC (2.33 fold increase), and VTA (3.78 fold increase) compared to vehicle treatment, while box A treatment blocked these METH-induced increases in IL-1 β to levels comparable to vehicle treatment in these brain regions. Interestingly, box A failed to block the effect of METH on IL-6 expression (NAcc and PFC) as well as NF- κ B α (NAcc, PFC, and VTA). This differential effect of box A suggests that DAMPs other HMGB1 (e.g. heat shock proteins) may be involved in the neuroinflammatory response to METH and thus unaffected by box A. Furthermore, the IL-1 β specific effects of box A treatment point to a unique relationship between IL-1 β and HMGB1. Formation of the NLRP3 inflammasome and activation of caspase-1, which regulate IL-1 β processing and release, have also been implicated as regulatory mechanisms of

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4 HMGB1 release (Lamkanfi et al., 2010) suggesting that HMGB1 may be released
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6 in a manner similar to IL-1 β or co-released with HMGB1. Moreover, HMGB1 is
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8 capable of binding IL-1 β protein and potentiating IL-1 β signaling through the IL-1
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10 type 1 receptor (Hreggvidsdottir et al., 2009; Sha et al., 2008), which highlights
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12 the role of HMGB1 as an endogenous adjuvant. Whether HMGB1 plays such a
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14 role in the neuroinflammatory effects of METH is unclear, but the possibility is
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16 intriguing. While the present findings suggest that HMGB1 mediates, in part, the
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18 neuroinflammatory effects of METH and thus implicates HMGB1 in the
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20 neuroinflammatory effects of METH, several key questions remain to be
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22 addressed.
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29 First, it is unclear which cell type(s) in the CNS releases HMGB1 in
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31 response to METH. It should be noted that here we did not directly measure
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33 cellular release of HMGB1 in the CNS, but only METH-induced increases of
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35 HMGB1. From the present data, we infer that METH induced the release of
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37 HMGB1 in the CNS because box A competitively antagonizes HMGB1 signaling
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39 through TLR4 (Yang et al., 2004), which requires the extra-cellular release of
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41 HMGB1. HMGB1 is primarily located in the nucleus of most cells (Yang et al.,
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43 2004). Within the nucleus it was originally identified as a non-histone DNA
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45 binding protein, which is loosely associated with chromatin and is involved in
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47 maintaining nucleosome structure, regulating gene transcription, and modulating
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49 the transcriptional activity of steroid hormone receptors (Gerlitz et al., 2009).
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51 HMGB1 is released from cells through two primary mechanisms, one involving
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53 passive release from necrotic or damaged cells and the other involving active
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4 secretion from immuno-competent cells (Bianchi and Manfredi, 2007). A number
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6 of studies have found that neurons are a primary source of HMGB1 in several
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8 neuroinflammatory conditions including ischemia, traumatic brain injury, seizure
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10 and chronic ethanol exposure (Frank et al., 2015). Of relevance here, chronic
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12 ethanol treatment has been found to induce the neuronal release of HMGB1,
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14 which mediated the pro-inflammatory effects of ethanol (Zou and Crews, 2014),
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16 in particular IL-1 β . Zhou and Crews propose that ethanol induces the active
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18 release of HMGB1 from neurons, which signals through TLR4 on immuno-
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20 competent cells to induce pro-inflammatory cytokines (Zou and Crews, 2014). It
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22 is unclear from the present results whether METH induced the active or passive
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24 release of HMGB1. A review of the neurotoxic effects of METH in rats found that
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26 a chronic dosing regimen of METH at concentrations ranging from 0.125 mg/kg
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28 to 50 mg/kg are typically required to induce neuronal damage or death (Krasnova
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30 and Cadet, 2009). However, Imam and Ali found that a single 10 mg/kg dose of
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32 METH given IP induced neuronal damage in striatum 4h post-injection (Imam
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34 and Ali, 2001). Clearly, the preponderance of evidence suggests that chronic
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36 high dose METH is typically required to induce neuronal damage/death.
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38 Therefore, the effects observed here of acute METH on HMGB1 are most likely
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40 pharmacological in nature, thereby inducing the active release of HMGB1 from
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42 neurons. Nevertheless, given that the dopamine transporter on dopaminergic
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44 neurons is the main target of METH (Wang et al., 2015), it is likely that
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46 dopaminergic neurons are the source of METH-induced HMGB1, but the mode of
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48 release remains to be clarified. The parallels between the findings here and the
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4 findings of Crews and colleagues raise the possibility that induction and release
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6 of DAMPs in the CNS may serve as a general mechanism of innate immune
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8 recognition of xenobiotics (i.e. drugs of abuse), which are thus "seen" as
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10 dangerous to the organism.
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15 Another key issue that remains is the molecular form of HMGB1 that
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17 mediates the neuroinflammatory effects of METH. Recent studies have found
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19 that the redox state of HMGB1 is a key determinant of its receptor interaction and
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21 immunological function. HMGB1 contains three critical cysteine residues (C23,
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23 C45, and C106) that are the site of post-translational modification (oxidation) to
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25 create three distinct redox forms of HMGB1, each with unique functional
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27 properties (Antoine et al., 2014; Venereau et al., 2012). A fully-reduced form and
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29 a disulfide form mediate the chemotactic and pro-inflammatory effects of
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31 HMGB1, respectively, while a fully oxidized form has no known biological activity
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33 (Venereau et al., 2012). In the present study, the redox state of HMGB1 was not
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35 characterized. However, box A has been shown to block the pro-inflammatory
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37 effects of disulfide HMGB1 (Yang et al., 2015), therefore the present results of
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39 box A blocking the neuroinflammatory effects of METH suggest that METH
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41 induced the disulfide form of HMGB1. Of relevance here, METH induces
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43 oxidative stress (Cadet and Krasnova, 2009) as part of its neurotoxic effects.
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45 Partial oxidation of HMGB1 is required to convert fully reduced HMGB1 into
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47 disulfide HMGB1 (Venereau et al., 2012). Because the redox state of HMGB1
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49 was not characterized here, it is unclear which receptor(s)(i.e. TLR4, TLR2
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51 and/or RAGE) mediated the neuroinflammatory effects of HMGB1. However, we
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4 speculate that a possible mechanism of METH-induced neuroinflammation may
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6 entail the oxidative conversion of HMGB1 into its disulfide form, which then is
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8 passively released to induce pro-inflammatory cytokines via TLR4 on microglia or
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10 other myeloid cells. Of note, the present results do not exclude the possibility that
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12 the reduced form of HMGB1 may also play a role in the neuroinflammatory
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14 effects of METH.
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20 Taken together, the present findings suggest that HMGB1 may play a
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22 pivotal role in the neuroinflammatory effects of METH and thus may be a
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24 pharmacological target to ameliorate the neuroinflammatory and neurotoxic
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26 effects of METH exposure.
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35 and SFM.
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Table 1 Legend

Abbreviations: GFAP, glial fibrillary acidic protein; IL, interleukin; Iba-1, ionized calcium-binding adaptor molecule-1; MHCII, Major histocompatibility complex II; NF- κ B α , nuclear factor kappa light chain enhancer of activated B cells inhibitor alpha; TNF α , tumor necrosis factor- α

Figure Captions

Fig. 1. Effects of METH on neuroinflammatory mediators. Rats were injected IP with vehicle (0.9% saline) or METH (10 mg/kg). At 2, 4 and 6h post-treatment, neuroinflammatory mediators were measured (mRNA) in brain reward-related structures including the NAcc, PFC, and VTA (N = 4/experimental group). Data are presented as the mean + SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the 2h vehicle control group for each respective cytokine.

Fig. 2. Effect of METH on IL-1 β protein. Rats were injected IP with vehicle (0.9% saline) or METH (10 mg/kg). At 4h post-treatment, IL-1 β protein was measured in brain reward-related structures including the NAcc, PFC, and VTA (N = 5/experimental group). Data are presented as the mean + SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the vehicle control group for each respective brain region.

Fig. 3. *In vitro* effects of METH on microglia. Whole brain primary microglia were isolated from naïve animals and exposed to varying concentrations of METH (0, 0.1, 1, 10, 100 and 1000 μ M) for 24h and pro-inflammatory mediators (mRNA and protein) measured. Data are presented as the mean + SEM and represent 4 independent replications. * $p < 0.05$ and *** $p < 0.001$ compared to the media (0 μ M) control group for each dependent measure.

Fig. 4. Effect of METH on striatal HMGB1 and IL-1 β protein levels. Rats were injected IP with vehicle (0.9% pyrogen-free saline) or METH (10 mg/kg). 4h post-treatment, protein levels of HMGB1 (**A**: Top panel, relative quantitation of

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4 HMGB1 to β -actin; bottom panel, representative Western blot of HMGB1 and β -
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6
7 actin) and IL-1 β (**B**) were measured in whole striatum (N = 4/experimental
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9 group). Data are presented as the mean + SEM. ** p < 0.01, and *** p < 0.001
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11 compared to the vehicle control group.
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15 **Fig. 5. Effect of the HMGB1 antagonist box A on the neuroinflammatory**
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17 **effects of METH.** Rats were injected ICM with vehicle (5 μ l pyrogen-free sterile
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19 water) or box A (10 μ g). 24h post-ICM injection, animals were injected IP with
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21 vehicle (0.9% pyrogen-free saline) or METH (10 mg/kg). 4h post-IP injection,
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23 neuroinflammatory mediators were measured (IL-1 β mRNA) in NAcc, PFC, and
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25 VTA (N = 5-6/experimental group). Data are presented as the mean + SEM. * p <
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27 0.05, ** p < 0.01, and *** p < 0.001 compared to the vehicle/METH treatment
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29 group for each respective brain region.
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Table 1. Primer Description and Sequence

Gene	Primer Sequence 5' → 3'	Function
β-Actin	F: TTCCTTCCTGGGTATGGAAT R: GAGGAGCAATGATCTTGATC	Cytoskeletal protein (Housekeeping gene)
CD163	F: GTAGTAGTCATTCAACCCTCAC R: CGGCTTACAGTTTCCTCAAG	Macrophage antigen not expressed by microglia
GFAP	F: AGATCCGAGAAACCAGCCTG R: CCTTAATGACCTCGCCATCC	Astrocyte antigen
IL-1β	F: CCTTGTGCAAGTGTCTGAAG R: GGGCTTGGAAGCAATCCTTA	Pro-inflammatory cytokine
IL-6	F: AGAAAAGAGTTGTGCAATGGCA R: GGCAAATTTCTGTTATATCC	Pro-inflammatory cytokine
Iba-1	F: GGCAATGGAGATATCGATAT R: AGAATCATTCTCAAGATGGC	Microglia/Macrophage antigen
MHCII	F: AGCACTGGGAGTTTGAAGAG R: AAGCCATCACCTCCTGGTAT	Microglia/Macrophage antigen
NF-κBα	F: CACCAACTACAACGGCCACA R: GCTCCTGAGCGTTGACATCA	Induced by NFκB to inhibit NFκB function
TNFα	F: CAAGGAGGAGAAGTTCCCA R: TTGGTGGTTTGCTACGACG	Pro-inflammatory cytokine

Figure 1 NAcc

Figure 1 PFC

Figure 1 VTA

Figure 2

Figure 3

Figure 4A

Figure 4B

Figure 5