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14. ABSTRACT Drug abuse and misuse is a major health hazard in the military as well as in the population more generally. There have been major recent advances in our understanding of the alterations in the brain produced by drugs of abuse, and in how the "addicted brain" differs from the normal brain. However, many individuals who experience, or are exposed to a drug of abuse do not develop addiction, or abuse the drug. For example, the overwhelming majority of patients that receive opiates for pain relief while hospitalized do not develop opiate addiction. That is, use does not always develop into abuse, and the factors that mediate this transition are largely unknown. The development of an understanding of the factors and brain mechanisms that throw the balance towards the development of abuse from use would be a major step in the development of therapies that can ameliorate addiction. The core hypothesis is that stressors, via their production of increased glucocorticoids (GCs), sensitize microglia so that these cells produce excessively high levels of inflammatory mediators such as IL-1 when acted upon by drugs of abuse, and that this process is responsible, in whole or in part, for the increased vulnerability to drug abuse produced by stressful experiences. This is a novel, and previously unexplored hypothesis. The work, if successful, could lead to a re-conceptualization of GCs as a *sensitization factor* that induces a vulnerability to neuroinflammatory processes and thereby open a new field of investigation into the role of stress and GCs in the etiology of substance abuse disorders.

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1. Introduction

Drug abuse and misuse is a major health hazard in the military as well as in the population more generally. There have been major recent advances in our understanding of the alterations in the brain produced by drugs of abuse, and in how the "addicted brain" differs from the normal brain. However, many individuals who experience, or are exposed to a drug of abuse neither develop addiction, nor abuse the drug. For example, the overwhelming majority of patients that receive opiates for pain relief while hospitalized do not develop opiate addiction. That is, use does not always develop into abuse, and the factors that mediate this transition are largely unknown. The development of an understanding of the factors and brain mechanisms that throw the balance towards the development of abuse from use would be a major step in the development of therapies that can ameliorate addiction.

Drug abuse is exacerbated by deployment in war zones, and particularly by exposure to trauma, resulting in high co-morbidity with PTSD. Although there is a wealth of human and animal data clearly demonstrating that exposure to stressful conditions potentiates drug taking, the development of addiction, and the reinstatement of extinguished drug self-administration, the mechanisms involved are poorly understood, and consequently there are few, if any, preventative or curative treatments. The hallmark of the stress response is an increase in adrenal glucocorticoids (GCs) (cortisol in the human, corticosterone in the rodent), and it is known that the GC response to stressors is involved in the facilitation of addictive processes produced by stress. Thus, for example, adrenalectomy prevents stressor-induced potentiation of drug self-administration, as well as the augmentation of drug-induced dopamine release in reward-related areas of the brain produced by prior stress. However, the mechanism(s) by which stress and GCs exaggerate behavioral and neurochemical responses to drugs of abuse are poorly understood, and so therapeutic targets have correspondingly not been identified. The goals of the present proposal are to further our understanding of how stress and/or GCs potentiate responses to drugs of abuse, and to identify therapeutic targets that would allow the blockade of stress effects on drug use and addiction.

Over the course of this project, we have made significant advances in our understanding of how stress potentiates the neuroinflammatory effects of drugs of abuse, specifically methamphetamine (METH) and identified a therapeutic target (i.e., high mobility group box-1; HMGB1), which we consider a pivotal mediator of the neuroinflammatory effects of stress and METH.

2. Key Words

drug abuse; methamphetamine; morphine; cocaine; stress; neuroinflammation; microglia; danger-associated molecular pattern; HMGB1; cytokines; interleukin.

3. Overall Project Summary

To provide a complete and clear summary of the work accomplished over the 3 year project period, we have organized the body of the final report with research results presented for each completed specific aim followed by a brief discussion of the results.

Specific Aim I. Do stress and/or GCs potentiate neuroinflammatory responses to drugs of abuse?
2a. IA. Acute stress and acute rises in GC
2a1. IA1. Stress.

This sub-aim addressed whether prior exposure to a severe acute stressor potentiates the neuroinflammatory effects of several drugs of abuse including morphine, cocaine and METH. Briefly, animals were exposed to a single session of inescapable tailshock (IS), which consists of 100, 1.0 mA, 5 s tailshocks delivered via fixed electrodes while restrained in Plexiglas tubes or animals served as home cage controls (HCC). 24 hours after stressor exposure, animals were treated with morphine (8 mg/kg ip), methamphetamine (10 mg/kg ip), cocaine (10 mg/kg ip) or vehicle (0.9% saline). As proposed in the original research design, the neuroinflammatory effects of these drugs were assessed 4 h after drug treatment in the prefrontal cortex (PFC), nucleus accumbens (NAcc) and ventral tegmental area (VTA). Transcriptional profiling of 14 genes involved in neuroinflammatory or indicative of neuroinflammatory processes were measured using real time PCR (See year 1 annual report for details).

Main Results

We found at this time-point post drug treatment that only the neuroinflammatory effects of METH were potentiated by prior stress exposure (See year 1 annual report). The neuroinflammatory effects of cocaine and morphine were not potentiated by prior stress exposure at 4 h post-drug treatment.

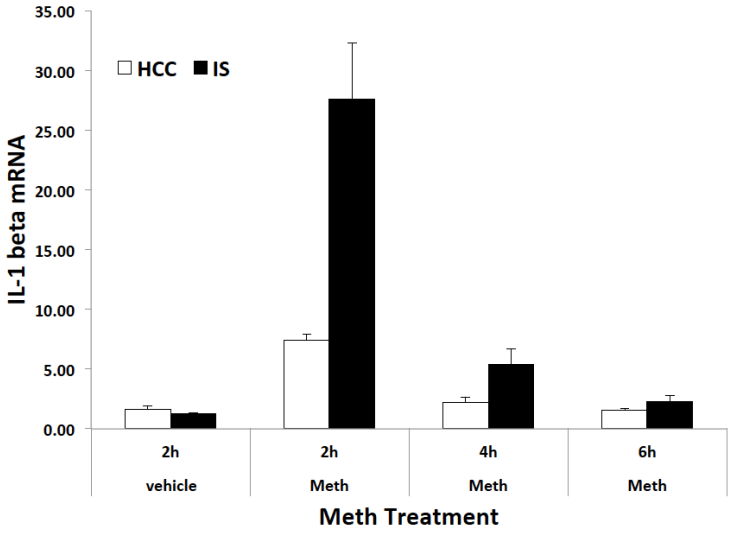

Fig. 1. Effect of prior stress on METH-induced IL-1 in NAcc

As detailed in our year 1 annual report, we raised the caveat that assessment of the neuroinflammatory effects of drugs of abuse at a single time-point post drug treatment may have precluded observing the neuroinflammatory effects of cocaine and morphine because of the highly dynamic kinetics of neuroinflammatory processes. Therefore, we changed the research strategy and measured neuroinflammatory processes at several time-points (2 and 6 h) after drug treatment. Inclusion of additional

time-points post-drug exposure revealed that stress also potentiated the neuroinflammatory effects of morphine, but the neuroinflammatory effects of cocaine were not potentiated by prior stress exposure. It should be noted that these studies generated an enormous amount of valuable data, which provided a transcriptional profile of 14 neuroinflammatory-related genes as a function of stress condition (HCC vs IS), drug treatment (vehicle, cocaine, morphine and METH), time post-drug treatment (2h, 4h and 6h) and brain region (PFC, NAcc and VTA). Further, we expanded the time-course for cocaine to include a 30 min time-point post-drug exposure to determine whether the neuroinflammatory effects of cocaine occur rapidly. As noted in our year 1 annual report, cocaine, morphine and METH induced neuroinflammatory effects independent of stress exposure and these effects varied considerably as a function of brain region and time post-drug exposure. Particularly noteworthy were the effects of prior stress exposure on METH induction of IL-1β. Prior stress resulted in a profound potentiation in METH induction of IL-1β mRNA, particularly in the NAcc (Fig. 1) and PFC (Fig. 2). As shown in Fig. 3, the effects of prior stress on METH-induced IL-1β were of lesser magnitude in the VTA. As a pro-inflammatory cytokine, IL-1β is of particular interest because the formation and secretion of IL-1β is a critical step in the neuroinflammatory cascade and IL-1β has been termed the “master regulator" of neuroinflammation (Basu

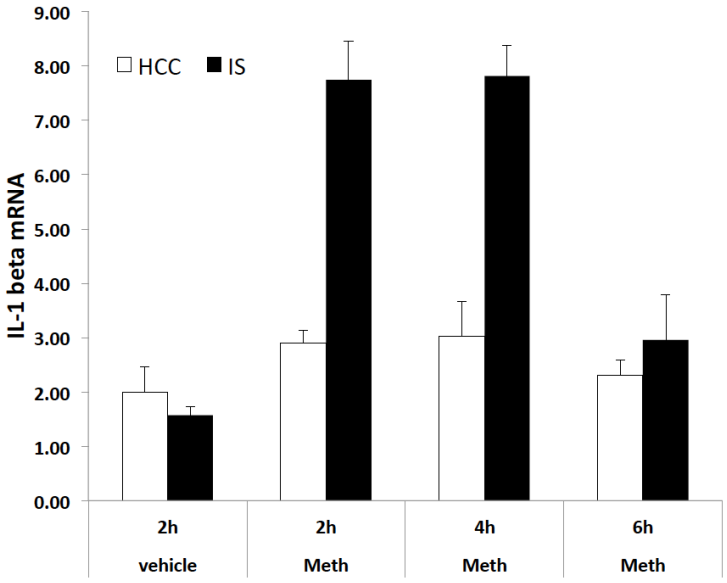

Fig. 2. Effect of prior stress on METH-induced IL-1 in PFC

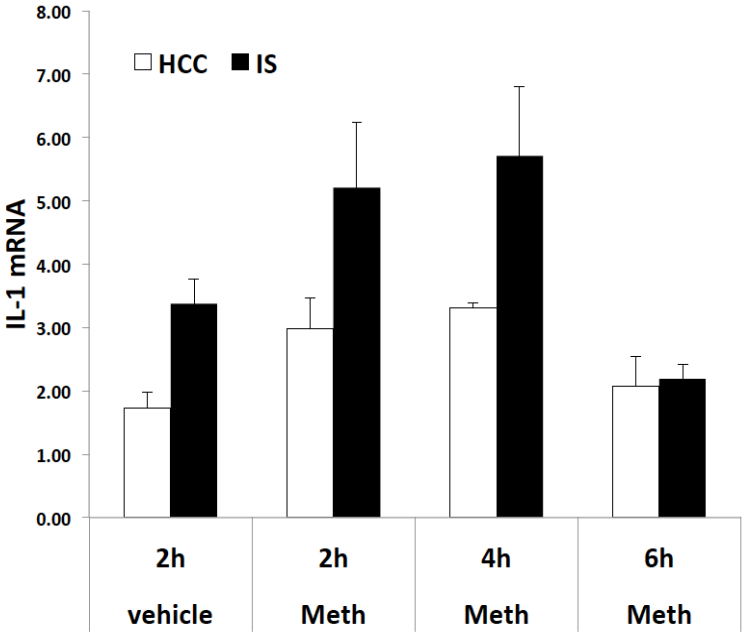

Fig. 3. Effect of prior stress on METH-induced IL-1 in VTA

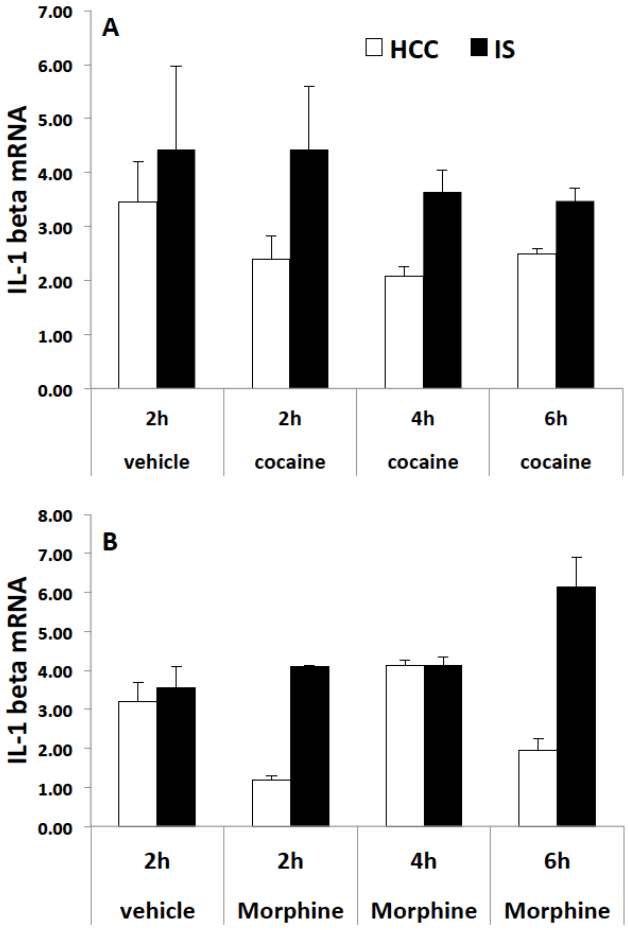

Fig. 4. Effect of prior stress on cocaine (A) and morphine (B)-induced IL-1 in NAcc

et al., 2004), given its pleiotropic role in the induction of central inflammatory processes as well as its critical role in the manifestation of the sickness response (Goshen and Yirmiya, 2009). Thus, the induction, processing and signaling of IL-1β is under tight regulation (Dinarello, 2011). IL-1β is transcribed as a larger pro-hormone, pro-IL-1β, and pro-IL-1β must be cleaved by caspase-1 to form the biologically active, mature form of IL-1β. As mentioned, IL-1β is a key mediator of the sickness response, which includes cognitive (memory), affective (mood), vegetative (sleep and eating), sensory (pain) and physiological (fever) changes, which play an adaptive role in an organism's host defense against infection, trauma, and injury (Dantzer, 2009). Of note, there is a remarkable similarity between the hallmarks of the sickness response and clinical endophenotypes typically observed in several psychiatric disorders including major depression and anxiety disorders. For example, anhedonia is a symptom typically observed in major depression and is also a hallmark of the sickness response to infection or injury (Dantzer et al., 2008). Indeed, a considerable number of findings suggest that inflammatory processes may play a role in the etiology of major depression (Raison and Miller, 2013). Therefore, prior stress exposure may potentiate the sickness response to METH and thus predispose individuals to developing psychiatric disorders such as major depression.

These effects of stress on IL-1β appear to be unique to METH as we did not observe similar effects with cocaine or morphine treatment after stress exposure. Given the large amount of data collected, we present here representative data demonstrating the relatively minor effects of prior stress exposure on cocaine and morphine-induce IL-1β mRNA in the NAcc (Fig. 4). Clearly, stress failed to significantly potentiate the IL-1β response to cocaine and morphine in NAcc. It should be noted that cocaine and morphine were capable of inducing neuroinflammatory responses independent of stress (see year 1 annual report), but these responses were by and large not potentiated by prior stress exposure.

Discussion

Prior stress exposure clearly primed the neuroinflammatory response to METH, which raised a key question with regard to mechanism. Specifically, did prior stress exposure prime microglia to METH? We have found that prior stress exposure sensitizes microglia to pro-inflammatory stimuli *ex vivo* (Frank et al., 2007). Briefly, the general phenomenon involves exposing rats to our standard stress protocol (described above). 24h after stress exposure, microglia are isolated from discrete brain region using a Percoll density gradient as previously described in a prior publication from our laboratory (Frank et al., 2006). Microglia are then directly exposed to a pro-inflammatory agent such as lipopolysaccharide (LPS), which is a component of the cell wall of gram-negative bacteria. LPS signals through pattern recognition receptors (toll-like receptor 4; TLR4) on microglia to induce the NF-kB signal transduction pathway and transcription of pro-inflammatory cytokines including IL-1β. We have found that prior stress exposure potentiates the pro-inflammatory cytokine response to LPS in isolated hippocampal microglia (Frank et al., 2007) suggesting that prior stress exposure primes microglia to pro-inflammatory stimuli. In light of these prior findings, we addressed the question of whether prior stress exposure would potentiate the pro-inflammatory response of isolated microglia to METH, which led us to focus on specific aim IIA1.

Specific Aim II. Do microglia mediate the effects of stress and glucocorticoids on neuroinflammatory responses to drugs of abuse?
IIA. Microglial sensitization.
IIA1. Basic effect

As detailed in our year 2 annual report, animals were exposed to our standard stress protocol or served as HCC. 24 h post-stress exposure, microglia were isolated from whole striatum, which encompasses the NAcc. Microglia were isolated and exposed to METH at several concentrations and pro-inflammatory mediators measured. This *ex vivo* study was restricted to studying METH because 1) stress largely only potentiated the neuroinflammatory response to METH and 2) data from specific aim IV (see below) demonstrated that prior stress potentiated only the behavioral effects of METH and not the effects of morphine.

Main Results

Unexpectedly, we found that 24h after stress exposure, isolated striatal microglia from both stressed and HCC animals failed to respond to direct METH treatment (see year 2 annual report). Most notable about these findings is that METH independent of stress condition failed to induce a pro-inflammatory response. Several replications of this finding were conducted first in striatal microglia and then in whole brain microglia. We recently published this data as part of a larger dataset demonstrating that the neuroinflammatory effects of METH are mediated by the danger-associated molecular pattern HMGB1 (Frank et al., 2015a; attached in Appendix 1). We address this study in detail below.

In Fig. 5, we present data from whole brain microglia from naive animals exposed to METH for 24h, and pro-inflammatory mediators as well as cell viability were measured. Interestingly, METH failed to induce a pro-inflammatory response, rather

METH treatment resulted in a decrease in IL-1β protein as well as cell viability. It should be emphasized that microglia were exposed to METH for 24h to ensure that the pro-inflammatory effects of METH could be observed. Clearly, the results presented in Fig. 5 demonstrate that under these *in vitro* conditions METH failed to induce a pro-inflammatory response in microglia.

Discussion

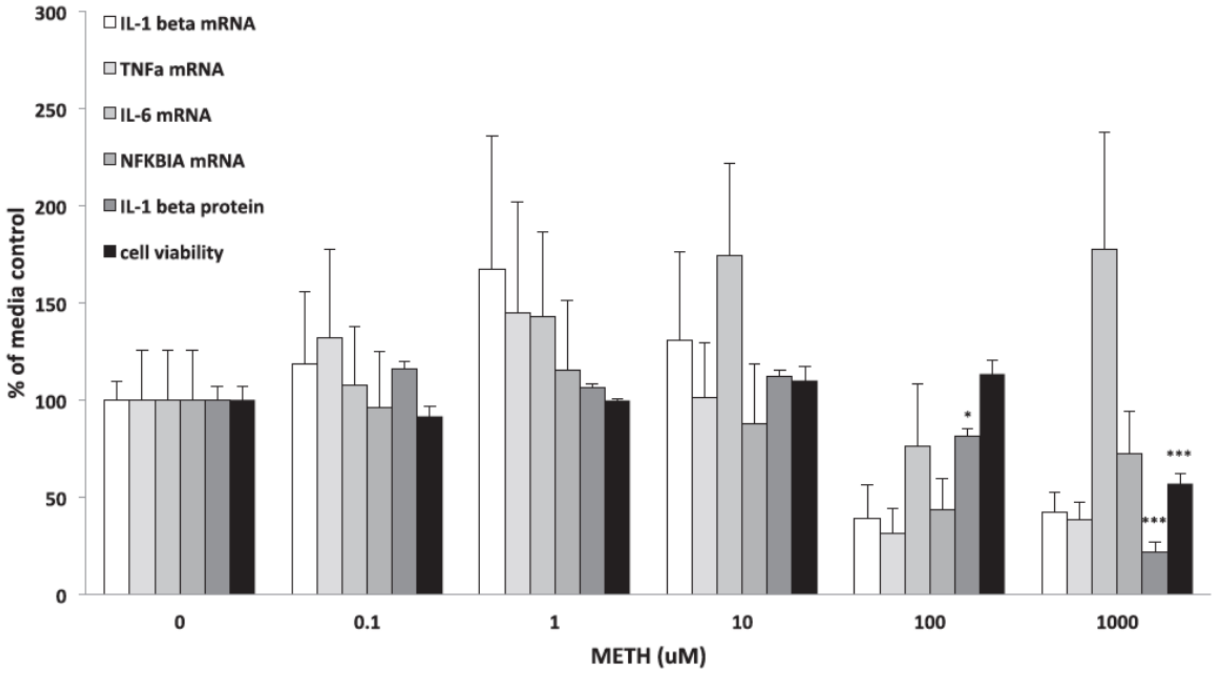

Fig. 5. Effect of METH on whole brain microglia

These perplexing findings prompted us to address the basic mechanistic question of how METH induces a neuroinflammatory response. It is important to note here that the neuroinflammatory mechanism(s) of METH action in the CNS was largely unknown when we conducted these experiments. The data presented in Fig. 5 suggests that the neuroinflammatory effects of METH are not due to direct effect on microglia, but rather through an indirect effect on microglia.

A considerable number of findings show that METH is neurotoxic, particularly at high concentrations, resulting in neuronal damage and death (Krasnova & Cadet, 2009). In addition, METH induces an array of toxic mediators such as reactive oxygen species, which can be pro-inflammatory. In light of the neurotoxic effects of METH, we explored the possibility that the neuroinflammatory effects of METH may be due to METH induced release of danger-associated molecular patterns (DAMPs) from damaged or dying neuronal cells. Our thinking was that these neuronal DAMPs may then target microglia and induce a neuroinflammatory response.

Our interest centered on the DAMP HMGB-1, which stemmed from our initial finding that pharmacologic blockade of TLR2 and TLR4 during stress exposure abrogates stress-induced priming of the neuroinflammatory response to a subsequent immune challenge (Weber et al., 2013). Please note that later in this report, we address the role of HMGB-1 in stress-induced priming of the neuroinflammatory response. Briefly, HMGB-1 is a ubiquitous nuclear DNA binding protein, which under normal conditions, is not present in the extracellular space (Kang et al., 2014). The primary structure of HMGB1 is comprised of two DNA binding domains, an A box and B box, and a negatively charged acidic tail (C-terminus)(Yang and Tracey, 2005). The B box domain contains the cytokine-inducing region of HMGB1, whereas the A box binds to receptor targets, but does not initiate receptor signaling (Li et al., 2003). Interestingly, the A box fragment functions to competitively antagonize the pro-inflammatory effects of HMGB1 since it binds to receptors without initiating signaling (Yang et al., 2004). It should be noted that the A Box fragment has not been detected *in vivo*, and so it is unclear whether the antagonistic properties of the A box domain play a physiological role in the regulation of HMGB1 signaling. The A box fragment is commercially available (HMGBiotech, Milan, Italy) and has been used to block the epileptogenic effects of HMGB1 in the CNS (Maroso et al., 2010).

Under necrotic conditions, HMGB-1 is passively released into the extracellular milieu to serve as an inflammatory signal (Scaffidi et al., 2002). The pattern recognition receptors TLR2 and TLR4 mediate the pro-inflammatory effects of HMGB-1, although some recent studies now suggest that the pro-inflammatory effects of HMGB-1 are mediated predominately through TLR4 (Yang et al., 2013). The receptor for advanced glycation end products (RAGE) and the chemokine receptor CXCR4 are thought to mediate the chemotactic function of HMGB-1 (Yang et al., 2013). Interestingly, immunocompetent cells also have the unique ability to actively release HMGB-1 in the absence of cell death (Bonaldi et al., 2003).

There are three distinct forms of HMGB-1 characterized by post-transcriptional modification of the redox state of three critical cysteine residues (C23, C45, and C106). Fully reduced (fr) HMGB-1 is the predominant form that occurs under non-oxidizing conditions. fr-HMGB-1 has chemotactic properties produced by the formation of a hetero-complex with the chemokine CXCL12, which then signals through the chemokine receptor CXCR4 (Schiraldi et al., 2012). The pro-inflammatory form occurs under increased oxidizing conditions, in which a disulfide bridge forms between C23 and C45 (disulfide (ds) HMGB-1). This redox form interacts with TLR4 to induce synthesis and secretion of pro-inflammatory cytokines (Antoine et al., 2014). Finally, the fully oxidized form of HMGB-1 has no known biological activity (Venereau et al., 2012; Yang et al., 2015).

Given the different immunological properties of these redox forms, we conducted a set of studies characterizing the neuroinflammatory, priming and behavioral effects of ds-HMGB-1 and fr-HMGB1. Importantly, the neuroinflammatory effects of these redox forms were completely unknown when we conducted these studies. We pursued these studies to test whether these forms are sufficient to induce neuroinflammatory processes and in doing so provide insight into the mechanisms of stress-induced neuroinflammatory priming as well as METH-induced neuroinflammatory processes. Our thinking here is that HMGB-1 is the proximal signal mediating stress-induced

priming of microglia as well as the signal METH induction of neuroinflammation. In our revised SOW, we proposed to address HMGB-1 as a mediator of stress-induced priming of the neuroinflammatory response to METH and thus these studies of the redox forms of HMGB1 directly related to aim **2a2**. **IA2**. Instead of addressing whether glucocorticoids (GCs) are sufficient to prime the neuroinflammatory response to METH, we addressed the question of whether HMGB-1 is sufficient to prime the neuroinflammatory response.

Specific Aim I. Do stress and/or GCs potentiate neuroinflammatory responses to drugs of abuse?
2a2. **IA2**. **HMGB-1**

In our initial studies, animals were administered intra-cisterna magna (ICM) a single dose of fr-HMGB1 (10 µg), ds-HMGB1 (10 µg) or vehicle and basal pro-inflammatory effects were measured 2h and 24h post-injection in hippocampus (Fig. 6). To assess the neuroinflammatory priming effects of these molecular forms, animals were administered ICM a single dose of fr-HMGB1 (10 µg), ds-HMGB1 (10 µg) or vehicle and 24h after injection, animals were challenged with LPS (10 µg/kg IP) or vehicle. Neuroinflammatory mediators (Fig. 7) were measured 2h after immune challenge. It should be noted that we used LPS in these initial studies to ensure a robust pro-inflammatory response and thus provide a strong test of the priming effects of HMGB-1. We recently published the findings from this study (Frank et al., 2015b; attached in Appendix 1).

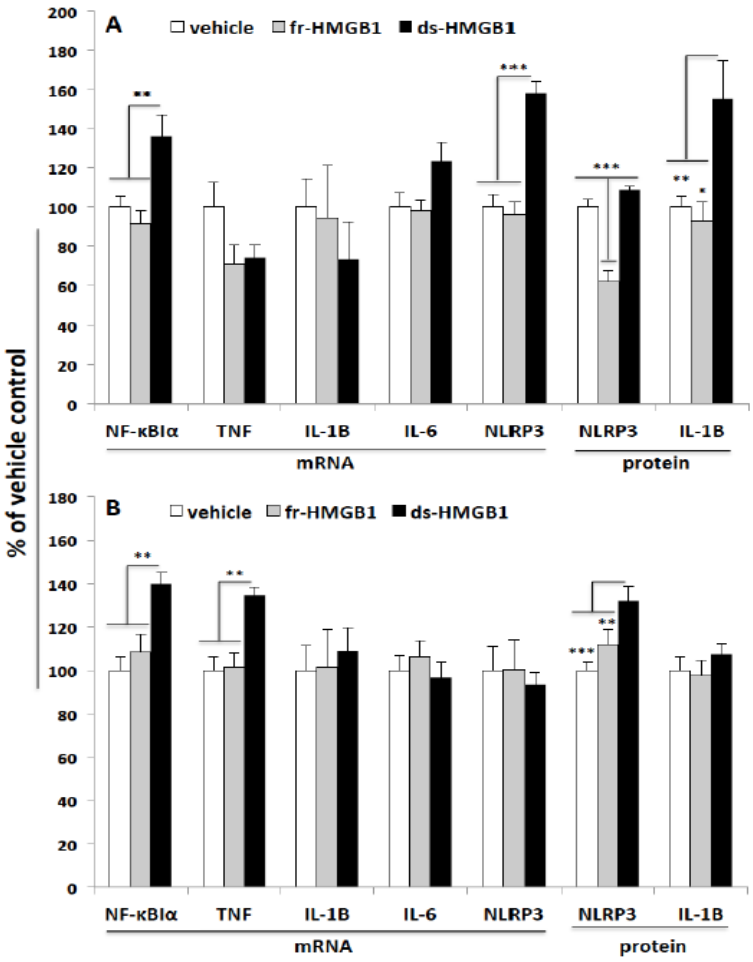

Fig. 6. The effects of fr-HMGB1 and ds-HMGB1 on hippocampal neuroinflammation.

Main Results

We present some of the main findings here from our recent publication. Fig. 6 clearly demonstrates that ds-HMGB1 is sufficient to induce neuroinflammatory processes, while fr-HMGB1 failed to significantly alter expression of these analytes. At 2h post-ds-HMGB1 treatment, NF-kBIA mRNA, NLRP3 mRNA and

IL-1β protein were increased. At 24h post-ds-HMGB1 treatment, NF-kBIA mRNA, TNFa mRNA and NLRP3 protein were increased. These results suggest that the disulfide redox form of HMGB1 is sufficient to induce a neuroinflammatory response. A subsequent study was performed to determine whether ds-HMGB1 is capable of priming the neuroinflammatory response to a pro-inflammatory challenge. In Fig. 7, data is presented demonstrating that ds-HMGB1 potentiates the neuroinflammatory response to the pro-inflammatory stimulus LPS. We found that fr-HMGB1 failed to potentiate the neuroinflammatory response to LPS.

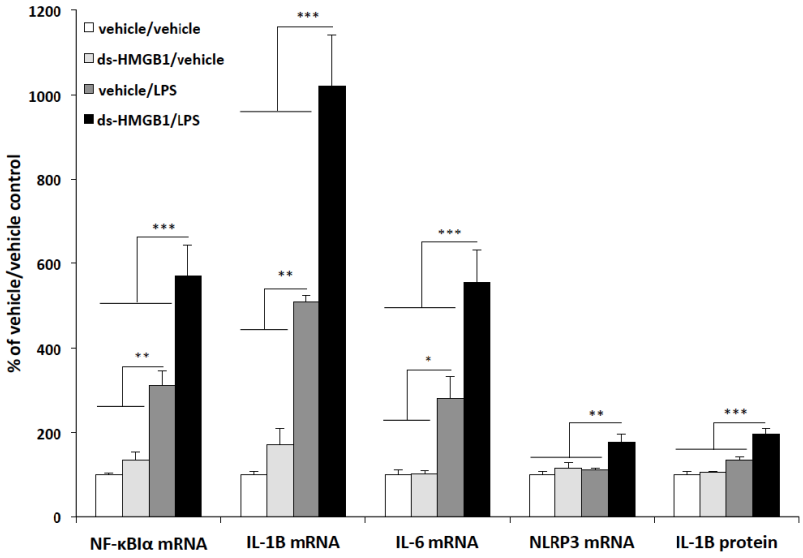

Fig. 7. ds-HMGB1 potentiates the neuroinflammatory response to LPS

Discussion

These data suggest that ds-HMGB1 primes the neuroinflammatory response to pro-inflammatory stimuli and provided valuable insight into the role of the redox state of HMGB1 in neuroinflammatory processes. These findings suggested to us that ds-HMGB1 could play a pivotal role in stress-induced priming of the neuroinflammatory response to METH as well as the neuroinflammatory effects of METH. In parallel with these studies of the redox state of HMGB1, we conducted a set of studies directly relevant to specific aim 2, in which we examined the mechanisms of stress-induced priming of the neuroinflammatory response to a proinflammatory challenge.

Specific Aim I. Do stress and/or GCs potentiate neuroinflammatory responses to drugs of abuse?
2c1. IC1. Acute Stress.

In this set of studies, we addressed the mediating role of HMGB1 in stress-induced neuroinflammatory priming. An extensive set of studies was conducted, of which we present the most relevant findings here. It should be noted that the full set studies has been recently published (Weber et al., 2015; attached in Appendix 1) and was the first reported finding of a DAMP playing a role in stress-induced neuroinflammatory priming. Briefly, animals were exposed to IS and HMGB1 protein was measured in hippocampus immediately and 24 h after stress (Fig. 8). To assess the mediating role of HMGB1 in stress-induced neuroinflammatory priming, animals were administered an HMGB1 antagonist (Box A, 10 ug) or vehicle ICM to HCC animals or immediately prior to IS. 24 h after stress exposure, hippocampal microglia were

isolated and challenged with LPS to assess the effect of Box A on stress-induced microglial priming (Fig. 9).

Main Results

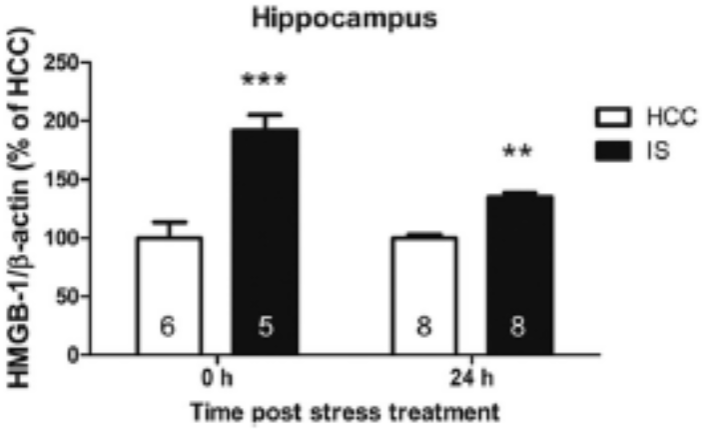

Fig. 8. Effect of stress on HMGB1 protein in hippocampus

Exposure to stress increased protein levels of HMGB1 in hippocampus at 0h and 24h after termination of the stressor (Fig. 8). It is important to clarify that the redox state of HMGB1 could not be determined in this experiment because the monoclonal antibody used to detect HMGB1 using Western blot does not distinguish between the redox states of HMGB1. Although not presented here, but detailed in Weber et al. (2015), we found that prior stress exposure induced the release of HMGB1 from hippocampal microglia. In light of these findings, we addressed the mediating role of HMGB1 in stress-induced neuroinflammatory priming. In Fig. 9, the IL-1 mRNA response to LPS in hippocampal microglia is presented. Consistent with our prior studies (Frank et al., 2007), we found that prior exposure to IS potentiated the IL-1 response to LPS in isolated microglia (black bar, 100 ng/ml LPS) compared to the IL-1 response in HCC animals (white bar, 100 ng/ml LPS). When Box A was administered prior to IS, IS failed to potentiate the IL-1 response to LPS (gray bar, 100 ng/ml LPS) compared to the IL-1 response in HCC animals (white bar). It should be noted that a similar pattern of effects was observed for NF-kBIA and NLRP3 mRNA. In addition, we reported in Weber et al. (2015) that ICM administration of the disulfide form of HMGB1 potentiated the microglial pro-inflammatory response to LPS.

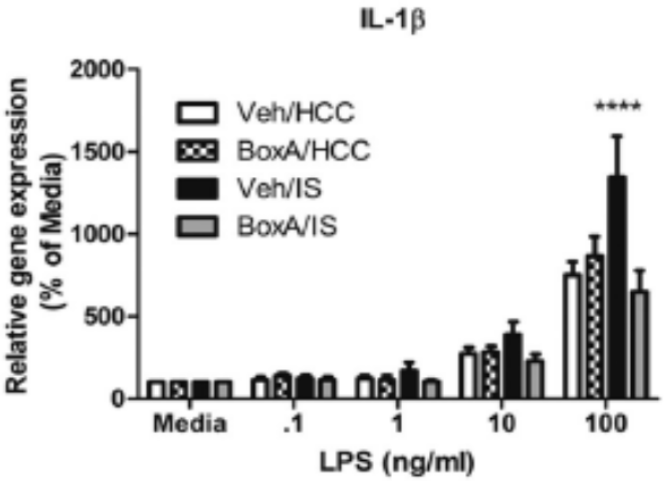

Fig. 9. Effect of Box A on stress-induced priming of hippocampal microglia

Discussion

The finding presented in Fig. 8 suggests that exposure to stress increases HMGB1 levels in the hippocampus. Further, the effect of Box A on stress-induced microglial priming (Fig. 9) suggests that HMGB1 mediated these priming effects of stress. These results dovetail with the results presented in Fig. 7 to suggest that the disulfide form of HMGB1 is

most likely the redox form that mediates stress-induced neuroinflammatory priming.

Taken together, the results presented thus far in this summary raise 2 key questions. First, does HMGB1 mediate the neuroinflammatory effects of METH? Second, does HMGB1 mediate the stress-induced priming of the neuroinflammatory response to METH. Indeed, the data presented thus far suggested to us that HMGB1 mediates both the process of METH induction of neuroinflammation independent of stress as well as neuroinflammatory priming. A key methodological issue in testing these questions is being able to distinguish the priming effects of HMGB1 from the direct neuroinflammatory effect of HMGB1. Thus, we proceeded with conducting experiments to answer the first question of whether HMGB1 mediates the neuroinflammatory effects of METH independent of stress.

Specific Aim I. Do stress and/or GCs potentiate neuroinflammatory responses to drugs of abuse?
2c1. IC1. Acute Stress.

We conducted an extensive set of experiments, which addressed whether HMGB1 mediates the neuroinflammatory effects of METH. These experiments were directly relevant to Specific Aim 1 because the results would provide insight into the feasibility of experimentally testing whether HMGB1 mediates the stress-induced priming of the neuroinflammatory response to METH. The full set of experiments is detailed in Frank et al. (2015a), of which we present the key findings here. Briefly, to confirm that METH induces a neuroinflammatory response, animals were injected IP with METH (10 mg/kg) or vehicle (0.9% saline) and IL-1 protein was measured in NAcc, VTA and PFC (Fig. 10). To test the mediating role of HMGB1 in the neuroinflammatory effects of METH, animals were injected ICM 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 (Fig. 11).

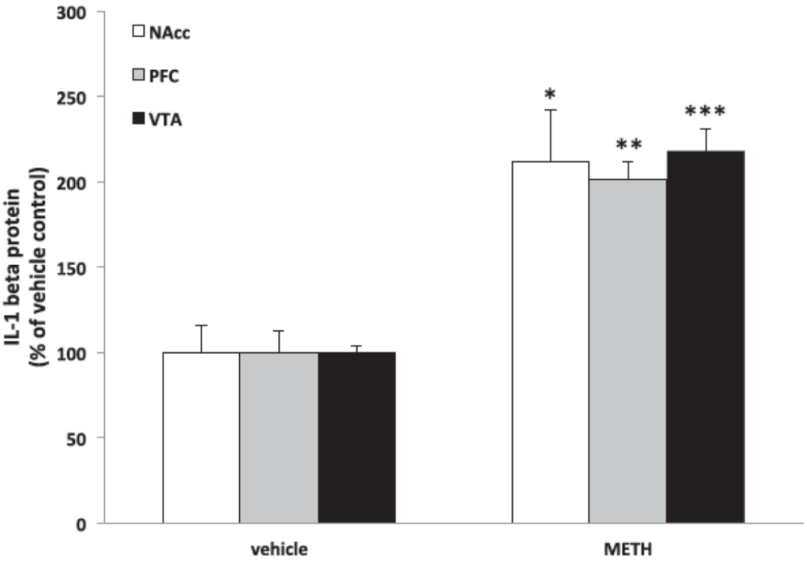

Fig. 10. Effect of METH on IL-1 beta in NAcc, PFC and VTA.

Main Results

Consistent with the results presented in Fig. 1-3 as well as 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), we found that METH treatment induced a robust IL-1β protein response in NAcc, PFC

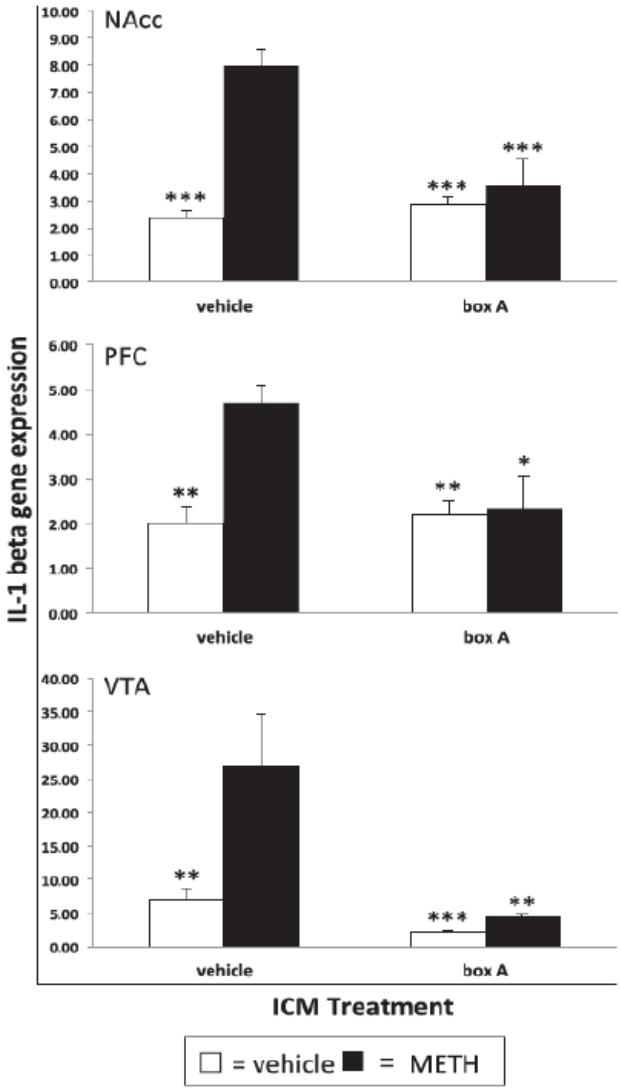

Fig. 11. Effect of HMGB1 antagonist Box A on the neuroinflammatory effects of METH

and VTA (Fig. 10). As noted in our discussion of data presented in Fig. 5, this neuroinflammatory effect of METH is most likely not due to a direct effect on microglia. Rather, we considered the distinct possibility that METH, via its neurotoxic effects, induces the release of DAMPs such as HMGB1 from damaged neurons, which then targets pattern recognition receptors (i.e. TLR4) on microglia to induce a neuroinflammatory response. Indeed, as shown in Fig. 11, pretreatment with the HMGB1 antagonist Box A completely abrogated the METH induction of IL-1β mRNA in NAcc, PFC and VTA.

Discussion

The finding presented in Fig. 11 suggests that HMGB1 is a pivotal mediator of the neuroinflammatory effects of METH. Of note, this finding is one of the first demonstrations of a mechanism by which METH induces a neuroinflammatory response and represents a key milestone of this project. METH can induce profound neurotoxic and neuroinflammatory effects depending largely on METH dosage. Importantly, METH-induced neuroinflammatory mediators are also neurotoxic, which then may lead to an amplification of neuronal death and damage, and release of DAMPS, thereby establishing a positive feedback loop between the neurotoxic and neuroinflammatory effects of METH. The present findings suggest that HMGB1 is a proximal mediator of the neuroinflammatory effects of METH and thus HMGB1 may be a therapeutic target to attenuate or block the induction of the neurotoxic/neuroinflammatory cascade as a consequence of METH exposure.

Specific Aim III. Do microglial activation and consequent neuroinflammatory responses mediate the effects of stress and glucocorticoids on drug abuse behavior?

IIIA. Progressive ratio (PR) responding.

In our year 2 progress report we detail the effects of prior stress exposure on drug abuse behavior. Here, we highlight the main findings of stress-induced potentiation of METH self-administration. Briefly, Rats were exposed to IS or served as non-stressed controls. Approximately 1 week post-stress, rats were trained to self-administer METH (0.1 mg/kg/infusion) in daily 2-h sessions, first on fixed ratio (FR)1 and then switched to FR5. Once stable self-administration behavior was maintained, rats self-administered 0.01, 0.03, 0.05 and 0.1 mg/kg/infusion (2 sessions at each dose; doses were counterbalanced).

Main Results

Fig. 12 shows that stressed and non-stressed rats both acquired self-administration of METH, and stress increased METH self-administration, particularly at the 0.03 mg/kg/inf dose (significant at 0.03; trend towards significance at 0.05).

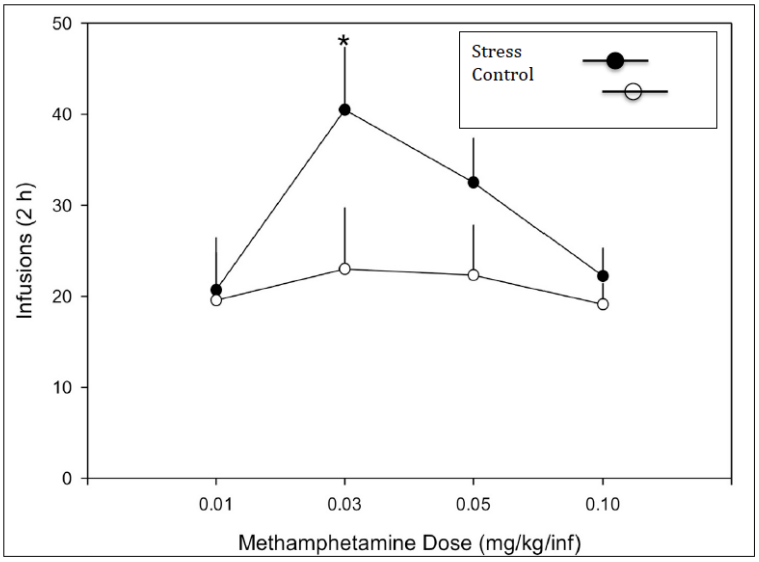

Fig. 12. Effect of prior stress on METH self-administration

Discussion

Data presented in Fig. 12 suggests that prior exposure to stress potentiates drug abuse behavior. The findings presented above clearly indicate that stress potentiates the neuroinflammatory effects of METH, which are mediated by the DAMP HMGB1. The results presented in Fig. 12 raises the interesting question of whether the neuroinflammatory effects of METH play a role in drug abuse behavior. Although, we did not address this question experimentally, there are a number of findings suggesting that pro-inflammatory mediators may indeed play this role (Frank et al., 2011).

4. Key Research Accomplishments

The DAMP HMGB1 was found to mediate the stress-induced priming of neuroinflammatory processes as well as the neuroinflammatory effects of METH. Of note, these findings are unique and establish for the first time the importance of DAMPs

playing a causal role in the neuroinflammatory effects of stress and drugs of abuse. From a clinical perspective, this key outcome of this project identifies a novel target, HMGB1, to attenuate the neuroinflammatory effects of drugs of abuse.

5. Conclusion

Drug abuse is exacerbated by deployment in war zones, and particularly by exposure to trauma, resulting in high co-morbidity with PTSD. The goals of the present proposal were to further our understanding of how stress potentiates responses to drugs of abuse, and to identify therapeutic targets that would allow the blockade of stress effects on drug use and addiction. Indeed, the results of the present project have identified a therapeutic target, HMGB1, which could be targeted in military personnel to ameliorate the neuroinflammatory effects of war trauma as well as drugs of abuse. Though this project has been completed, our future plans are clearly centered around understanding how HMGB1 mediates stress-induced priming of neuroinflammatory processes. In particular, we are pursuing the novel hypothesis that stress-induced glucocorticoids induce the release of HMGB1 within CNS. These studies are ongoing.

6. Publications, Abstracts and Presentations

1. Lay Press: N/A

2. Peer-Reviewed Scientific Journals

Frank MG, Adhikary S, Sobesky JL, Weber MD, Watkins LR, Maier SF. The danger-associated molecular pattern HMGB1 mediates the neuroinflammatory effects of methamphetamine. Brain Behav Immun 2015 (Epub ahead of print). DOI: 10.1016/j.bbi.2015.08.001.

Frank MG, Weber MD, Fonken LK, Hershman SA, Watkins LR, Maier SF. The redox state of the alarmin HMGB1 is a pivotal factor in neuroinflammatory and microglial priming: A role for the NLRP3 inflammasome. Brain Behav Immun 2015 (Epub ahead of print). DOI: 10.1016/j.bbi.2015.10.009.

Weber MD, Frank MG, Tracey KJ, Watkins LR, Maier SF. Stress Induces the Danger-Associated Molecular Pattern HMGB-1 in the Hippocampus of Male Sprague Dawley Rats: A Priming Stimulus of Microglia and the NLRP3 Inflammasome. J Neurosci 2015; 35: 316-324. 10.1523/JNEUROSCI.3561-14.2015.

3. Invited Articles: N/A

4. Abstracts:

M. G. FRANK[1], S. A. HERSHMAN[2], M. D. WEBER[2], L. R. WATKINS[2], S. F. MAIER[2]. The endogenous danger signal high mobility group box-1 (HMGB1) sensitizes microglia

to pro-inflammatory stimuli: in vitro characterization of the disulfide and fully reduced forms. Society for Neuroscience, 2014.

M. G. Frank, S. Adhikary, J. L. Sobesky, M. D. Weber, L. R. Watkins, S. F. Maier. The danger-associated molecular pattern HMGB1 mediates the neuroinflammatory effects of methamphetamine. Society for Neuroscience, 2014.

7. Inventions, Patents and Licenses

Nothing to report.

8. Reportable Outcomes

Nothing to report.

9. Other Achievements

Nothing to report.

10. List of Personnel Receiving Pay

Matthew G. Frank, PhD
Jasmine Yap, PhD
Julia Sobesky, PhD

11. References

Antoine, D.J., Harris, H.E., Andersson, U., Tracey, K.J., Bianchi, M.E., 2014. A systematic nomenclature for the redox states of high mobility group box (HMGB) proteins. Mol Med 20, 135-137.

Basu A, Krady JK, Levison SW. Interleukin-1: a master regulator of neuroinflammation. J Neurosci Res 2004;78:151-156.

Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, et al. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. The EMBO journal 2003;22:5551-5560.

Coutinho A, Flynn C, Burdo TH, Mervis RF, Fox HS. Chronic methamphetamine induces structural changes in frontal cortex neurons and upregulates type I interferons. J Neuroimmune Pharmacol 2008;3:241-245.

Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 2008;9:46-56.

Dantzer R. Cytokine, sickness behavior, and depression. Immunol Allergy Clin North Am 2009;29:247-264.

Dinarello CA. Interleukin-1. Cytokine Growth Factor Rev 1997;8:253-265.

Flora G, Lee YW, Nath A, Hennig B, Maragos W, Toborek M. Methamphetamine potentiates HIV-1 Tat protein-mediated activation of redox-sensitive pathways in discrete regions of the brain. Exp Neurol 2003;179:60-70.

Flora G, Lee YW, Nath A, Maragos W, Hennig B, Toborek M. Methamphetamine-induced TNF-alpha gene expression and activation of AP-1 in discrete regions of mouse brain: potential role of reactive oxygen intermediates and lipid peroxidation. Neuromolecular Med 2002;2:71-85.

Frank MG, Wieseler-Frank JL, Watkins LR, Maier SF. Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: immunophenotypic and functional characteristics. J Neurosci Methods 2006;151:121-130.

Frank MG, Baratta MV, Sprunger DB, Watkins LR, Maier SF. Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. Brain Behav Immun 2007;21:47-59.

Frank MG, Watkins LR, Maier SF. Stress- and glucocorticoid-induced priming of neuroinflammatory responses: potential mechanisms of stress-induced vulnerability to drugs of abuse. Brain, behavior, and immunity 2011;25 Suppl 1:S21-28.

Frank MG, Adhikary S, Sobesky JL, Weber MD, Watkins LR, Maier SF. The danger-associated molecular pattern HMGB1 mediates the neuroinflammatory effects of methamphetamine. Brain Behav Immun 2015a.

Frank MG, Weber MD, Fonken LK, Hershman SA, Watkins LR, Maier SF. The redox state of the alarmin HMGB1 is a pivotal factor in neuroinflammatory and microglial priming: A role for the NLRP3 inflammasome. Brain Behav Immun 2015b.

Goshen I, Yirmiya R. Interleukin-1 (IL-1): a central regulator of stress responses. Front Neuroendocrinol 2009;30:30-45.

Kang R, Chen R, Zhang Q, Hou W, Wu S, Cao L, et al. HMGB1 in health and disease. Molecular aspects of medicine 2014.

Kelly KA, Miller DB, Bowyer JF, O'Callaghan JP. Chronic exposure to corticosterone enhances the neuroinflammatory and neurotoxic responses to methamphetamine. Journal of neurochemistry 2012;122:995-1009.

Krasnova IN, Cadet JL. Methamphetamine toxicity and messengers of death. Brain Res Rev 2009;60:379-407.

Lai YT, Tsai YP, Cherng CG, Ke JJ, Ho MC, Tsai CW, et al. Lipopolysaccharide mitagates methamphetamine-induced striatal dopamine depletion via modulating local TNF-alpha and dopamine transporter expression. J Neural Transm 2009;116:405-415.

Li, J., Kokkola, R., Tabibzadeh, S., Yang, R., Ochani, M., Qiang, X., Harris, H.E., Czura, C.J., Wang, H., Ulloa, L., Wang, H., Warren, H.S., Moldawer, L.L., Fink, M.P., Andersson, U., Tracey, K.J., Yang, H., 2003. Structural basis for the proinflammatory cytokine activity of high mobility group box 1. Mol Med 9, 37-45.

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Aronica, E., Iyer, A.M., Rossetti, C., Molteni, M., Casalgrandi, M., Manfredi, A.A., Bianchi, M.E., Vezzani, A., 2010. Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nature medicine 16, 413-419.

Nakajima A, Yamada K, Nagai T, Uchiyama T, Miyamoto Y, Mamiya T, et al. Role of tumor necrosis factor-alpha in methamphetamine-induced drug dependence and neurotoxicity. J Neurosci 2004;24:2212-2225.

Raison CL, Miller AH. Role of inflammation in depression: implications for phenomenology, pathophysiology and treatment. Modern trends in pharmacopsychiatry 2013;28:33-48.

Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002;418:191-195.

Schiraldi, M., Raucci, A., Munoz, L.M., Livoti, E., Celona, B., Venereau, E., Apuzzo, T., De Marchis, F., Pedotti, M., Bachi, A., Thelen, M., Varani, L., Mellado, M., Proudfoot, A., Bianchi, M.E., Uguccioni, M., 2012. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. Journal of Experimental Medicine 209, 551-563.

Venereau, E., Casalgrandi, M., Schiraldi, M., Antoine, D.J., Cattaneo, A., De Marchis, F., Liu, J., Antonelli, A., Preti, A., Raeli, L., Shams, S.S., Yang, H., Varani, L., Andersson, U., Tracey, K.J., Bachi, A., Uguccioni, M., Bianchi, M.E., 2012. Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. The Journal of experimental medicine 209, 1519-1528.

Weber MD, Frank MG, Sobesky JL, Watkins LR, Maier SF. Blocking toll-like receptor 2 and 4 signaling during a stressor prevents stress-induced priming of neuroinflammatory responses to a subsequent immune challenge. Brain, behavior, and immunity 2013.

Weber et al., 2015. Stress Induces the Danger-Associated Molecular Pattern HMGB-1 in the Hippocampus of Male Sprague Dawley Rats: A Priming Stimulus of Microglia and the NLRP3 Inflammasome. J Neurosci 35, 316-324.

Yamaguchi T, Kuraishi Y, Minami M, Nakai S, Hirai Y, Satoh M. Methamphetamine-induced expression of interleukin-1 beta mRNA in the rat hypothalamus. Neurosci Lett 1991;128:90-92.

Yamaguchi T, Kuraishi Y, Yabuuchi K, Minami M, Satoh M. In situ hybridization analysis of the induction of interleukin-1beta mRNA by methamphetamine in the rat hypothalamus. Mol Cell Neurosci 1991;2:259-265.

Yang, H., Tracey, K.J., 2005. High mobility group box 1 (HMGB1). Critical care medicine 33, S472-474.

Yang H, Antoine DJ, Andersson U, Tracey KJ. The many faces of HMGB1: molecular structure-functional activity in inflammation, apoptosis, and chemotaxis. J Leukoc Biol 2013;93:865-873.

Yang, H., Wang, H., Ju, Z., Ragab, A.A., Lundback, P., Long, W., Valdes-Ferrer, S.I., He, M., Pribis, J.P., Li, J., Lu, B., Gero, D., Szabo, C., Antoine, D.J., Harris, H.E., Golenbock, D.T., Meng, J., Roth, J., Chavan, S.S., Andersson, U., Billiar, T.R., Tracey, K.J., Al-Abed, Y., 2015. MD-2 is required for disulfide HMGB1-dependent TLR4 signaling. The Journal of experimental medicine 212, 5-14.

11. Appendix 1: Original Copies of Journal Articles

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The danger-associated molecular pattern HMGB1 mediates the 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 2 h, 4 h and 6 h 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 μM) for 24 h and pro-inflammatory cytokines measured. The effect of METH on HMGB1 and IL-1β 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 μg) or vehicle (sterile water). 24 h post-injection, animals were injected IP with METH (10 mg/kg) or vehicle (0.9% saline) and 4 h 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-inflammatory analyte measured. In particular, METH induced profound effects on IL-1β in NAcc (2 h) and PFC (2 h and 4 h). Exposure of microglia to METH *in vitro* failed to induce a pro-inflammatory response, but rather induced significant cell death as well as a decrease in IL-1β. METH treatment increased HMGB1 in parallel with IL-1β in striatum. Pre-treatment with the HMGB1 antagonist box A blocked the neuroinflammatory effects (IL-1β) of METH in NAcc, VTA and PFC. The present results suggest that HMGB1 mediates, in part, the neuroinflammatory effects of METH and thus may alert CNS innate immune cells to the toxic effects of METH.

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, 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)-1β (Yamaguchi et al., 1991a,b). 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-1β (Flora et al., 2003, 2002). Likewise, Kelly and colleagues found that acute METH up-regulated the expression of pro-inflammatory cytokines and

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chemokines, including TNFα, IL-6, IL-1β, 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 TNFα and NF-kB (Lai et al., 2009), as well as TNFα 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 TNFα, TNFr1, TNFr2, IL-1α 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 end products, 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 4 h after treatment in striatal tissue (Imam and Ali, 2001). Therefore, we conducted an initial timecourse experiment (2, 4 and 6 h 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 4 h 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 μM) for 24 h, and pro-inflammatory mediators (mRNA and protein) measured. A 24 h 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-1β expression

Rats were injected IP with vehicle (0.9% pyrogen-free saline; $N = 4$) or METH (10 mg/kg; $N = 4$). 4 h 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 μl pyrogen-free sterile water) or box A (10 μg; 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). 24 h post-ICM injection, animals were injected IP with vehicle (0.9% pyrogen-free saline) or METH (10 mg/kg). 4 h post-IP injection, neuroinflammatory mediators were measured (mRNA) in NAcc, PFC, and VTA. Group sample sizes: ICM veh/IP veh ($N = 6$), ICM veh/IP METH ($N = 5$), ICM boxA/IP veh ($N = 6$) and ICM boxA/IP METH ($N = 6$).

2.3. General procedures

2.3.1. Brain tissue collection

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

2.3.2. ICM injection

ICM injection was selected as an intra-cerebral route of drug administration because this procedure obviates the potential neuroinflammatory effects caused by cannulation, minimizes anesthesia exposure and effectively delivers compounds throughout the CNS (Proescholdt et al., 2000). Rats were briefly anesthetized with isoflurane (~3 min). The dorsal aspect of the skull was shaved and swabbed with 70% EtOH. A 27-gauge needle, attached via polyethylene-50 tubing to a 25 μl Hamilton syringe, was inserted into the cisterna 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

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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: GGCAAATTTCCTGGTTATATCC	Pro-inflammatory cytokine
Iba-1	F: GGCAATGGAGATATCGATAT R: AGAATCATTCTCAAGATGGC	Microglia/macrophage antigen
MHCII	F: AGCACTGGGAGTTTGAAGAG R: AAGCCATCACCTCCTGGTAT	Microglia/Macrophage antigen
NF-κBIα	F: CACCAACTACAACGGCCACA R: GCTCCTGAGCGTTGACATCA	Induced by NFκB to inhibit NFκB function
TNFα	F: CAAGGAGGAGAAGTTCCCA R: TTGGTGGTTTGCTACGACG	Pro-inflammatory cytokine

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

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% CO_2 under the experimental conditions described above. Supernatants were collected for protein assay and cells were washed in ice-cold 1× 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 Island, NY). Gene expression of proinflammatory cytokines was measured using real-time RT-PCR.

2.3.4. MTT assay of microglia viability

Microglia were incubated with 12 mM MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide; Life Technologies) at 37 °C for 4 h. Dimethyl sulfoxide (50 μl) was added to each well and incubated at 37 °C for 10 min. Absorbance was measured at 540 nm, and cell viability was determined according to the protocol of the manufacturer.

2.3.5. RNA extraction, cDNA synthesis and real time RT-PCR

RNA was extracted using a standard Trizol protocol (Chomczynski and Sacchi, 1987). cDNA was synthesized using Superscript II reverse transcriptase according to the manufacturer's protocol (Life Technologies). A detailed description of the PCR amplification protocol has been published previously (Frank et al., 2006). cDNA sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Primer sequences were designed using the Eurofins MWG Operon Oligo Analysis and Plotting Tool (http://www.operon.com/technical/toolkit.aspx) and tested for sequence specificity using the Basic Local Alignment Search Tool at the NCBI (Altschul et al., 1997). Primers were obtained from Invitrogen. Primer specificity was verified by melt curve analysis. All primers were designed to span exon/exon boundaries and thus exclude amplification of genomic DNA (for primer description and sequences, see Table 1). PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA). Formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Relative gene expression was determined by taking the expression ratio of the gene of interest to the housekeeping gene β-actin.

2.3.6. Western blot

Striatum was sonicated in a mixture containing extraction buffer (Life Technologies) and protease inhibitors (Sigma). Ice-cold tissue samples were centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was removed, and the protein concentration for each sample was quantified using the Bradford method. Samples were heated to 75 °C for 10 min and loaded into a standard polyacrylamide Bis–Tris gel (Life Technologies). SDS–PAGE was performed in 3-(N-morpholino)-propanesulfonic acid running buffer (Life Technologies) at 175 V for 1.25 h. Protein was transferred onto a nitrocellulose membrane using the iblot dry transfer system (Life Technologies). The membrane was blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h and incubated with a primary antibody in blocking buffer overnight at 4 °C. The following day, the membrane was washed in 1× PBS containing Tween 20 (0.1%) and then incubated in blocking buffer containing either goat anti-rabbit or goat anti-mouse (LI-COR) IRDye 800CW secondary antibody at a concentration of 1:10,000 (LI-COR) for 1 h at room temperature. Protein expression was quantified using an Odyssey Infrared Imager (LI-COR) and expressed as a ratio to the housekeeping protein. Primary antibodies included rabbit anti-rat HMGB-1 (1:4000 dilution; Abcam) and mouse anti-rat β-actin (1:200,000 dilution; Sigma–Aldrich), which served as a housekeeping protein.

2.3.7. Enzyme-linked immunosorbent assay (ELISA)

A standard sandwich ELISA was used to measure IL-1β 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.

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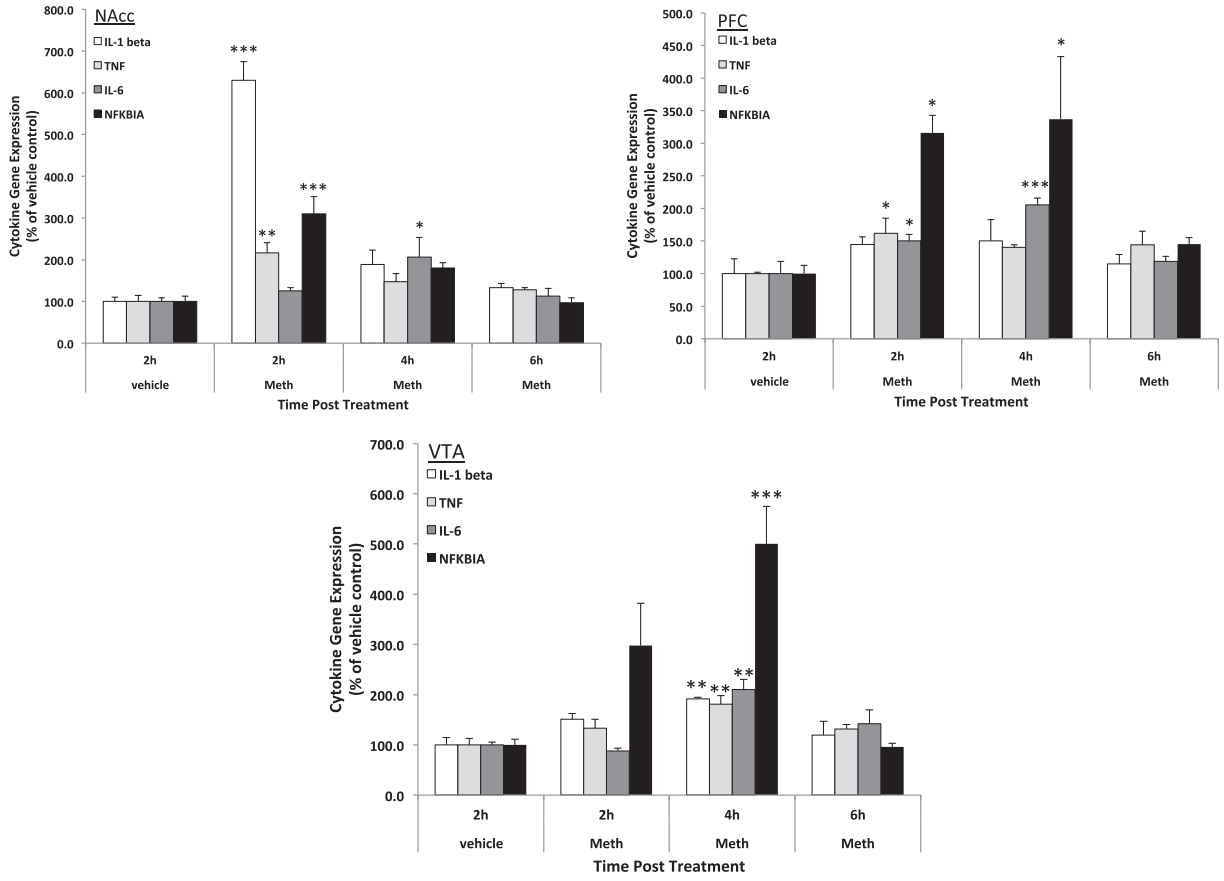

Fig. 1. Effects of METH on neuroinflammatory mediators. Rats were injected IP with vehicle (0.9% saline) or METH (10 mg/kg). At 2, 4 and 6 h post-treatment, neuroinflammatory mediators were measured (mRNA) in brain reward-related structures including the NAcc, PFC, and VTA (N = 4/experimental group).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

2.3.8. Statistical analysis

Data is presented as the mean ± 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-1β (df = 3, 12, $F = 71.65$, $p < 0.0001$), TNFα (df = 3, 12, $F = 7.86$, $p = 0.003$) and NF-κBIα (df = 3, 12, $F = 18.18$, $p < 0.0001$) gene expression. Post-hoc comparisons show that METH increased IL-1β ($p < 0.001$), TNFα ($p < 0.01$) and NF-κBIα ($p < 0.001$) at 2 h post-treatment compared to vehicle control.

In PFC, METH induced a significant change in IL-6 (df = 3, 12, $F = 13.68$, $p = 0.004$) and NF-κBIα (df = 3, 12, $F = 5.61$, $p = 0.012$) gene expression. Post-hoc comparisons show that METH increased IL-6 at 2 h ($p < 0.05$) and 4 h ($p < 0.001$) and NF-κBIα at 2 h ($p < 0.05$) and 4 h ($p < 0.05$) post-treatment compared to vehicle control.

In VTA, METH induced a significant change in IL-1β (df = 3, 12, $F = 5.87$, $p = 0.01$), TNFα (df = 3, 12, $F = 5.07$, $p = 0.017$), IL-6 (df = 3, 12, $F = 9.85$, $p = 0.001$) and NF-κBIα (df = 3, 12, $F = 11.54$, $p = 0.0008$) gene expression. IL-1β ($p < 0.01$), TNFα ($p < 0.01$), IL-6 ($p < 0.01$) and NF-κBIα ($p < 0.001$) were significantly increased at 4 h post-treatment compared to vehicle control.

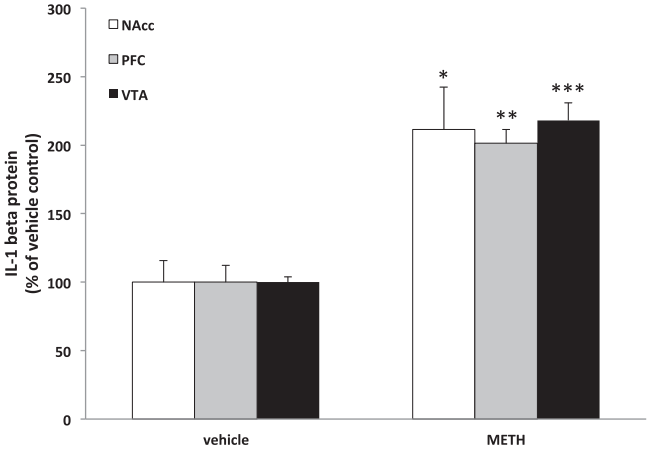

Fig. 2. Effect of METH on IL-1β protein. Rats were injected IP with vehicle (0.9% saline) or METH (10 mg/kg). At 4 h post-treatment, IL-1β protein was measured in brain reward-related structures including the NAcc, PFC, and VTA (N = 5/experimental group). Data are presented as the mean + SEM. $^{*}p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

To determine whether the neuroinflammatory effects of METH extended to the level of protein expression, the above experiment was repeated at the 4 h time-point post-METH treatment and IL-1β protein levels measured in NAcc, PFC and VTA (Fig. 2). METH

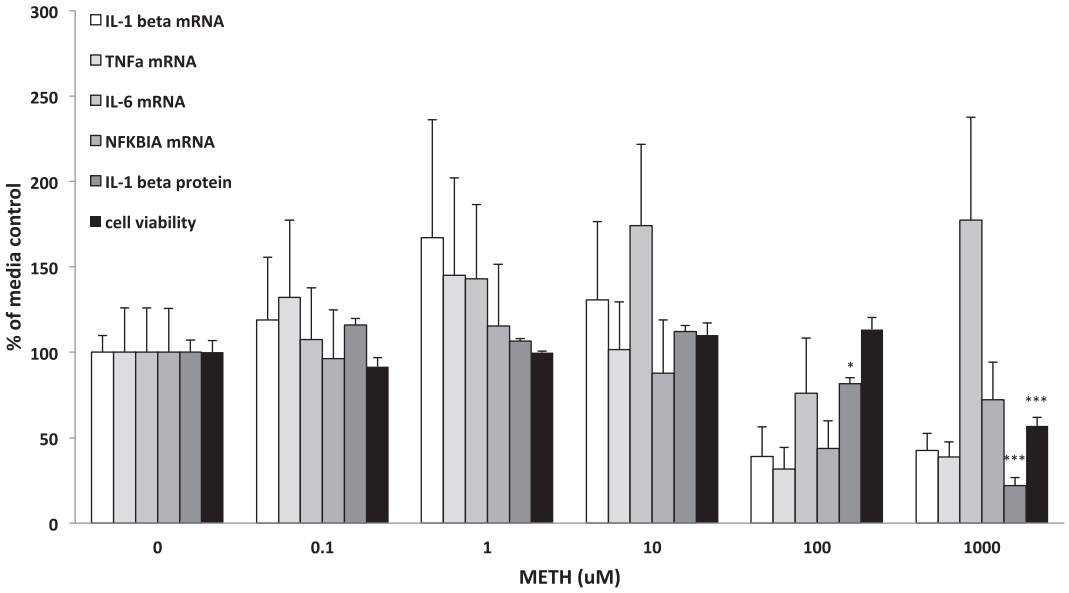

Fig. 3. *In vitro* effects of METH on microglia. Whole brain primary microglia were isolated from naïve animals and exposed to varying concentrations of METH (0, 0.1, 1, 10, 100 and 1000 μM) for 24 h and pro-inflammatory mediators (mRNA and protein) measured. Data are presented as the mean + SEM and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

significantly increased IL-1β protein levels in NAcc (df = 8, $t = 3.21$, $p = 0.012$), PFC (df = 8, $t = 6.39$, $p = 0.0002$) and VTA (df = 8, $t = 3.58$, $p = 0.0071$), compared to vehicle control.

3.2. In vitro effects of METH on microglia

In light of these observed neuroinflammatory effects of METH, we sought to test whether METH directly induces proinflammatory responses in isolated microglia. Initially, primary whole brain microglia were utilized to conduct *in vitro* experiments because sufficient numbers of microglia could not be isolated from CNS micropunches (Fig. 3). METH failed to significantly modulate the gene expression of IL-1β (df = 5, 18, $F = 1.76$, $p = 0.17$), IL-6 (df = 5, 18, $F = 0.99$, $p = 0.45$), TNFα (df = 5, 18, $F = 1.91$, $p = 0.14$) and NF-κBIα (df = 5, 18, $F = 0.84$, $p = 0.54$). Interestingly, METH significantly modulated IL-1β protein levels in cell culture supernatants (df = 5, 18, $F = 63.64$, $p < 0.0001$). Post-hoc comparisons show that METH significantly reduced IL-1β protein at 100 μM ($p < 0.05$) and 1000 μM ($p < 0.001$) compared to media control. To assess whether this effect of METH on IL-1β 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 μM ($p < 0.001$) 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-1β 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 to induce a pro-inflammatory response. Towards exploring this possible mechanism, we examined the effect of METH on striatal HMGB1 levels at 4 h post-treatment. Whole striatum was used here because of technical challenges of reliably measuring HMGB1 protein levels in brain tissue micropunches. METH treatment resulted in a significant upregulation of HMGB1 (Fig. 4A) protein compared to vehicle control (df = 6, $t = 5.00$, $p = 0.0025$). Similarly, METH also induced a significant increase in IL-1β protein (Fig. 4B) compared to vehicle control (df = 6, $t = 6.43$, $p = 0.0007$).

3.4. Effect of the HMGB1 antagonist box A on the neuroinflammatory effects of METH

The results of experiment III demonstrated that METH induces HMGB1 in parallel with increases in IL-1β protein, therefore, we tested whether METH-induced HMGB1 mediates the neuroinflammatory effects of METH (Fig. 5).

In NAcc, box A treatment significantly modulated the effect of METH on IL-1β (interaction df = 1, 19, $F = 16.28$, $p = 0.0007$), but not TNFα (interaction df = 1, 19, $F = 0.0473$, $p = 0.83$), IL-6 (interaction df = 1, 19, $F = 2.73$, $p = 0.115$) or NF-κBIα (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-1β compared to vehicle/vehicle control ($p < 0.001$), while box A treatment significantly reduced METH-induced IL-1β expression compared to vehicle/METH treatment ($p < 0.001$). The main effect of METH was significant for IL-6 (df = 1, 19, $F = 5.27$, $p = 0.03$) and NF-κBIα (df = 1, 19, $F = 6.32$, $p = 0.02$) 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-1β (interaction df = 1, 19, $F = 6.89$, $p = 0.0039$), but not TNFα (interaction df = 1, 19, $F = 1.73$, $p = 0.2$), IL-6 (interaction df = 1, 19, $F = 0.28$, $p = 0.6$) or NF-κBIα (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-1β compared to vehicle/vehicle control ($p < 0.01$), while box A/METH treatment significantly reduced IL-1 beta expression

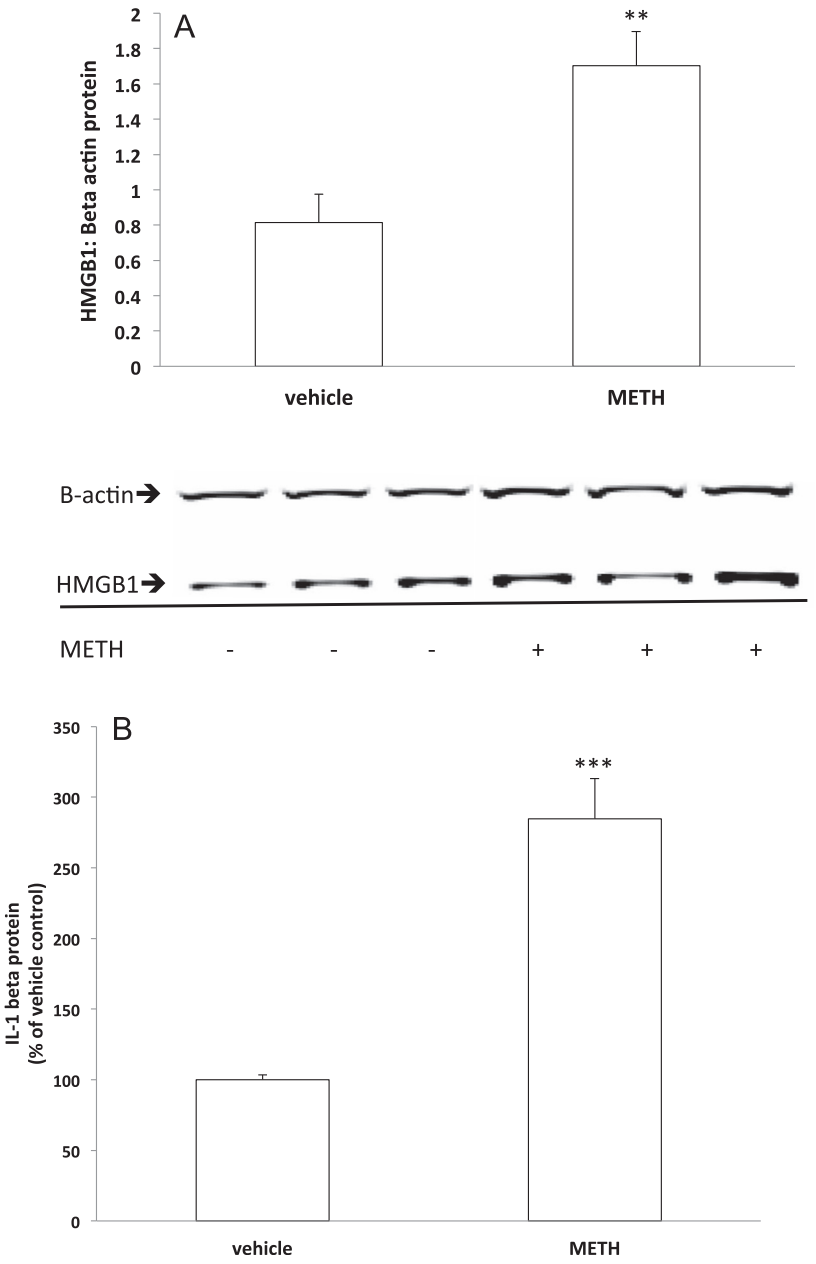

Fig. 4. Effect of METH on striatal HMGB1 and IL-1β protein levels. Rats were injected IP with vehicle (0.9% pyrogen-free saline) or METH (10 mg/kg). 4 h post-treatment, protein levels of HMGB1 (A: Top panel, relative quantitation of HMGB1 to β-actin; bottom panel, representative Western blot of HMGB1 and β-actin) and IL-1β (B) were measured in whole striatum ($N = 4$/experimental group). Data are presented as the mean + SEM. $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the vehicle control group.

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-κBIα (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-1β (interaction df = 1, 19, $F = 6.64$, $p = 0.0185$), but not TNFα (interaction df = 1, 19, $F = 1.73$, $p = 0.2$), IL-6 (interaction df = 1, 19, $F = 0.28$, $p = 0.6$) or NF-κBIα (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-1β compared to vehicle/vehicle control ($p < 0.01$), while box A/METH treatment significantly reduced IL-1β expression compared to vehicle/METH treatment ($p < 0.01$). The main effect of METH was significant for NF-κBIα (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, 2002; Kelly et al., 2012; Lai et al., 2009; Nakajima et al., 2004; Yamaguchi et al., 1991a,b), 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-1β mRNA and protein as well as NF-κBIα gene expression, which is induced by the transcription factor NF-κB (Sun et al., 1993). This finding suggests that METH activates the NF-κB pathway, resulting in the induction of pro-inflammatory cytokines, most notably IL-1β.

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

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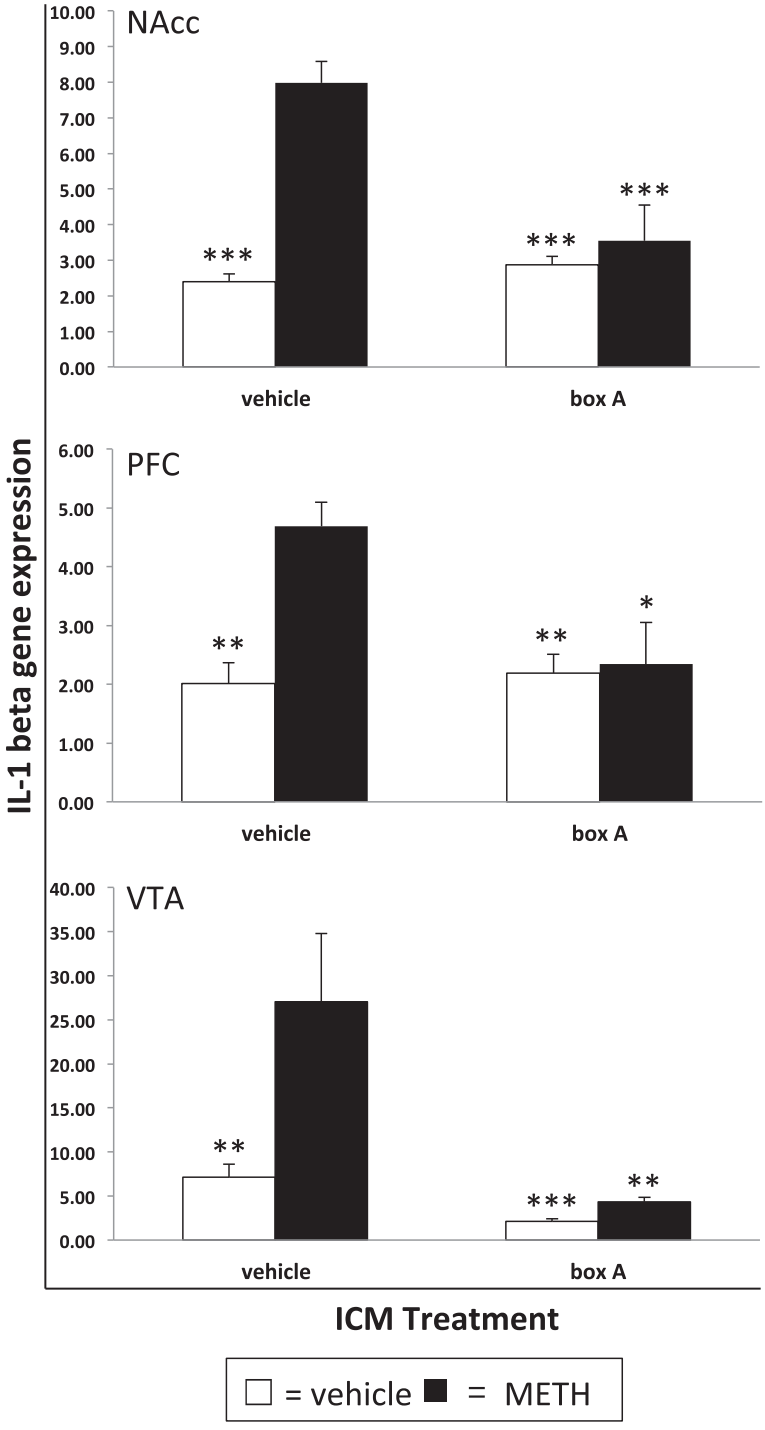

Fig. 5. Effect of the HMGB1 antagonist box A on the neuroinflammatory effects of METH. Rats were injected ICM with vehicle (5 μl pyrogen-free sterile water) or box A (10 μg). 24 h post-ICM injection, animals were injected IP with vehicle (0.9% pyrogen-free saline) or METH (10 mg/kg). 4 h post-IP injection, neuroinflammatory mediators were measured (IL-1β mRNA) in NAcc, PFC, and VTA (N = 5–6/experimental group). Data are presented as the mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the vehicle/METH treatment group for each respective brain region.

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-1β 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-1β 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

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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-1β 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-1β 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-1β 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-κBIα (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-1β specific effects of box A treatment point to a unique relationship between IL-1β and HMGB1. Formation of the NLRP3 inflammasome and activation of caspase-1, which regulate IL-1β processing and release, have also been implicated as regulatory mechanisms of HMGB1 release (Lamkanfi et al., 2010) suggesting that HMGB1 may be released in a manner similar to IL-1β or co-released with HMGB1. Moreover, HMGB1 is capable of binding IL-1β protein and potentiating IL-1β signaling through the IL-1 type 1 receptor (Hreggvidsdottir et al., 2009; Sha et al., 2008), which highlights the role of HMGB1 as an endogenous adjuvant. Whether HMGB1 plays such a role in the neuroinflammatory effects of METH is unclear, but the possibility is intriguing. While the present findings suggest that HMGB1 mediates, in part, the neuroinflammatory effects of METH and thus implicates HMGB1 in the neuroinflammatory effects of METH, several key questions remain to be addressed.

First, it is unclear which cell type(s) in the CNS releases HMGB1 in response to METH. It should be noted that here we did not directly measure cellular release of HMGB1 in the CNS, but only METH-induced increases of HMGB1. From the present data, we infer that METH induced the release of HMGB1 in the CNS because box A competitively antagonizes HMGB1 signaling through TLR4 (Yang et al., 2004), which requires the extra-cellular release of HMGB1. HMGB1 is primarily located in the nucleus of most cells (Yang et al., 2004). Within the nucleus it was originally identified as a non-histone DNA binding protein, which is loosely associated with chromatin and is involved in maintaining nucleosome structure, regulating gene transcription, and modulating the transcriptional activity of steroid hormone receptors (Gerlitz et al., 2009). HMGB1 is released from cells through two primary mechanisms, one involving passive release from necrotic or damaged cells and the other involving active secretion from immuno-competent cells (Bianchi and Manfredi, 2007). A number of studies have found that neurons are a primary source of HMGB1 in several neuroinflammatory conditions including ischemia, traumatic brain injury, seizure and chronic ethanol exposure (Frank et al., 2015). Of relevance here, chronic ethanol treatment has been found to induce the neuronal release of HMGB1, which mediated the pro-inflammatory effects of ethanol (Zou and Crews, 2014), in particular IL-1β. Zhou and Crews propose that ethanol induces the active release of HMGB1 from neurons, which signals through TLR4 on immuno-competent cells to induce pro-inflammatory cytokines (Zou and Crews, 2014). It is unclear from the present results whether METH induced the active or passive release of HMGB1. A review of the neurotoxic effects of METH in rats found that a chronic dosing regimen of METH at concentrations ranging from 0.125 mg/kg to 50 mg/kg are typically required to induce neuronal damage or death (Krasnova and Cadet, 2009). However, Imam and Ali found that a single 10 mg/kg dose of METH given IP induced neuronal damage in striatum 4 h post-injection (Imam and Ali, 2001). Clearly, the preponderance of evidence suggests that chronic high dose METH is typically required to induce neuronal damage/death. Therefore, the effects observed here of acute METH on HMGB1 are most likely pharmacological in nature, thereby inducing the active release of HMGB1 from neurons. Nevertheless, given that the dopamine transporter on dopaminergic neurons is the main target of METH (Wang et al., 2015), it is likely that dopaminergic neurons are the source of METH-induced HMGB1, but the mode of release remains to be clarified. The parallels between the findings here and the findings of Crews and colleagues raise the possibility that induction and release of DAMPs in the CNS may serve as a general mechanism of innate immune recognition of xenobiotics (i.e., drugs of abuse), which are thus "seen" as dangerous to the organism.

Another key issue that remains is the molecular form of HMGB1 that mediates the neuroinflammatory effects of METH. Recent studies have found that the redox state of HMGB1 is a key determinant of its receptor interaction and immunological function. HMGB1 contains three critical cysteine residues (C23, C45, and C106) that are the site of post-translational modification (oxidation) to create three distinct redox forms of HMGB1, each with unique functional properties (Antoine et al., 2014; Venereau et al., 2012). A fully-reduced form and a disulfide form mediate the chemotactic and pro-inflammatory effects of HMGB1, respectively, while a fully oxidized form has no known biological activity (Venereau et al., 2012). In the present study, the redox state of HMGB1 was not characterized. However, box A has been shown to block the pro-inflammatory effects of disulfide HMGB1 (Yang et al., 2015), therefore the present results of box A blocking the neuroinflammatory effects of METH suggest that METH induced the disulfide form of HMGB1. Of relevance here, METH induces oxidative stress (Cadet and Krasnova, 2009) as part of its neurotoxic effects. Partial oxidation of HMGB1 is required to convert fully reduced HMGB1 into disulfide HMGB1 (Venereau et al., 2012). Because the redox state of HMGB1 was not characterized here, it is unclear which receptor(s) (i.e., TLR4, TLR2 and/or RAGE) mediated the neuroinflammatory effects of HMGB1. However, we speculate that a possible mechanism of METH-

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induced neuroinflammation may entail the oxidative conversion of HMGB1 into its disulfide form, which then is passively released to induce pro-inflammatory cytokines via TLR4 on microglia or other myeloid cells. Of note, the present results do not exclude the possibility that the reduced form of HMGB1 may also play a role in the neuroinflammatory effects of METH.

Taken together, the present findings suggest that HMGB1 may play a pivotal role in the neuroinflammatory effects of METH and thus may be a pharmacological target to ameliorate the neuroinflammatory and neurotoxic effects of METH exposure.

Acknowledgments

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Antoine, D.J., Harris, H.E., Andersson, U., Tracey, K.J., Bianchi, M.E., 2014. A systematic nomenclature for the redox states of high mobility group box (HMGB) proteins. Mol. Med. 20, 135–137.

Bianchi, M.E., Manfredi, A.A., 2007. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. Immunol. Rev. 220, 35–46.

Bowyer, J.F., Ali, S., 2006. High doses of methamphetamine that cause disruption of the blood–brain barrier in limbic regions produce extensive neuronal degeneration in mouse hippocampus. Synapse 60, 521–532.

Buchanan, J.B., Sparkman, N.L., Johnson, R.W., 2010. A neurotoxic regimen of methamphetamine exacerbates the febrile and neuroinflammatory response to a subsequent peripheral immune stimulus. J. Neuroinflammation 7, 82.

Cadet, J.L., Krasnova, I.N., 2009. Molecular bases of methamphetamine-induced neurodegeneration. Int. Rev. Neurobiol. 88, 101–119.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Coelho-Santos, V., Goncalves, J., Fontes-Ribeiro, C., Silva, A.P., 2012. Prevention of methamphetamine-induced microglial cell death by TNF-alpha and IL-6 through activation of the JAK-STAT pathway. J. Neuroinflammation 9, 103.

Coutinho, A., Flynn, C., Burdo, T.H., Mervis, R.F., Fox, H.S., 2008. Chronic methamphetamine induces structural changes in frontal cortex neurons and upregulates type I interferons. J. Neuroimmune Pharmacol. 3, 241–245.

Escubedo, E., Guitart, L., Sureda, F.X., Jimenez, A., Pubill, D., Pallas, M., Camins, A., Camarasa, J., 1998. Microgliosis and down-regulation of adenosine transporter induced by methamphetamine in rats. Brain Res. 814, 120–126.

Fantegrossi, W.E., Ciullo, J.R., Wakabayashi, K.T., De La Garza 2nd, R., Traynor, J.R., Woods, J.H., 2008. A comparison of the physiological, behavioral, neurochemical and microglial effects of methamphetamine and 3,4-methylenedioxymethamphetamine in the mouse. Neuroscience 151, 533–543.

Flora, G., Lee, Y.W., Nath, A., Maragos, W., Hennig, B., Toborek, M., 2002. Methamphetamine-induced TNF-alpha gene expression and activation of AP-1 in discrete regions of mouse brain: potential role of reactive oxygen intermediates and lipid peroxidation. Neuromol. Med. 2, 71–85.

Flora, G., Lee, Y.W., Nath, A., Hennig, B., Maragos, W., Toborek, M., 2003. Methamphetamine potentiates HIV-1 Tat protein-mediated activation of redox-sensitive pathways in discrete regions of the brain. Exp. Neurol. 179, 60–70.

Frank, M.G., Wieseler-Frank, J.L., Watkins, L.R., Maier, S.F., 2006. Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: immunophenotypic and functional characteristics. J. Neurosci. Methods 151, 121–130.

Frank, M.G., Weber, M.D., Watkins, L.R., Maier, S.F., 2015. Stress sounds the alarmin. The role of the danger-associated molecular pattern HMGB1 in stress-induced neuroinflammatory priming. Brain Behav. Immun.

Gerlitz, G., Hock, R., Ueda, T., Bustin, M., 2009. The dynamics of HMG protein-chromatin interactions in living cells. Biochem. Cell Bio. = Biochim. Biol. Cell. 87, 127–137.

Goncalves, J., Baptista, S., Martins, T., Milhazes, N., Borges, F., Ribeiro, C.F., Malva, J. O., Silva, A.P., 2010. Methamphetamine-induced neuroinflammation and neuronal dysfunction in the mice hippocampus: preventive effect of indomethacin. Eur. J. Neurosci. 31, 315–326.

Guilarte, T.R., Nihei, M.K., McGlothan, J.L., Howard, A.S., 2003. Methamphetamine-induced deficits of brain monoaminergic neuronal markers: distal axotomy or neuronal plasticity. Neuroscience 122, 499–513.

Hreggvidsdottir, H.S., Ostberg, T., Wahamaa, H., Schierbeck, H., Aveberger, A.C., Klevenvall, L., Palmblad, K., Ottosson, L., Andersson, U., Harris, H.E., 2009. The alarmin HMGB1 acts in synergy with endogenous and exogenous danger signals to promote inflammation. J. Leukoc. Biol. 86, 655–662.

Imam, S.Z., Ali, S.F., 2001. Aging increases the susceptiblity to methamphetamine-induced dopaminergic neurotoxicity in rats: correlation with peroxynitrite production and hyperthermia. J. Neurochem. 78, 952–959.

Kelly, K.A., Miller, D.B., Bowyer, J.F., O'Callaghan, J.P., 2012. Chronic exposure to corticosterone enhances the neuroinflammatory and neurotoxic responses to methamphetamine. J. Neurochem. 122, 995–1009.

Krasnova, I.N., Cadet, J.L., 2009. Methamphetamine toxicity and messengers of death. Brain Res. Rev. 60, 379–407.

Ladenheim, B., Krasnova, I.N., Deng, X., Oyler, J.M., Polettini, A., Moran, T.H., Huestis, M.A., Cadet, J.L., 2000. Methamphetamine-induced neurotoxicity is attenuated in transgenic mice with a null mutation for interleukin-6. Mol. Pharmacol. 58, 1247–1256.

Lai, Y.T., Tsai, Y.P., Cherng, C.G., Ke, J.J., Ho, M.C., Tsai, C.W., Yu, L., 2009. Lipopolysaccharide mitigates methamphetamine-induced striatal dopamine depletion via modulating local TNF-alpha and dopamine transporter expression. J. Neural. Transm. 116, 405–415.

Lamkanfi, M., Sarkar, A., Vande Walle, L., Vitari, A.C., Amer, A.O., Wewers, M.D., Tracey, K.J., Kanneganti, T.D., Dixit, V.M., 2010. Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. J. Immunol. 185, 4385–4392.

LaVoie, M.J., Card, J.P., Hastings, T.G., 2004. Microglial activation precedes dopamine terminal pathology in methamphetamine-induced neurotoxicity. Exp. Neurol. 187, 47–57.

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Aronica, E., Iyer, A.M., Rossetti, C., Molteni, M., Casalgrandi, M., Manfredi, A.A., Bianchi, M.E., Vezzani, A., 2010. Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat. Med. 16, 413–419.

Nakajima, A., Yamada, K., Nagai, T., Uchiyama, T., Miyamoto, Y., Mamiya, T., He, J., Nitta, A., Mizuno, M., Tran, M.H., Seto, A., Yoshimura, M., Kitaichi, K., Hasegawa, T., Saito, K., Yamada, Y., Seishima, M., Sekikawa, K., Kim, H.C., Nabeshima, T., 2004. Role of tumor necrosis factor-alpha in methamphetamine-induced drug dependence and neurotoxicity. J. Neurosci. 24, 2212–2225.

Proescholdt, M.G., Hutto, B., Brady, L.S., Herkenham, M., 2000. Studies of cerebrospinal fluid flow and penetration into brain following lateral ventricle and cisterna magna injections of the tracer [14C]inulin in rat. Neuroscience 95, 577–592.

Pubill, D., Canudas, A.M., Pallas, M., Camins, A., Camarasa, J., Escubedo, E., 2003. Different glial response to methamphetamine- and methylenedioxymethamphetamine-induced neurotoxicity. Naunyn Schmiedebergs Arch. Pharmacol. 367, 490–499.

Ransohoff, R.M., Perry, V.H., 2009. Microglial physiology: unique stimuli, specialized responses. Annu. Rev. Immunol. 27, 119–145.

Sekine, Y., Ouchi, Y., Sugihara, G., Takei, N., Yoshikawa, E., Nakamura, K., Iwata, Y., Tsuchiya, K.J., Suda, S., Suzuki, K., Kawai, M., Takebayashi, K., Yamamoto, S., Matsuzaki, H., Ueki, T., Mori, N., Gold, M.S., Cadet, J.L., 2008. Methamphetamine causes microglial activation in the brains of human abusers. J. Neurosci. 28, 5756–5761.

Sha, Y., Zmijewski, J., Xu, Z., Abraham, E., 2008. HMGB1 develops enhanced proinflammatory activity by binding to cytokines. J. Immunol. 180, 2531–2537.

Sharma, H.S., Kiyatkin, E.A., 2009. Rapid morphological brain abnormalities during acute methamphetamine intoxication in the rat: an experimental study using light and electron microscopy. J. Chem. Neuroanat. 37, 18–32.

Sriram, K., Miller, D.B., O'Callaghan, J.P., 2006. Minocycline attenuates microglial activation but fails to mitigate striatal dopaminergic neurotoxicity: role of tumor necrosis factor-alpha. J. Neurochem. 96, 706–718.

Sun, S.C., Ganchi, P.A., Ballard, D.W., Greene, W.C., 1993. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science 259, 1912–1915.

Theodore, S., Stolberg, S., Cass, W.A., Maragos, W.F., 2006. Human immunodeficiency virus-1 protein tat and methamphetamine interactions. Ann. N. Y. Acad. Sci. 1074, 178–190.

Thomas, D.M., Kuhn, D.M., 2005. Attenuated microglial activation mediates tolerance to the neurotoxic effects of methamphetamine. J. Neurochem. 92, 790–797.

Thomas, D.M., Dowgiert, J., Geddes, T.J., Francescutti-Verbeem, D., Liu, X., Kuhn, D. M., 2004. Microglial activation is a pharmacologically specific marker for the neurotoxic amphetamines. Neurosci. Lett. 367, 349–354.

Thomas, D.M., Francescutti-Verbeem, D.M., Kuhn, D.M., 2008. Methamphetamine-induced neurotoxicity and microglial activation are not mediated by fractalkine receptor signaling. J. Neurochem. 106, 696–705.

Tocharus, J., Khonthun, C., Chongthammakun, S., Govitrapong, P., 2010. Melatonin attenuates methamphetamine-induced overexpression of pro-inflammatory cytokines in microglial cell lines. J. Pineal Res. 48, 347–352.

Venereau, E., Casalgrandi, M., Schiraldi, M., Antoine, D.J., Cattaneo, A., De Marchis, F., Liu, J., Antonelli, A., Preti, A., Raeli, L., Shams, S.S., Yang, H., Varani, L., Andersson, U., Tracey, K.J., Bachi, A., Uguccioni, M., Bianchi, M.E., 2012. Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. J. Exp. Med. 209, 1519–1528.

Wang, K.H., Penmatsa, A., Gouaux, E., 2015. Neurotransmitter and psychostimulant recognition by the dopamine transporter. Nature.

Weber, M.D., Frank, M.G., Tracey, K.J., Watkins, L.R., Maier, S.F., 2015. Stress induces the danger-associated molecular pattern HMGB-1 in the hippocampus of male sprague dawley rats: a priming stimulus of microglia and the NLRP3 inflammasome. J. Neurosci. 35, 316–324.

Yamaguchi, T., Kuraishi, Y., Minami, M., Nakai, S., Hirai, Y., Satoh, M., 1991a. Methamphetamine-induced expression of interleukin-1 beta mRNA in the rat hypothalamus. Neurosci. Lett. 128, 90–92.

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Yamaguchi, T., Kuraishi, Y., Yabuuchi, K., Minami, M., Satoh, M., 1991b. In situ hybridization analysis of the induction of interleukin-1 beta mRNA by methamphetamine in the rat hypothalamus. Mol. Cell. Neurosci. 2, 259–265.

Yang, H., Tracey, K.J., 2005. High mobility group box 1 (HMGB1). Crit. Care Med. 33, S472–S474.

Yang, H., Ochani, M., Li, J., Qiang, X., Tanovic, M., Harris, H.E., Susarla, S.M., Ulloa, L., Wang, H., DiRaimo, R., Czura, C.J., Wang, H., Roth, J., Warren, H.S., Fink, M.P., Fenton, M.J., Andersson, U., Tracey, K.J., 2004. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. Proc. Natl. Acad. Sci. USA 101, 296–301.

Yang, H., Wang, H., Ju, Z., Ragab, A.A., Lundback, P., Long, W., Valdes-Ferrer, S.I., He, M., Pribis, J.P., Li, J., Lu, B., Gero, D., Szabo, C., Antoine, D.J., Harris, H.E., Golenbock, D.T., Meng, J., Roth, J., Chavan, S.S., Andersson, U., Billiar, T.R., Tracey, K.J., Al-Abed, Y., 2015. MD-2 is required for disulfide HMGB1-dependent TLR4 signaling. J. Exp. Med. 212, 5–14.

Zou, J.Y., Crews, F.T., 2014. Release of neuronal HMGB1 by ethanol through decreased HDAC activity activates brain neuroimmune signaling. PLoS ONE 9, e87915.

Please cite this article in press as: Frank, M.G., et al. The danger-associated molecular pattern HMGB1 mediates the neuroinflammatory effects of methamphetamine. Brain Behav. Immun. (2015), http://dx.doi.org/10.1016/j.bbi.2015.08.001

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The redox state of the alarmin HMGB1 is a pivotal factor in neuroinflammatory and microglial priming: A role for the NLRP3 inflammasome

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ABSTRACT

The alarmin high mobility group box-1 (HMGB1) has been implicated as a key factor mediating neuroinflammatory processes. Recent findings suggest that the redox state of HMGB1 is a critical molecular feature of HMGB1 such that the reduced form (fr-HMGB1) is chemotactic, while the disulfide form (ds-HMGB1) is pro-inflammatory. The present study examined the neuroinflammatory effects of these molecular forms as well as the ability of these forms to prime the neuroinflammatory and microglial response to an immune challenge. To examine the neuroinflammatory effects of these molecular forms in vivo, animals were administered intra-cisterna magna (ICM) a single dose of fr-HMGB1 (10 μg), ds-HMGB1 (10 μg) or vehicle and basal pro-inflammatory effects were measured 2 and 24 h post-injection in hippocampus. Results of this initial experiment demonstrated that ds-HMGB1 increased hippocampal pro-inflammatory mediators at 2 h (NF-κBIα mRNA, NLRP3 mRNA and IL-1β protein) and 24 h (NF-κBIα mRNA, TNFα mRNA, and NLRP3 protein) after injection. fr-HMGB1 had no effect on these mediators. These neuroinflammatory effects of ds-HMGB1 suggested that ds-HMGB1 may function to prime the neuroinflammatory response to a subsequent immune challenge. To assess the neuroinflammatory priming effects of these molecular forms, animals were administered ICM a single dose of fr-HMGB1 (10 μg), ds-HMGB1 (10 μg) or vehicle and 24 h after injection, animals were challenged with LPS (10 μg/kg IP) or vehicle. Neuroinflammatory mediators and the sickness response (3, 8 and 24 h after injection) were measured 2 h after immune challenge. We found that ds-HMGB1 potentiated the neuroinflammatory (NF-κBIα mRNA, TNFα mRNA, IL-1β mRNA, IL-6 mRNA, NLRP3 mRNA and IL-1β protein) and sickness response (reduced social exploration) to LPS challenge. fr-HMGB1 failed to potentiate the neuroinflammatory response to LPS. To examine whether these molecular forms of HMGB1 directly induce neuroinflammatory effects in isolated microglia, whole brain microglia were isolated and treated with fr-HMGB1 (0, 1, 10, 100, or 1000 ng/ml) or ds-HMGB1 (0, 1, 10, 100, or 1000 ng/ml) for 4 h and pro-inflammatory mediators measured. To assess the effects of these molecular forms on microglia priming, whole brain microglia were pre-exposed to these forms of HMGB1 (0, 1, 10, 100, or 1000 ng/ml) and subsequently challenged with LPS (10 ng/ml). We found that ds-HMGB1 increased expression of NF-κBIα mRNA and NLRP3 mRNA in isolated microglia, and potentiated the microglial pro-inflammatory response (TNFα mRNA, IL-1β mRNA and IL-1β protein) to LPS. fr-HMGB1 failed to potentiate the microglial pro-inflammatory response to LPS. Consistent with prior reports, the present findings demonstrate that the disulfide form of HMGB1 not only potentiates the neuroinflammatory response to a subsequent immune challenge in vivo, but also potentiates the sickness response to that challenge. Moreover, the present findings demonstrate for the first time that ds-HMGB1 directly potentiates the microglia pro-inflammatory response to an immune challenge, a finding that parallels the effects of ds-HMGB1 in vivo. In addition, ds-HMGB1 induced expression of NLRP3 and NF-κBIα in vivo and in vitro suggesting that the NLRP3 inflammasome may play role in the priming effects of ds-HMGB1. Taken together, the present results

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suggest that the redox state of HMGB1 is a critical determinant of the priming properties of HMGB1 such that the disulfide form of HMGB1 induces a primed immunophenotype in the CNS, which may result in an exacerbated neuroinflammatory response upon exposure to a subsequent pro-inflammatory stimulus.

1. Introduction

The alarmin high mobility group box-1 (HMGB1) has been implicated as a key factor mediating neuroinflammatory processes in several pathophysiological conditions including seizure (Maroso et al., 2010), ischemia (Kim et al., 2006), chronic pain (Agalave et al., 2014) and alcohol-induced neuroinflammation (Zou and Crews, 2014). Recently, we have shown that HMGB1 is also a critical mediator of stress-induced priming of the microglial pro-inflammatory response to a subsequent immune challenge (Weber et al., 2015). However, the mechanism(s) by which HMGB1 exerts these neuroinflammatory effects has not been clarified.

HMGB1 is a nuclear protein that functions as a danger associated molecular pattern (DAMP), which is released into the extracellular milieu to signal cellular damage, cellular stress, or pathogen insult (Bianchi, 2007). The primary structure of HMGB1 is composed of an A box domain, which functions as an HMGB1 antagonist and a B box domain, which exhibits pro-inflammatory properties (Klune et al., 2008). In addition, HMGB1 can function either as a chemotactic or pro-inflammatory mediator depending on the redox state of three critical cysteines (Venereau et al., 2012). HMGB1 functions as a chemotactic factor if cysteines C23, C45, and C106 remain in a thiol state (fully reduced HMGB1; fr-HMGB1), but in this state lacks pro-inflammatory properties. Alternatively, HMGB1 exerts pro-inflammatory effects if cysteines C23 and C45 become oxidized, while C106 remains in a thiol state. Oxidation of C23 and C45 results in the formation of a disulfide bond (disulfide HMGB1; ds-HMGB1), which is a critical determinant of the cytokine stimulating capacity of HMGB1 (Yang et al., 2012). Further, ds-HMGB1 lacks chemotactic properties, which suggests that fr- and ds-HMGB1 are mutually exclusive molecular forms (Venereau et al., 2012). Notably, oxidation of all three cysteines abrogates both the chemotactic and pro-inflammatory activity of HMGB1 (Yang et al., 2012).

The fr-HMGB1 forms a complex with the chemokine C-X-C motif ligand 12 (CXCL12), which then signals through the chemokine receptor, C-X-C chemokine receptor type 4 (CXCR4), to mediate chemotaxis, while the pro-inflammatory effects of ds-HMGB1 are mediated by the pattern recognition receptor, Toll-like receptor 4 (TLR4) (Lu et al., 2013). TLR4-mediated effects of HMGB1 are dependent upon the thiol state of cysteine C106 (Yang et al., 2012). Interestingly, the box A domain of HMGB1 can by itself function to competitively antagonize the pro-inflammatory effects of HMGB1 (Yang et al., 2004), presumably through TLR4.

Very few studies have investigated the role of HMGB1 redox states in neuroinflammatory processes. Balosso and colleagues found that the ds-HMGB1 increased NMDA-induced neuronal cell death and potentiated kainate-induced seizures (Balosso et al., 2014). A recent study by Liesz and colleagues found that cerebral ischemia induced the release of the fr-HMGB1 from necrotic brain lesions and that HMGB1 in a disulfide redox state was released into serum (Liesz et al., 2015). Recently, we found that box A administered into the CNS blocks stress-induced sensitization of the microglial pro-inflammatory response to lipopolysaccharide (LPS) *ex vivo*, suggesting that stress induces the release of HMGB1 in the CNS, which then functions to sensitize neuroinflammatory processes (Weber et al., 2015). Although it is unclear which form of HMGB1 mediated stress-induced sensitization of microglia, we found that ds-HMGB1 administered into the CNS *in vivo* was sufficient to prime microglia, whereas fr-HMGB1 failed to induce priming. Several key questions arising from this study and addressed here pertain to (1) the ability of ds-HMGB1 to induce neuroinflammatory effects *in vivo* independent of priming, (2) the ability of ds-HMGB1 to prime neuroinflammatory processes *in vivo* to a subsequent immune challenge, (3) whether the neuroinflammatory priming effects of ds-HMGB1 are behaviorally relevant, (4) whether ds-HMGB1 directly acts upon microglia to induce a primed state, and (5) the mechanism by which ds-HMGB1 primes microglia and the neuroinflammatory response to a subsequent immune challenge.

Several lines of evidence raise the possibility that the nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain containing 3 (NLRP3) inflammasome might mediate ds-HMGB1-induced neuroinflammatory priming. The NLRP3 inflammasome is a multiprotein complex that mediates the processing and maturation of the pro-inflammatory cytokine interleukin (IL)-1β (Lamkanfi and Kanneganti, 2010). We and others have shown that stress induces NLRP3 (Pan et al., 2014; Weber et al., 2015) and NLRP3 is considered a sensor of a diverse array of DAMPs (Leemans et al., 2011). Of particular relevance here, formation of the NLRP3 inflammasome requires both a priming step and an activating step for the processing of IL-1β to proceed (Hornung and Latz, 2010). Therefore, the present investigation explored the effects of ds-HMGB1 on NLRP3 *in vivo* and *in vitro* as a mechanistic basis of the neuroinflammatory priming effects of ds-HMGB1.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (60–90 day-old; Harlan Sprague–Dawley, Inc., Indianapolis, IN, USA) 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 07:00 h). All rats were allowed 1 week of acclimatization to the colony rooms before experimentation. All experimental procedures were conducted in accordance with the University of Colorado Institutional Animal Care and Use Committee.

2.2. Reagents

Lyophilized fr-HMGB1 and ds-HMGB1 were obtained from HMGBiotech (Milan, IT), suspended in pyrogen-free sterile water and are certified LPS-free. LPS (*E. coli* serotype O111:B4) was obtained from Sigma (St. Louis, MO).

2.3. Intra-cisterna magna (ICM) injections of fr-HMGB1 and ds-HMGB1

Rats were anesthetized with isoflurane (~3 min). The dorsal aspect of the skull was shaved and swabbed with 70% EtOH, a 27-gauge needle, attached via PE50 tubing to a 25 μl Hamilton syringe, was inserted into the cisterna magna. To verify entry into the cisterna magna, cerebrospinal fluid (CSF) was withdrawn (~2 μl) and visually inspected for the presence of red blood cells. Clear

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CSF indicated entry in the cisterna magna. fr-HMGB1 (1 μg in 10 μl), ds-HMGB1 (1 μg in 10 μl) or vehicle (10 μl pyrogen-free sterile water) was injected ICM. ICM administration was used to avoid implanting cannulae, which itself produces enduring neuroinflammation (Holguin et al., 2007). Hippocampus was dissected 2 and 24 h after ICM injection. Hippocampus was a focus here because we have found this brain region to be particularly sensitive to stress-induced sensitization of neuroinflammatory processes (Frank et al., 2007; Johnson et al., 2002; Weber et al., 2015).

2.4. fr-HMGB1- and ds-HMGB1-induced priming in vivo

LPS (10 μg/kg IP) or vehicle (0.9% pyrogen-free saline) was injected 24 h post-ICM injection of fr-HMGB1 (1 μg in 10 μl), ds-HMGB1 (1 μg in 10 μl) or vehicle (10 μl pyrogen-free sterile water). 2 h post-LPS or vehicle injection, hippocampus was dissected.

2.5. Tissue collection

Rats were injected with a lethal dose of sodium pentobarbital. Upon deep anesthesia, rats underwent transcardial perfusion with ice-cold saline (0.9%) for 3 min to remove peripheral immune cells from the CNS vasculature. Brains were rapidly extracted and placed on ice. For *in vivo* experiments, hippocampus was dissected, flash frozen in liquid nitrogen and stored at −80 °C. For *in vitro* experiments, whole brain microglia were immediately isolated.

2.6. In vitro stimulation of microglia with fr-HMGB1 and ds-HMGB1

Whole brain microglia were isolated using a Percoll density gradient as previously described (Frank et al., 2006). We have previously shown (Frank et al., 2006) that this microglia isolation procedure yields highly pure (>95%) microglia (Iba-1+/MHCII+/CD163−/GFAP−). Immunophenotype and purity of microglia was assessed and verified using real time RT-PCR of MHCII, CD163, Iba-1 and GFAP gene expression. Microglia were routinely found to be MHCII+/Iba1+/CD163−/GFAP− (data not shown). Microglia were cultured in 100 μl DMEM + 10% FBS and microglia concentration determined by trypan blue exclusion. Microglia were plated in individual wells of a 96-well v-bottom plate and incubated at 37 °C, 5% CO_2. Microglia were exposed to fr-HMGB1 (0, 1, 10, 100, and 1000 ng/ml) or ds-HMGB1 (0, 1, 10, 100, and 1000 ng/ml) for 4 h. Plate was centrifuged at 1000× g for 10 min, 4 °C. Supernatants were collected for assay of cytokine release and cells were lysed under differing conditions (see below) for assay of protein or mRNA. To determine priming effects *in vitro*, microglia were exposed to fr-HMGB1 (0, 1, 10, 100, and 1000 ng/ml) or ds-HMGB1 (0, 1, 10, 100, and 1000 ng/ml) for 4 h. Cells were washed (2× in fresh media) free of HMGB1 by centrifuging at 1000× g for 10 min, 4 °C. Cells were suspended in fresh media (100 μl) and treated with LPS (10 ng/ml) or media control for 18 h. Supernatants and cells were processed as described above.

2.7. In vitro stimulation of peritoneal macrophages with fr-HMGB1 and ds-HMGB1

Peritoneal lavage was utilized to collect macrophages. An incision was made in the peritoneal cavity (~2 cm) and ice-cold Hank's Balanced Salt Solution (30 ml) was pipetted into the abdominal cavity. The abdomen was massaged for 30 s after which the lavage fluid was removed (~20 ml) and centrifuged at 1000 rpm for 5 min at 4 °C. The cells were treated with red blood cell lysis buffer (160 mM NH_4Cl, 12 mM $NaHCO_3$, 100 μM EDTA, dissolved in dH_2O, pH 7.3) for 2 min. The cells were suspended in 20 ml Iscove's medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin and 2 μM L-glutamine and centrifuged at 1000 rpm for 5 min. Cells were then suspended in Iscove's media and plated at 200,000 cells per well/200 μl media in a 96-well tissue culture plate and incubated for 2 h. The cells were then washed with warm Dulbecco's Phosphate Buffered Saline, to remove any non-adherent cells and suspended in 200 μl media. The cells were cultured for 4 h *ex vivo* with 0, 1, 10, 100, 1000 ng/ml of either ds-HMGB-1 or fr-HMGB-1 at 37 °C with 5% CO_2. Following incubation, cells were centrifuged, supernatant removed and Trizol added to lyse cells for mRNA analysis.

2.8. cDNA synthesis and real time RT-PCR

Total RNA was isolated from whole hippocampus and peritoneal macrophages utilizing a standard method of phenol:chloroform extraction (Chomczynski and Sacchi, 1987) and cDNA synthesis was performed using the SuperScript II cDNA synthesis kit (Invitrogen, Carlsbad, CA). For isolated microglia, cells were washed in 1× PBS. Cells were lysed/homogenized and cDNA synthesis was performed according to the manufacturer's protocol using the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen). Gene expression was measured using real time RT-PCR. A detailed description of the PCR amplification protocol has been published previously (Frank et al., 2006). cDNA sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Primer sequences were designed using the Eurofins MWG Operon Oligo Analysis & Plotting Tool (http://www.operon.com/technical/toolkit.aspx) and tested for sequence specificity using the Basic Local Alignment Search Tool at NCBI (Altschul et al., 1997). Primers were obtained from Invitrogen. Primer specificity was verified by melt curve analysis. All primers were designed to span exon/exon boundaries and thus exclude amplification of genomic DNA (see Table 1 for primer description and sequences). PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR Kit (Qiagen, Valencia, CA). Formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Relative gene expression was determined by taking the expression ratio of the gene of interest to β-actin.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Hippocampus was sonicated using a tissue extraction reagent (Invitrogen) supplemented with a protease inhibitor cocktail (Sigma). Homogenate was centrifuged (10 min, 14,000× g, 4 °C) and supernatant collected and stored at −20 °C. Total protein was quantified using a Bradford assay. IL-1β protein was measured using a commercially available ELISA (R & D Systems, Minneapolis, MN). For whole hippocampus, concentrations of IL-1β protein were scaled to total protein and expressed as pg/mg total protein. For cell culture supernatants, concentrations of IL-1β are expressed as pg/ml.

2.10. Western blot

Hippocampus was processed and total protein determined as described under ELISA. Samples were heated to 75 °C for 10 min and loaded into a standard polyacrylamide Bis–Tris gel (Invitrogen). SDS–PAGE was performed in MOPS running buffer (Invitrogen) at 175 V for 1.25 h. Protein was transferred onto a nitrocellulose membrane using the iBlot dry transfer system (Invitrogen). The membrane was blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h and incubated with a primary antibody in blocking buffer containing Tween 20 (0.2%) overnight at 4 °C. The following day, the membrane was washed in 1× PBS containing Tween 20 (0.1%) and then incubated in blocking

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Table 1
Primer sequence and gene function.

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: GGCAAATTTCCTGGTTATATCC	Pro-inflammatory cytokine
Iba-1	F: GGCAATGGAGATATCGATAT R: AGAATCATTCTCAAGATGGC	Microglia/macrophage antigen
MHCII	F: AGCACTGGGAGTTTGAAGAG R: AAGCCATCACCTCCTGGTAT	Microglia/macrophage antigen
NF-κBIα	F: CACCAACTACAACGGCCACA R: GCTCCTGAGCGTTGACATCA	Induced by NFκB to inhibit NFκB function
NLRP3	F: AGAAGCTGGGGTTGGTGAATT R: GTTGTCTAACTCCAGCATCTG	Inflammasome component mediating caspase-1/IL-1β activation
TNFα	F: CAAGGAGGAGAAGTTCCCA R: TTGGTGGTTTGCTACGACG	Pro-inflammatory cytokine

Abbreviations: GFAP, glial fibrillary acidic protein; IL, interleukin; Iba-1, ionized calcium-binding adaptor molecule-1; MHCII, major histocompatibility complex II; NF-κBIα, nuclear factor kappa light chain enhancer of activated B cells inhibitor alpha; NLRP3, nucleotide-binding domain and leucine-rich repeat containing family, pyrin domain containing 3; TNFα, tumor necrosis factor-α.

buffer (0.2% Tween 20) containing either goat anti-rabbit or goat anti-mouse (LI-COR) IRDye 800CW secondary antibody at a concentration of 1:10,000 (LI-COR) for 1 h at RT. The membrane was washed in 1x PBS containing Tween 20 (0.1%). Protein expression was quantified using an Odyssey Infrared Imager (LI-COR) and expressed as a ratio to the housekeeping protein (β-actin). Primary antibodies included rabbit anti-rat NLRP3 monoclonal antibody (1:4000, Abcam, Cambridge, MA) and mouse anti-rat β-actin monoclonal antibody (1:200,000, Sigma).

2.11. Social exploration test

Each experimental subject was transferred to a novel cage with shaved wood bedding in a dimly lit room (40 lx). After a 15 min habituation period, a 28–32 day old juvenile male rat was introduced to the subject's cage for 5 min and exploratory behaviors (sniffing, pinning and allo-grooming) were timed by an observer blind to treatment condition. After the test, the juvenile was removed and the experimental adult rat was returned to its homecage. Although juvenile stimulus rats were reused for multiple tests, the adult was never re-tested with the same juvenile. Baseline social exploration was measured 24 h prior to ICM injection of ds-HMGB1 (1 μg in 10 μl) or vehicle (10 μl pyrogen-free sterile water). 24 h post-ICM treatment, vehicle (0.9% pyrogen-free saline) or LPS (10 μg/kg) was injected IP. Social exploration was then measured 3, 8, and 24 h after IP injection.

2.12. Statistical analysis and data presentation

All data are presented as mean + SEM. Statistical analyses consisted of ANOVA followed by post-hoc tests (Newman–Keuls) using Prism 5 (Graphpad Software, Inc., La Jolla, CA). A one-way ANOVA was used to assess the effects of fr-HMGB1 and ds-HMGB1 *in vivo* and *in vitro*. A two-way ANOVA was used to assess the main effects and interaction of HMGB1 and LPS *in vivo*. All data met the assumptions of ANOVA including normality of data and homogeneity of variance. Omnibus *F*-values are reported for each ANOVA and serve as a criterion for performing post-hoc analyses. Post-hoc comparisons are provided in figures. Threshold for statistical significance was set at $\alpha = 0.05$. 6–10 animals per experimental group were used in each experiment *in vivo*. 3–4 replications were performed for each *in vitro* experiment.

3. Results

3.1. Effect of fr-HMGB1 and ds-HMGB1 on pro-inflammatory mediators in hippocampus

To determine whether the redox state of HMGB1 differentially modulates the expression of pro-inflammatory mediators, fr-HMGB1, ds-HMGB1 and vehicle control were injected ICM. At 2 and 24 h post-injection, the gene expression and protein levels of several pro-inflammatory mediators were measured in hippocampus (Fig. 1).

3.1.1. 2h Post-ICM injection

HMGB1 treatment failed to significantly modulate the gene expression of IL-1β (df = 2, 15, $F = 0.46$, $p = 0.64$), TNFα (df = 2, 18, $F = 3.17$, $p = 0.07$), and IL-6 (df = 2, 17, $F = 3.17$, $p = 0.07$). However, ds-HMGB1 treatment significantly modulated the gene expression of NF-κBIα (df = 2, 18, $F = 8.42$, $p = 0.003$) and NLRP3 (df = 2, 18; $F = 27.01$, $p < 0.0001$) (Fig. 1A). ds-HMGB1 significantly increased NF-κBIα and NLRP3 mRNA compared to fr-HMGB1 and vehicle control treatment. NLRP3 protein was significantly changed by fr-HMGB1 treatment (df = 2, 18; $F = 27.04$, $p < 0.0001$), such that fr-HMGB1 treatment significantly decreased NLRP3 protein levels compared to vehicle and ds-HMGB1 treatment. ds-HMGB1 treatment also significantly altered IL-1β protein levels (df = 2, 17; $F = 6.72$, $p = 0.01$), whereby ds-HMGB1 significantly increased IL-1β protein compared to fr-HMGB1 and vehicle control groups (Fig. 1A).

3.1.2. 24h Post-ICM injection

IL-1β (df = 2, 16; $F = 0.13$, $p = 0.87$), IL-6 (df = 2, 17; $F = 0.43$, $p = 0.66$), and NLRP3 (df = 2, 18; $F = 0.11$, $p = 0.89$) gene expression was not significantly changed by HMGB1 treatment. ds-HMGB1 treatment did significantly modulate the expression of NF-κBIα (df = 2, 16; $F = 9.31$, $p = 0.002$) and TNFα (df = 2, 18; $F = 9.85$, $p = 0.001$) (Fig. 1B). ds-HMGB1 increased the expression of NF-κBIα and TNFα compared to fr-HMGB1 and vehicle control. IL-1β protein was unchanged by HMGB1 treatment (df = 2, 19; $F = 0.67$,

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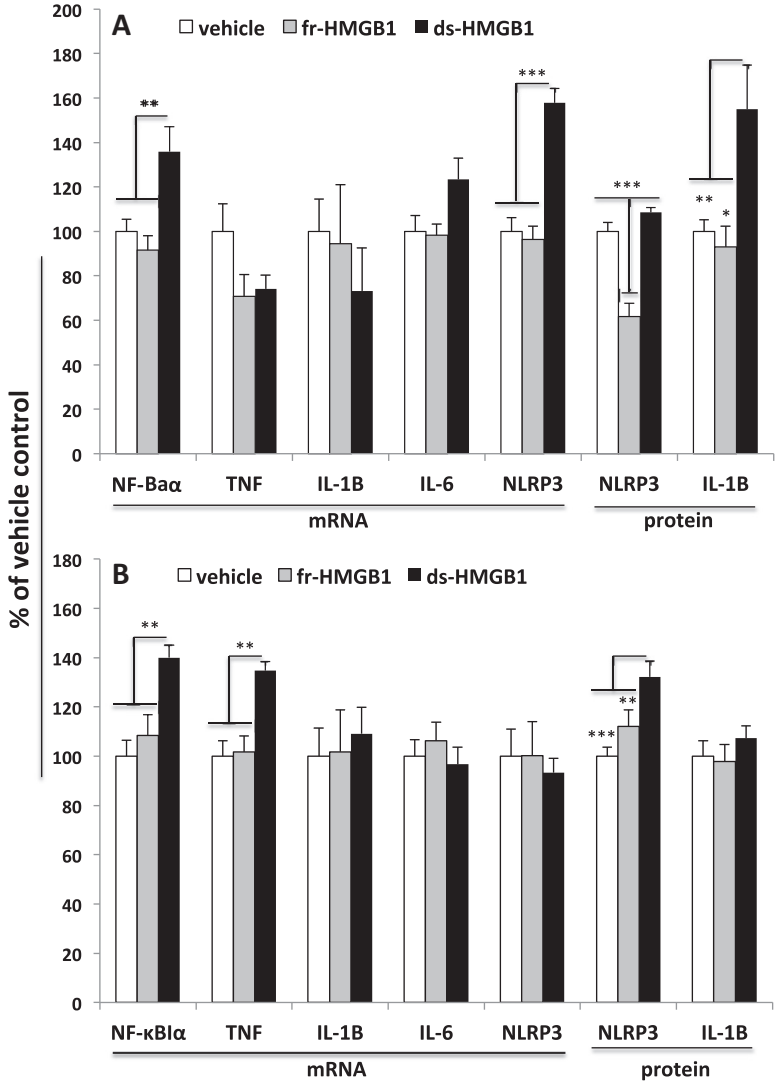

Fig. 1. Effect of fr-HMGB1 and ds-HMGB1 on pro-inflammatory mediators in hippocampus. Vehicle, fr-HMGB1 (1 μg), or ds-HMGB1 (1 μg) was injected ICM and pro-inflammatory mediators measured 2 h (A) and 24 h (B) post-injection. A: fr-HMGB1 significantly reduced protein levels of NLRP3 protein compared to vehicle and ds-HMGB1 treatments, but failed to modulate the expression level of all other analytes. ds-HMGB1 induced a significant increase in NF-κBIα mRNA, NLRP3 mRNA and IL-1β protein compared to vehicle and fr-HMGB1 treatments. B: fr-HMGB1 failed to significantly modulate the expression level of all analytes tested. ds-HMGB1 significantly increased NF-κBIα mRNA, TNFα mRNA, and NLRP3 protein compared to vehicle and fr-HMGB1 treatments. $N = 6–10$ animals per experimental group. Data are presented as the mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

$p = 0.52$), whereas NLRP3 protein was significantly changed (df = 2, 17; $F = 10.23$, $p = 0.001$) (See Fig. S1 in supplementary data for Western blot data). ds-HMGB1 treatment resulted in a significant increase in NLRP3 protein compared to fr-HMGB1 and vehicle control treatments (Fig. 1B).

3.2. Effect of fr-HMGB1 and ds-HMGB1 on priming of the hippocampal neuroinflammatory response

During an inflammatory response, changes in NF-κBIα expression reflect the transcriptional activity of NF-κB (Sun et al., 1993), which induces NLRP3 protein as part of the priming step of the NLRP3 inflammasome (Bauernfeind et al., 2009). Therefore, the observation that ds-HMGB1 increased the expression of NF-κBIα mRNA and NLRP3 protein at 24 h post-treatment suggests that ds-HMGB1 may prime the neuroinflammatory response to a subsequent immune challenge. To test this possibility, fr-HMGB1 and ds-HMGB1 were injected ICM. 24 h post-injection, LPS or vehicle were injected peripherally and pro-inflammatory mediators measured in hippocampus 2 h post-LPS injection.

The interaction between fr-HMGB1 treatment and LPS was not statistically significant for NF-κBIα (df = 1, 20; $F = 1.26$, $p = 0.27$), IL-1β (df = 1, 20; $F = 0.013$, $p = 0.91$), IL-6 (df = 1, 20; $F = 0.30$, $p = 0.59$), TNFα (df = 1, 20; $F = 0.075$, $p = 0.79$) or NLRP3 (df = 1, 20; $F = 1.05$, $p = 0.32$) gene expression (data not shown). However, LPS treatment, irrespective of fr-HMGB1 treatment, significantly increased NF-κBIα (df = 1, 20; $F = 64.72$, $p < 0.0001$), IL-1β (df = 1, 20; $F = 20.97$, $p = 0.0002$), IL-6 (df = 1, 20; $F = 51.06$, $p < 0.0001$) and TNFα (df = 1, 20; $F = 5.7$, $p < 0.0001$) gene expression (data not shown). Similarly, fr-HMGB1 failed to differentially modulate the LPS induction of IL-1β protein levels (df = 1, 20; $F = 0.15$, $p = 0.70$), however LPS significantly increased IL-1β protein (df = 1, 20; $F = 8.31$, $p = 0.009$) (data not shown).

ds-HMGB1 treatment had no effect on any measures by itself, but it potentiated the pro-inflammatory effects of LPS on NF-κBIα (df = 1, 20; $F = 7.27$, $p = 0.014$), IL-1β (df = 1, 20; $F = 11.58$, $p = 0.003$), IL-6 (df = 1, 20; $F = 8.8$, $p = 0.008$), and NLRP3 (df = 1, 20; $F = 4.77$, $p = 0.04$) gene expression (Fig. 2). Likewise, the interaction between ds-HMGB1 and LPS on IL-1β protein was significant (df = 1, 20; $F = 7.24$, $p = 0.014$) (Fig. 2). ds-HMGB1 failed

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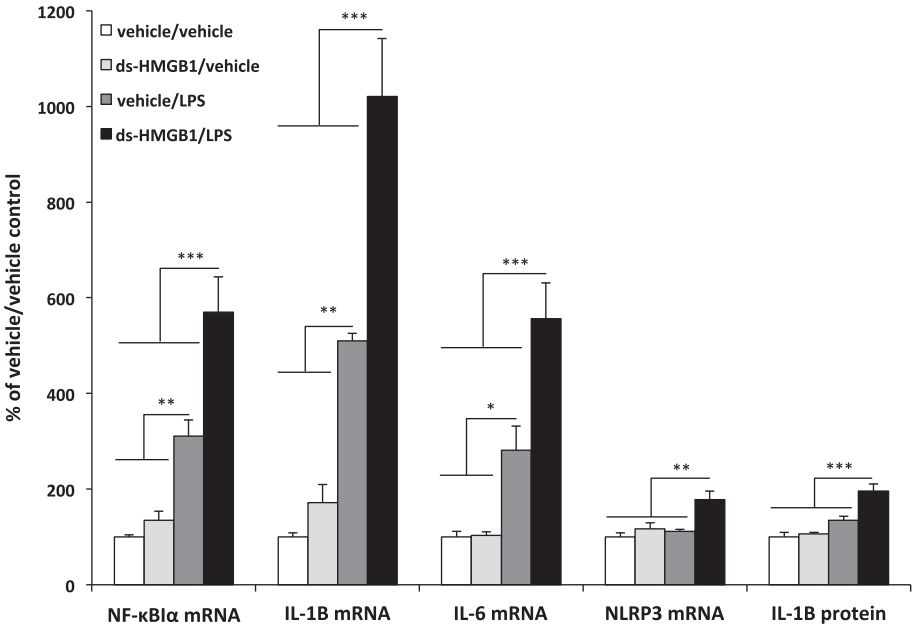

Fig. 2. Effect of ds-HMGB1 on priming of the hippocampal neuroinflammatory response. Vehicle or ds-HMGB1 (1 μg) was injected ICM. 24 h post-ICM injection, vehicle or LPS (10 μg/kg) was injected IP and 2 h post-LPS or vehicle treatment, pro-inflammatory cytokines were measured in hippocampus. ds-HMGB1 treatment potentiated the LPS induction of NF-κBIα mRNA ($^{***}p < 0.001$), IL-1β mRNA ($^{***}p < 0.001$), IL-6 mRNA ($^{***}p < 0.001$), NLRP3 mRNA ($^{***}p < 0.001$), and IL-1β protein ($^{***}p < 0.001$) compared to vehicle/LPS, ds-HMGB1/vehicle, and vehicle/vehicle treatment groups. Vehicle/LPS treatment significantly increased NF-κBIα mRNA ($^{**}p < 0.01$), IL-1β mRNA ($^{**}p < 0.01$), and IL-6 mRNA ($^{*}p < 0.05$) compared to the ds-HMGB1/vehicle and vehicle/vehicle treatment groups. $N = 6$ animals per experimental group. Data are presented as the mean + SEM.

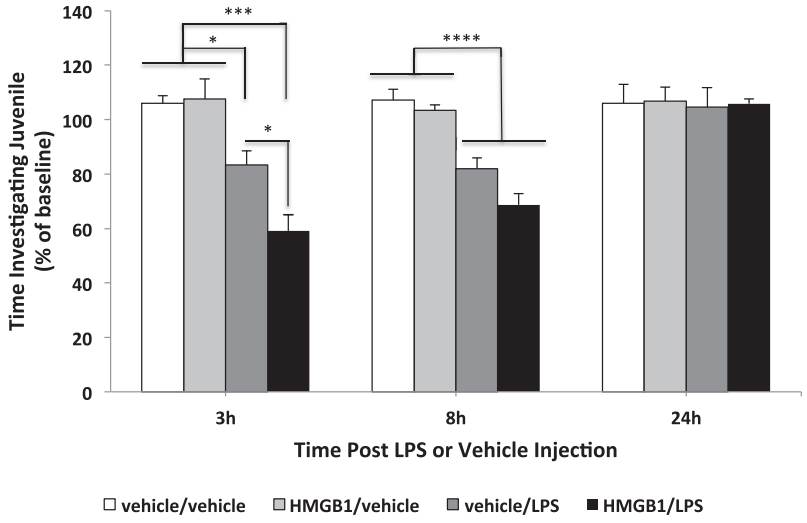

Fig. 3. Effect of ds-HMGB1 on priming of the sickness response to LPS. Vehicle or ds-HMGB1 (1 μg) was injected ICM and 24 h post-ICM injection, vehicle or LPS (10 μg/kg) was injected IP. Social exploration of a con-specific juvenile by an adult treated animal was used as a behavioral measure of the sickness response to LPS. Social exploration (time investigating juvenile) was measured 24 h prior to ICM injection (baseline) and 3, 8, and 24 h post-LPS or vehicle injection. At 3 h post-LPS treatment, ds-HMGB1 treatment significantly potentiated the LPS-induced decrease in social exploration compared to vehicle/LPS ($^{*}p < 0.05$), ds-HMGB1/vehicle ($^{***}p < 0.001$), and vehicle/vehicle ($^{***}p < 0.001$) treatment groups. Vehicle/LPS treatment also significantly reduced social exploration compared to ds-HMGB1/vehicle ($^{*}p < 0.05$) and vehicle/vehicle ($^{*}p < 0.05$) treatment groups. At 8 h post-LPS treatment, LPS independent of ds-HMGB1 treatment reduced social exploration compared to non-LPS treated animals ($^{****}p < 0.0001$). $N = 7–8$ animals per experimental group. Data are presented as the mean + SEM.

to differentially modulate the effect of LPS on TNFα gene expression (df = 1, 20; $F = 1.51$, $p = 0.23$), however LPS induced a significant increase in TNFα independent of ds-HMGB1 treatment (df = 1, 20; $F = 9.36$, $p = 0.006$) (data not shown).

3.3. Effect of ds-HMGB1 on priming of the sickness response to LPS

To explore whether the ds-HMGB1-induced potentiation of the neuroinflammatory response has behavioral consequences, we investigated the effect of ds-HMGB1 on LPS-induced decrements in social exploration of a conspecific juvenile. Juvenile social exploration is a widely used behavioral measure of the sickness response (Goshen and Yirmiya, 2009). The effect of fr-HMGB1 on the sickness response to LPS was not explored here given its failure to potentiate the LPS-induced neuroinflammatory response. ICM administration of ds-HMGB1 by itself had no effect on social exploration at any timepoint. LPS reduced social investigation, but importantly, prior ICM administration of ds-HMGB1 potentiated the LPS-induced reductions in social exploration at 3 h post-LPS (LPS× ds-HMGB1 interaction; df = 1, 30; $F = 5.15$, $p = 0.03$), but

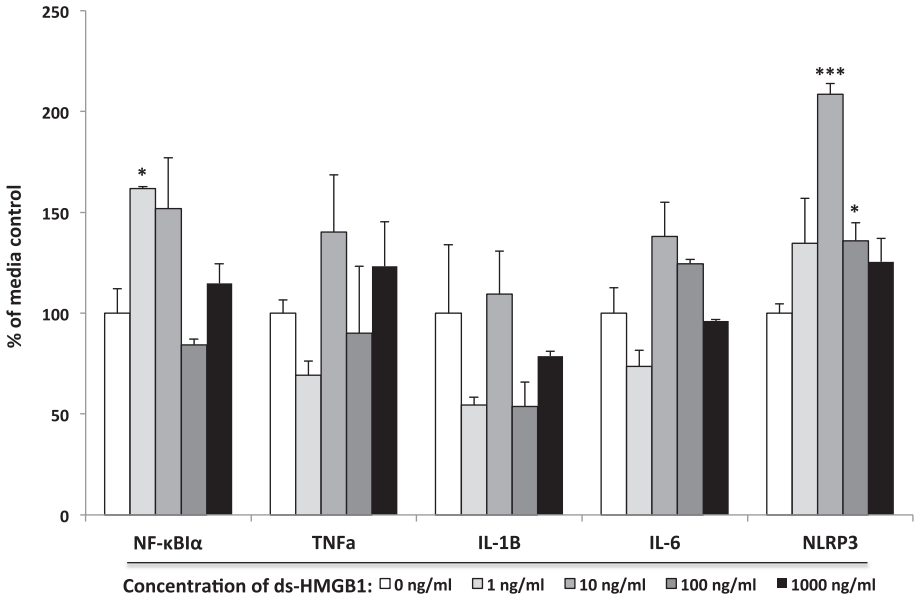

Fig. 4. Effect of ds-HMGB1 on pro-inflammatory mediators in isolated microglia. Whole brain microglia were isolated and directly exposed to several concentrations of ds-HMGB1 for 4 h and mRNA of pro-inflammatory mediators measured. ds-HMGB1 significantly increased NF-κBIα mRNA at 1 ng/ml ($^{*}p < 0.05$) and NLRP3 mRNA at 10 ($^{***}p < 0.001$) and 100 ng/ml ($^{*}p < 0.05$) compared to media control. $N = 4$ replications. Data are presented as the mean + SEM.

failed to potentiate LPS induced sickness behavior at 8 h and 24 h post-LPS injection (Fig. 3). At 8 h post-LPS, the main effect of LPS on social exploration was significant (df = 1, 30; $F = 66.73$, $p < 0.0001$) such that LPS decreased social exploration compared to the non-LPS treated animals. At 24 h post-LPS, exploratory behavior returned to levels comparable to baseline levels.

3.4. Effect of fr-HMGB1 and ds-HMGB1 on pro-inflammatory mediators in isolated microglia and peritoneal macrophages

The observation that ds-HMGB1 primed the neuroinflammatory response as well as the sickness response raised the question of whether this priming effect is due to the direct action of ds-HMGB1 on microglia. To examine this possibility, whole brain microglia were treated with fr-HMGB1 or ds-HMGB1 at varying concentrations for 4 h and pro-inflammatory mediators measured.

ds-HMGB1 failed to significantly alter TNFα (df = 4, 14; $F = 1.55$, $p = 0.26$) and IL-1β (df = 4, 14; $F = 1.84$, $p = 0.2$) gene expression. In addition, ds-HMGB1 failed to alter the concentration of IL-1β protein in the supernatant (df = 4, 14; $F = 0.66$, $p = 0.63$) (Fig. 4). However, the effect of ds-HMGB1 on microglia was significant for NF-κBIα (df = 4, 14; $F = 6.17$, $p = 0.009$), IL-6 (df = 4, 14; $F = 6.3$, $p = 0.008$) and NLRP3 (df = 4, 14; $F = 10.81$, $p = 0.001$) mRNA (Fig. 4) ds-HMGB1 significantly increased NF-κBIα (1 ng/ml ds-HMGB1) and NLRP3 (10 and 100 ng/ml ds-HMGB1) compared to media control. Though the one-way ANOVA for IL-6 was significant, post-hoc tests failed to show any group differences between media control and ds-HMGB1 treatment.

fr-HMGB1 failed to modulate the level of NF-κBIα (df = 4, 15; $F = 0.49$, $p = 0.74$), TNFα (df = 4, 15; $F = 0.35$, $p = 0.84$), IL-1β (df = 4, 15; $F = 0.35$, $p = 0.84$), IL-6 (df = 4, 15; $F = 1.01$, $p = 0.43$) and NLRP3 (df = 4, 15; $F = 0.44$, $p = 0.78$) mRNA. IL-1β protein levels in supernatant were also unchanged by fr-HMGB1 treatment (df = 4, 15; $F = 1.46$, $p = 0.26$) (data not shown).

In light of these results, we examined the effect of ds-HMGB1 and fr-HMGB1 on pro-inflammatory mediators in peritoneal macrophages to determine whether this pattern of pro-inflammatory effects was unique to microglia. In peritoneal macrophages, ds-HMGB1 induced a pattern of changes in pro-inflammatory mediators similar to that observed in microglia. While ds-HMGB1 failed to significantly alter the gene expression of IL-1β (df = 4, 10; $F = 0.41$, $p = 0.8$) and TNFα (df = 4, 10; $F = 0.22$, $p = 0.92$), ds-HMGB1 treatment had a significant effect on NF-κBIα (df = 4, 10; $F = 6.83$, $p = 0.0064$) and NLRP3 (df = 4, 10; $F = 4.42$, $p = 0.016$) mRNA (see Fig. S2 in supplementary data). fr-HMGB1 failed to significantly modulate the gene expression of NF-κBIα (df = 4, 10; $F = 0.12$, $p = 0.97$), TNFα (df = 4, 10; $F = 0.7$, $p = 0.61$), IL-1β (df = 4, 10; $F = 0.94$, $p = 0.48$), and NLRP3 (df = 4, 10; $F = 2.6$, $p = 0.1$) mRNA (data not shown).

3.5. Effect of ds-HMGB1 on priming of the microglial pro-inflammatory response

The effect of ds-HMGB1 on NF-κBIα and NLRP3 *in vitro* paralleled the effects of ds-HMGB1 *in vivo* suggesting that ds-HMGB1 may directly induce a primed immunophenotype in microglia. fr-HMGB1 was not examined in this context given its inability to prime neuroinflammatory responses *in vivo* or induce NF-κBIα and NLRP3 *in vitro*. Therefore, we examined whether prior exposure to ds-HMGB1 potentiates the microglial pro-inflammatory response to LPS.

As before, microglia exposed to ds-HMGB1 alone did not alter TNFα mRNA, IL-1β mRNA, IL-6 mRNA or IL-1β protein. However, prior exposure of microglia to ds-HMGB1 differentially modulated the TNFα mRNA (df = 4, 20; $F = 16.85$, $p < 0.0001$), IL-1β mRNA (df = 4, 20; $F = 38.24$, $p < 0.0001$) and IL-1β protein (df = 4, 20; $F = 4.86$, $p = 0.007$) response to LPS (Fig. 5). ds-HMGB1 potentiated the TNFα mRNA response (1, 10 and 100 ng/ml ds-HMGB1), IL-1β mRNA response (1 and 100 ng/ml ds-HMGB1) and IL-1β protein response (1 and 10 ng/ml ds-HMGB1) to LPS (Fig. 5). The IL-6 mRNA response to LPS was unchanged by ds-HMGB1 (df = 4, 20; $F = 0.72$, $p = 0.59$), however the main effect of LPS was significant (df = 4, 20; $F = 725.2$, $p < 0.0001$) (data not shown).

4. Discussion

The redox state of HMGB1 is a critical determinant of its immunomodulatory properties in peripheral innate immune cells

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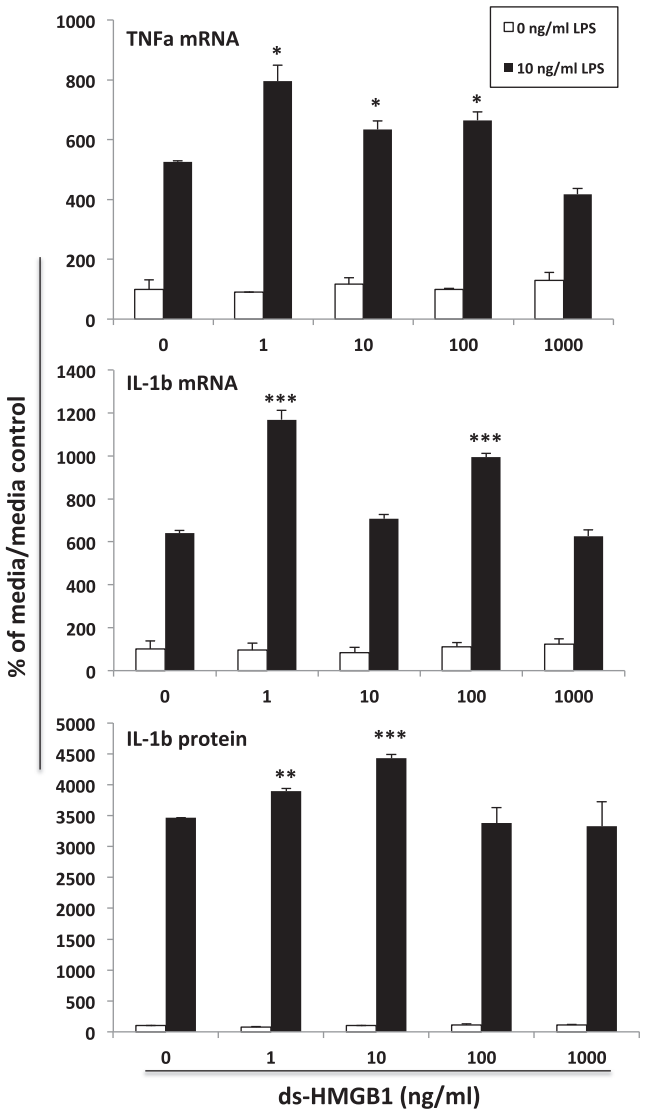

Fig. 5. Effect of ds-HMGB1 on priming of the microglial pro-inflammatory response. Whole brain microglia were isolated and directly exposed to several concentrations of ds-HMGB1 for 4 h. Cells were washed free of ds-HMGB1 and treated with LPS or media for 18 h and pro-inflammatory mediators measured. ds-HMGB1 potentiated the LPS induction of TNFα mRNA (1, 10, and 100 ng/ml), IL-1β mRNA (1 and 100 ng/ml) and extra-cellular IL-1β protein (1 and 10 ng/ml) compared to LPS alone. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the LPS/0 ng/ml ds-HMGB1 condition. $N = 3$ replications. Data are presented as the mean + SEM.

(Venereau et al., 2012), such that the disulfide form is proinflammatory, whereas the reduced form is chemotactic. Our interest here was to further characterize how these different molecular forms of HMGB1 influence neuroinflammatory priming, and more specifically, microglia priming. Indeed, we found here that the redox state of HMGB1 is a critical molecular feature for determining the neuroinflammatory effects of HMGB1.

Prior studies have found that ds-HMGB1 is pro-inflammatory in both primary macrophages as well as macrophage cell lines (Venereau et al., 2012; Yang et al., 2012). Consistent with these findings, we found that ds-HMGB1, when injected ICM, induced pro-inflammatory effects in the hippocampus at 2 h (NF-κBIα mRNA, NLRP3 mRNA and IL-1β protein) and 24 h (NF-κBIα mRNA, TNFα mRNA, and NLRP3 protein) after injection. However, fr-HMGB1 failed to significantly increase the expression level of these pro-inflammatory mediators, which is consistent with prior findings demonstrating that fr-HMGB1 is a chemotactic factor and lacks pro-inflammatory properties (Venereau et al., 2012). It is important to note that the present findings do not exclude the possibility that fr-HMGB1 induced chemotactic processes in the CNS. However, the failure of fr-HMGB1 to induce neuroinflammatory priming suggests that such chemotactic processes, if induced under the experimental conditions employed here, were not sufficient to play a role in the priming of the neuroinflammatory response.

Interestingly, we found that ds-HMGB1 induced a persistent increase in NF-κBIα expression suggesting that ds-HMGB1 treatment induced activation of the NF-κB pathway, which is considered a pivotal regulator of pro-inflammatory gene transcription through TLRs (Kawai and Akira, 2007). Further, the induction of NLRP3 mRNA and protein by ds-HMGB1 also suggests that the NF-κB pathway was activated because NF-kB has been found to induce NLRP3 protein as part of the priming step of the NLRP3 inflammasome (Bauernfeind et al., 2009). Thus, the effects of ds-HMGB1 on NF-κBIα and NLRP3 suggested that ds-HMGB1 induces a primed neuroinflammatory state. To examine this possibility, animals were pretreated with ds-HMGB1 and subsequently exposed to a sub-threshold dose of the pro-inflammatory stimulus LPS. Indeed, prior exposure to ds-HMGB1 potentiated the neuroinflammatory response to LPS. However, fr-HMGB1 failed to potentiate the neuroinflammatory effects of LPS, which is consistent with our observation that fr-HMGB1 failed to induce a shift in basal expression of hippocampal NF-κBIα or NLRP3. To address the behavioral consequences of ds-HMGB1-induced neuroinflammatory priming, we examined whether ds-HMGB1 would potentiate the sickness response to LPS. Such an outcome was expected since ds-HMGB1 potentiated the IL-1β increase produced by LPS, and IL-1β is a pivotal mediator of the sickness response to pathogens and pro-inflammatory stimuli (Goshen and Yirmiya, 2009). Indeed, prior exposure to ds-HMGB1 potentiated the sickness response to LPS (i.e. reduced social exploration of a juvenile). Therefore, the present findings suggest that the neuroinflammatory priming effects of ds-HMGB1 play an important role in its potentiation of the sickness response to pro-inflammatory stimuli.

Microglia are the predominant innate immune cell in the CNS and are thus considered a pivotal mediator of neuroinflammatory processes (Gehrmann et al., 1995). Microglia express TLRs, in particular TLR4 (Ransohoff and Perry, 2009), which has been found to mediate the pro-inflammatory effects of HMGB1 in peripheral innate immune cells (Yang et al., 2010, 2015). Therefore, we directly exposed primary microglia to ds-HMGB1 or fr-HMGB1 and assessed the induction of a pro-inflammatory/primed immunophenotype. Similar to our observations of the effect of ds-HMGB1 *in vivo*, ds-HMGB1 induced an inflammatory phenotype (increased NF-κBIα and NLRP3 mRNA, but not TNFα, IL-1β, or IL-6 mRNA) *in vitro* remarkably similar to the primed neuroinflammatory phenotype induced *in vivo*. Interestingly, the effect of ds-HMGB1 on NF-κBIα and NLRP3 was not dose dependent, but rather exhibited an inverted-U function. It is unclear what may account for this effect, but one possibility is that at high concentrations of ds-HMGB1, increased oxidative stress may have resulted in complete oxidation of ds-HMGB1, which would abrogate the pro-inflammatory properties of ds-HMGB1. fr-HMGB1 failed to alter the pro-inflammatory phenotype of microglia *in vitro*, which is consistent with our observations of the null effects of fr-HMGB1 *in vivo*. Notably, the pro-inflammatory effects of ds-HMGB1 *in vitro* were not unique to microglia as we found that ds-HMGB1 increased NF-κBIα and NLRP3 mRNA in primary peritoneal macrophages as it did in primary microglia. The ds-HMGB1-induced shift in basal expression of NF-κBIα and NLRP3 suggests that ds-HMGB1 directly induces a primed immunophenotype. To determine whether ds-HMGB1 induces a primed phenotype, microglia were pre-exposed to ds-HMGB1 and subsequently

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challenged with the TLR4 agonist LPS. Consistent with our observations *in vivo*, prior exposure to ds-HMGB1 potentiated the pro-inflammatory cytokine response to LPS in primary microglia. Interestingly, the effects of ds-HMGB1 on LPS-induced cytokines were not dose dependent, but exhibited a pattern (inverted-U response) consistent with the effects of ds-HMGB1 on basal expression of NF-κBIα and NLRP3.

There are few studies examining a role for HMGB1 in neuroinflammatory priming. We recently conducted an initial study showing that HMGB1 mediates stress-induced priming of microglia *ex vivo* (Weber et al., 2015). In this study, animals were administered (ICM) the HMGB1 antagonist box A and then exposed to a severe acute stressor. 24 h after termination of the stressor, hippocampal microglia were isolated and challenged with LPS. Consistent with our prior studies (Frank et al., 2007, 2012; Weber et al., 2013), pre-exposure to stress potentiated the proinflammatory response of microglia to LPS. However, box A treatment abrogated this effect of stress on microglia priming. Furthermore, ds-HMGB1 administered ICM was sufficient to prime the pro-inflammatory response of microglia *ex vivo* suggesting that ds-HMGB1 might directly prime microglia. Two key questions arose from this study: (1) does ds-HMGB1 prime the neuroinflammatory and sickness response to an immune challenge and more importantly (2) does ds-HMGB1 directly prime the microglial proinflammatory response. Indeed, the present findings demonstrate that ds-HMGB1 is sufficient to prime the neuroinflammatory and sickness response to a pro-inflammatory challenge. Further, ds-HMGB1 was capable of directly priming the microglia pro-inflammatory response to an immune challenge suggesting that the neuroinflammatory effects of ds-HMGB1 are mediated, in part, through direct actions on microglia. It is likely that the priming effects of ds-HMGB1 on microglia are mediated by the TLR4 signalosome given the findings of Yang and colleagues, who found that the TLR4 adaptor MD-2 is necessary for the pro-inflammatory effects of ds-HMGB1 (Yang et al., 2015). In parallel with its priming effects, ds-HMGB1 consistently induced an immunophenotype characterized by increased NF-κBIα and NLRP3 expression, which play pivotal roles in immune priming (Bauernfeind et al., 2009). These findings suggest that NF-kB induction of NLRP3 may play a key role in the neuroinflammatory priming effects of ds-HMGB1. However, it is important to note that our findings are correlative in nature and do not address a mediating role of the NLRP3 inflammasome in ds-HMGB1-induced priming. Also, it is important to note that our data suggests that ds-HMGB1 exhibits relatively minor pro-inflammatory effects per se compared to the observed priming effects of this molecular form. ds-HMGB1 induced small effects on IL-1β protein (2 h post injection) and TNFα mRNA (24 h post-injection). Further, these proinflammatory changes were not sufficient to elicit a sickness response in the social exploration test. Rather, ds-HMGB1 was only capable of *potentiating* the sickness response after a proinflammatory challenge with LPS. This finding again highlights the priming qualities of ds-HMGB1 under these experimental conditions, which involved a single ICM administration of one dose of ds-HMGB1. Clearly, an increase in dose and/or chronicity of ds-HMGB1 treatment might demonstrate the pro-inflammatory properties per se of ds-HMGB1. On this note, under more chronic neuroinflammatory conditions, HMGB1 has been found to play a mediating role in the neuroinflammatory response associated with seizure (Maroso et al., 2010), ischemia (Kim et al., 2006), chronic pain (Agalave et al., 2014), and alcohol-induced neuroinflammation (Zou and Crews, 2014) suggesting that prolonged HMGB1 signaling is sufficient to elicit a pro-inflammatory response.

Taken together, the present findings suggest that acute increases or exposure to ds-HMGB1, as may occur during acute stress or trauma, may induce a primed immunophenotype in the CNS, which might lead to an exacerbated neuroinflammatory response if exposure to a subsequent pro-inflammatory stimulus occurs.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2015.10.009.

References

Agalave, N.M., Larsson, M., Abdelmoaty, S., Su, J., Baharpoor, A., Lundback, P., Palmblad, K., Andersson, U., Harris, H., Svensson, C.I., 2014. Spinal HMGB1 induces TLR4-mediated long-lasting hypersensitivity and glial activation and regulates pain-like behavior in experimental arthritis. Pain 155, 1802–1813.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Balosso, S., Liu, J., Bianchi, M.E., Vezzani, A., 2014. Disulfide-containing high mobility group box-1 promotes N-methyl-D-aspartate receptor function and excitotoxicity by activating Toll-like receptor 4-dependent signaling in hippocampal neurons. Antioxid. Redox Signaling 21, 1726–1740.

Bauernfeind, F.G., Horvath, G., Stutz, A., Alnemri, E.S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B.G., Fitzgerald, K.A., Hornung, V., Latz, E., 2009. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J. Immunol. 183, 787–791.

Bianchi, M.E., 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. J. Leukocyte Biol. 81, 1–5.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Frank, M.G., Baratta, M.V., Sprunger, D.B., Watkins, L.R., Maier, S.F., 2007. Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. Brain Behav. Immun. 21, 47–59.

Frank, M.G., Thompson, B.M., Watkins, L.R., Maier, S.F., 2012. Glucocorticoids mediate stress-induced priming of microglial pro-inflammatory responses. Brain Behav. Immun. 26, 337–345.

Frank, M.G., Wieseler-Frank, J.L., Watkins, L.R., Maier, S.F., 2006. Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: Immunophenotypic and functional characteristics. J. Neurosci. Methods 151, 121–130.

Gehrmann, J., Matsumoto, Y., Kreutzberg, G.W., 1995. Microglia: intrinsic immuneffector cell of the brain. Brain Res. Brain Res. Rev. 20, 269–287.

Goshen, I., Yirmiya, R., 2009. Interleukin-1 (IL-1): a central regulator of stress responses. Front Neuroendocrinol 30, 30–45.

Holguin, A., Frank, M.G., Biedenkapp, J.C., Nelson, K., Lippert, D., Watkins, L.R., Rudy, J.W., Maier, S.F., 2007. Characterization of the temporo-spatial effects of chronic bilateral intrahippocampal cannulae on interleukin-1beta. J. Neurosci. Methods 161, 265–272.

Hornung, V., Latz, E., 2010. Critical functions of priming and lysosomal damage for NLRP3 activation. Eur. J. Immunol. 40, 620–623.

Johnson, J.D., O'Connor, K.A., Deak, T., Stark, M., Watkins, L.R., Maier, S.F., 2002. Prior stressor exposure sensitizes LPS-induced cytokine production. Brain Behav. Immun. 16, 461–476.

Kawai, T., Akira, S., 2007. Signaling to NF-kappaB by Toll-like receptors. Trends Mol. Med. 13, 460–469.

Kim, J.B., Sig Choi, J., Yu, Y.M., Nam, K., Piao, C.S., Kim, S.W., Lee, M.H., Han, P.L., Park, J.S., Lee, J.K., 2006. HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J. Neurosci. 26, 6413–6421.

Klune, J.R., Dhupar, R., Cardinal, J., Billiar, T.R., Tsung, A., 2008. HMGB1: endogenous danger signaling. Mol. Med. 14, 476–484.

Lamkanfi, M., Kanneganti, T.D., 2010. Nlrp3: an immune sensor of cellular stress and infection. Int. J. Biochem. Cell Biol. 42, 792–795.

Leemans, J.C., Cassel, S.L., Sutterwala, F.S., 2011. Sensing damage by the NLRP3 inflammasome. Immunol. Rev. 243, 152–162.

Liesz, A., Dalpke, A., Mracsko, E., Antoine, D.J., Roth, S., Zhou, W., Yang, H., Na, S.Y., Akhisaroglu, M., Fleming, T., Eigenbrod, T., Nawroth, P.P., Tracey, K.J., Veltkamp, R., 2015. DAMP signaling is a key pathway inducing immune modulation after brain injury. J. Neurosci. 35, 583–598.

Lu, B., Wang, H., Andersson, U., Tracey, K.J., 2013. Regulation of HMGB1 release by inflammasomes. Protein Cell 4, 163–167.

Maroso, M., Balosso, S., Ravizza, T., Liu, J., Aronica, E., Iyer, A.M., Rossetti, C., Molteni, M., Casalgrandi, M., Manfredi, A.A., Bianchi, M.E., Vezzani, A., 2010. Toll-like

receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat. Med. 16, 413–419.

Pan, Y., Chen, X.Y., Zhang, Q.Y., Kong, L.D., 2014. Microglial NLRP3 inflammasome activation mediates IL-1beta-related inflammation in prefrontal cortex of depressive rats. Brain Behav. Immun. 41, 90–100.

Ransohoff, R.M., Perry, V.H., 2009. Microglial physiology: unique stimuli, specialized responses. Annu. Rev. Immunol. 27, 119–145.

Sun, S.C., Ganchi, P.A., Ballard, D.W., Greene, W.C., 1993. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science 259, 1912–1915.

Venereau, E., Casalgrandi, M., Schiraldi, M., Antoine, D.J., Cattaneo, A., De Marchis, F., Liu, J., Antonelli, A., Preti, A., Raeli, L., Shams, S.S., Yang, H., Varani, L., Andersson, U., Tracey, K.J., Bachi, A., Uguccioni, M., Bianchi, M.E., 2012. Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. J. Exp. Med. 209, 1519–1528.

Weber, M.D., Frank, M.G., Sobesky, J.L., Watkins, L.R., Maier, S.F., 2013. Blocking toll-like receptor 2 and 4 signaling during a stressor prevents stress-induced priming of neuroinflammatory responses to a subsequent immune challenge. Brain Behav. Immun.

Weber, M.D., Frank, M.G., Tracey, K.J., Watkins, L.R., Maier, S.F., 2015. Stress induces the danger-associated molecular pattern HMGB-1 in the hippocampus of male sprague dawley rats: a priming stimulus of microglia and the NLRP3 inflammasome. J. Neurosci. 35, 316–324.

Yang, H., Hreggvidsdottir, H.S., Palmblad, K., Wang, H., Ochani, M., Li, J., Lu, B., Chavan, S., Rosas-Ballina, M., Al-Abed, Y., Akira, S., Bierhaus, A., Erlandsson-Harris, H., Andersson, U., Tracey, K.J., 2010. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc. Natl. Acad. Sci. U. S. A. 107, 11942–11947.

Yang, H., Lundback, P., Ottosson, L., Erlandsson-Harris, H., Venereau, E., Bianchi, M. E., Al-Abed, Y., Andersson, U., Tracey, K.J., Antoine, D.J., 2012. Redox modification of cysteine residues regulates the cytokine activity of high mobility group box-1 (HMGB1). Mol. Med. 18, 250–259.

Yang, H., Ochani, M., Li, J., Qiang, X., Tanovic, M., Harris, H.E., Susarla, S.M., Ulloa, L., Wang, H., DiRaimo, R., Czura, C.J., Wang, H., Roth, J., Warren, H.S., Fink, M.P., Fenton, M.J., Andersson, U., Tracey, K.J., 2004. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. Proc. Natl. Acad. Sci. U. S. A. 101, 296–301.

Yang, H., Wang, H., Ju, Z., Ragab, A.A., Lundback, P., Long, W., Valdes-Ferrer, S.I., He, M., Pribis, J.P., Li, J., Lu, B., Gero, D., Szabo, C., Antoine, D.J., Harris, H.E., Golenbock, D.T., Meng, J., Roth, J., Chavan, S.S., Andersson, U., Billiar, T.R., Tracey, K.J., Al-Abed, Y., 2015. MD-2 is required for disulfide HMGB1-dependent TLR4 signaling. J. Exp. Med. 212, 5–14.

Zou, J.Y., Crews, F.T., 2014. Release of neuronal HMGB1 by ethanol through decreased HDAC activity activates brain neuroimmune signaling. PloS one 9, e87915.

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Neurobiology of Disease

Stress Induces the Danger-Associated Molecular Pattern HMGB-1 in the Hippocampus of Male Sprague Dawley Rats: A Priming Stimulus of Microglia and the NLRP3 Inflammasome

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Exposure to acute and chronic stressors sensitizes the proinflammatory response of microglia to a subsequent immune challenge. However, the proximal signal by which stressors prime microglia remains unclear. Here, high mobility group box-1 (HMGB-1) protein was explored as a potential mediator of stress-induced microglial priming and whether HMGB-1 does so via the nucleotide-binding domain, leucine-rich repeat, pyrin domain containing protein 3 (NLRP3) inflammasome. Exposure to 100 inescapable tail shocks (ISs) increased HMGB-1 and NLRP3 protein in the hippocampus and led isolated microglia to release HMGB-1 *ex vivo*. To determine whether HMGB-1 signaling is necessary for stress-induced sensitization of microglia, the HMGB-1 antagonist BoxA was injected into the cisterna magna before IS. Hippocampal microglia were isolated 24 h later and stimulated with LPS *ex vivo* to probe for stress-induced sensitization of proinflammatory responses. Previous IS potentiated gene expression of NLRP3 and proinflammatory cytokines to LPS, that is, microglia were sensitized. Treatment with BoxA abolished this effect. To determine whether HMGB-1 is sufficient to prime microglia, IS was replaced with intracerebral administration of disulfide or fully reduced HMGB-1. Intracerebral disulfide HMGB-1 mimicked the effect of the stressor, because microglia isolated from HMGB-1-treated rats expressed exaggerated NLRP3 and proinflammatory cytokine expression after LPS treatment, whereas fully reduced HMGB-1 had no effect. The present results suggest that the CNS innate immune system can respond to an acute stressor as if it were cellular damage, thereby releasing the danger signal HMGB-1 in the brain to prime microglia by acting on the NLRP3 inflammasome, in preparation for a later immune challenge.

Key words: **DAMPS; HMGB-1; inflammasome; microglia; neuroinflammation; stress**

Introduction

Exposure to stressors can lead to a proinflammatory environment within the brain, an outcome thought to be critical to the potential psychopathological effect of stressors (Gadek-Michalska et al., 2013, Fillman et al., 2014). Both acute and chronic stressors prime microglia (Frank et al., 2007) and amplify the neuroinflammatory response to a subsequent peripheral (Johnson et al., 2002, Wohleb et al., 2012) or central (de Pablos et al., 2006; Espinosa-Oliva et al., 2011) inflammatory challenge. The mechanism(s) by which stressors have neuroinflammatory effects is essentially unknown. However, blockade of the pattern recognition receptors Toll-like receptor (TLR) 2 and TLR4 in the CNS during exposure to an acute stressor prevents the development of stress-induced exaggerated neuroinflammatory responses to later lipopolysaccharide (LPS; Weber et al., 2013). Thus, stressors must induce the release of an endogenous ligand within the CNS that signals via TLR2 and TLR4 to mediate neuroinflammatory sensitization produced by stressors. However, there are currently no known transmitters or modulators released in the brain by stressors that act at TLRs.

Although TLRs on peripheral immune cells were first thought to recognize only pathogen-associated molecular patterns, more recent findings suggest that a variety of conditions can lead to the release of endogenous molecules called alarmins or danger-associated molecular patterns (DAMPs; Bianchi, 2007) that can also signal at TLRs (Park et al., 2004). The concept has developed that innate immune receptors discriminate between danger and non-danger rather than self and non-self (Matzinger, 2002).

High mobility group box-1 protein (HMGB-1) is perhaps the most studied alarmin. When released in the periphery, HMGB-1 interacts with TLR2, TLR4, and the receptor for advanced glycation end products (RAGE) in which it acts as both a chemotactic and proinflammatory mediator (van Zoelen et al., 2009).

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The authors declare no competing financial interests.

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HMGB-1 plays a proinflammatory role in sterile injury and cellular stress in the periphery (Venereau et al., 2013) and is involved in a number of diseases (Kang et al., 2014).

Importantly, HMGB-1 has been reported to be present in the CNS and to mediate neuroinflammatory responses to ischemia and other injuries (Yang and Tracey, 2009). Here, we tested the novel idea that the CNS innate immune system responds to stress as "danger," thus releasing HMGB-1 in brain, and that HMGB-1 primes neuroinflammatory processes in the event of a later immune challenge. If so, this would provide a new perspective on the role of HMGB-1 and suggest that the meaning of danger as it applies to innate immunity needs to be expanded. In addition, a new mechanism by which stress acts on the brain would be indicated. We also explored the mechanism by which stress-induced HMGB-1 primes proinflammatory immune responses. HMGB-1 in the periphery has been demonstrated to induce the expression of the nucleotide-binding domain, leucine-rich repeat, pyrin domain containing protein 3 (NLRP3) inflammasome (Xiang et al., 2011). The NLRP3 inflammasome mediates maturation and secretion of IL-1β and is the only known inflammasome that requires a priming stimulus before it becomes active. Therefore, we also explored whether stress increases NLRP3 and whether stress-induced HMGB-1 modulates NLRP3 expression.

Materials and Methods

Animals

Male Sprague Dawley rats (60–90 d-old; Harlan Sprague Dawley) 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.). All rats were allowed 1 week of acclimatization to the colony rooms before experimentation. All experimental procedures were conducted in accordance with the University of Colorado Institutional Animal Care and Use Committee.

Experimental design

Experiment 1: effect of inescapable tail shock on hippocampal HMGB-1 and NLRP3. Rats were killed immediately (0 h) or 24 h after inescapable tail shock (IS) exposure or served as home cage controls (HCCs). Hippocampal HMGB-1, NLRP3, and nuclear factor-κB (NF-κB) protein levels were measured.

Experiment 2: effect of IS on hippocampal microglial release of HMGB-1. An increased level of HMGB-1 protein in hippocampal tissue does not necessarily indicate that HMGB-1 was secreted because it could have remained intracellular. Therefore, HMGB-1 release was measured from microglial cells of rats that had been exposed to IS or served as HCCs. Immediately after IS exposure (0 h), IS and HCC rats were killed, and hippocampal microglia were isolated. Equal numbers of microglia (5×10^4/100 μl) from each subject were incubated for 24 h, HMGB-1 protein was measured in supernatants, and microglial cell viability was determined.

Experiment 3: effect of the HMGB-1 antagonist BoxA on IS-induced priming of microglia. Previous work has shown that exposure to IS primes microglia as assessed *ex vivo* in the sense that these microglia produce exaggerated inflammatory mediators in response to LPS (Frank et al., 2007). Rats were anesthetized with isoflurane (~3 min). The dorsal aspect of the skull was shaved and swabbed with 70% EtOH, and a 27 gauge needle, attached via polyethylene-50 tubing to a 25 μl Hamilton syringe, was inserted into the cisterna magna [intracisterna magna (ICM)]. To verify entry into the cisterna magna, ~2 μl of clear CSF was drawn and gently pushed back. Ten micrograms of BoxA (HMGBiotech; certified LPS free) were administered, suspended in 5 μl of pyrogen-free, sterile H_2O. An equal volume of sterile H_2O was injected ICM for vehicle control rats. ICM administration was used to avoid implanting cannulae, which itself produces enduring neuroinflammation (Holguin et al., 2007). Rats were exposed to IS or served as HCCs immediately after full recovery from the brief anesthesia. Hippocampal microglia were isolated from IS and HCC rats 24 h after stressor termination. Microglia were suspended in media, and microglia concentration was determined by trypan blue exclusion. Microglia concentration was adjusted for each rat to yield an equal number of microglia (1×10^4/100 μl) for each *in vitro* condition across all rats. One hundred microliters were added to individual wells of a 96-well V-bottom plate. Cells were incubated with LPS (0, 0.1, 1, 10, or 100 ng/ml) for 4 h because we have determined previously that this concentration range and time of LPS exposure is optimal for microglial production of proinflammatory cytokines (Frank et al., 2006). Cells were washed in 1× PBS. Cells were lysed/homogenized and cDNA synthesis was performed according to the protocol of the manufacturer using SuperScript III CellsDirect cDNA Synthesis System (Invitrogen). Gene expression of proinflammatory cytokines was measured using real-time RT-PCR. Data from three cohorts were collected.

Experiment 4: effect of disulfide and fully reduced HMGB-1 on microglial proinflammatory response to LPS. Recent studies demonstrate that the redox state of HMGB-1 is a key determinant in the receptor interaction and immunological activity of HMGB-1. When cysteines in position C23, C45, and C106 are fully reduced (fully reduced HMGB-1), HMGB-1 functions as a chemotactic signaling protein but does not stimulate proinflammatory cytokines (Venereau et al., 2012). However, HMGB-1 functions as a proinflammatory mediator if a disulfide bond is formed between C23 and C45 under oxidizing conditions, whereas C106 remains in a reduced state (disulfide HMGB-1; Yang et al., 2012). Oxidation at all three cysteines abrogates both the chemotactic and proinflammatory properties of HMGB-1 (oxidized HMGB-1; Venereau et al., 2012). Thus, HMGB-1 orchestrates both chemotaxis and induction of inflammatory cytokines depending on the redox state of the protein. Therefore, both disulfide and fully reduced HMGB-1 were investigated. Oxidized HMGB-1 was omitted because it has not been associated with any *in vivo* function.

One microgram of disulfide or fully reduced HMGB-1 (HMGBiotech) was suspended in 10 μl of sterile water and administered ICM. Twenty-four hours later, equal numbers of hippocampal microglia (1×10^4 cells/LPS condition) were incubated with LPS (0, 0.1, 1, 10, or 100 ng/ml) for 4 h. The plate was washed in 1× PBS, cDNA synthesis performed, and gene expression of proinflammatory cytokines was measured using real-time RT-PCR.

General procedures

IS. Rats were placed in Plexiglas tubes (23.4 cm length × 7 cm diameter) and exposed to 100 1.6 mA, 5 s tail shocks with a variable intertrial interval (ITI) ranging from 30 to 90 s (average ITI, 60 s). All IS treatments occurred between 9:00 A.M. and 11:00 A.M. IS rats were returned to their home cages immediately after termination of shock. HCC rats remained undisturbed in their home cages.

Tissue collection. Rats were injected with a lethal dose of sodium pentobarbital. During deep anesthesia, rats were transcardially perfused with ice-cold saline (0.9%) for 3 min to remove peripheral immune cells from the CNS vasculature. Brains were rapidly extracted, placed on ice, and hippocampus dissected. For *in vivo* experiments, hippocampus was flash frozen in liquid nitrogen and stored at −80°C. For *ex vivo* experiments, microglia were isolated immediately (see below for procedure).

Western blot. Hippocampus was sonicated in a mixture containing extraction buffer (Invitrogen) and protease inhibitors (Sigma). Ice-cold tissue samples were centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was removed, and the protein concentration for each sample was quantified using the Bradford method. Samples were heated to 75°C for 10 min and loaded into a standard polyacrylamide Bis-Tris gel (Invitrogen). SDS-PAGE was performed in 3-(*N*-morpholino)-propanesulfonic acid running buffer (Invitrogen) at 175 V for 1.25 h. Protein was transferred onto a nitrocellulose membrane using the iblot dry transfer system (Invitrogen). The membrane was blocked with Odyssey blocking buffer (LI-COR Biosciences) for 1 h and incubated with a primary antibody in blocking buffer overnight at 4°C. The following day, the membrane was washed in 1× PBS containing Tween 20 (0.1%) and then incubated in blocking buffer containing either goat anti-rabbit or goat anti-mouse (LI-COR) IRDye 800CW secondary antibody at a concentration of 1:10,000 (LI-COR) for 1 h at room temperature. Protein expression was quantified using an Odyssey Infrared Imager (LI-COR)

Table 1. Primer description and sequences

Gene	Primer sequence 5′ → 3′ Forward	Reverse	Function
β-actin	TTCCTTCCTGGGTATGGAAT	GAGGAGCAATGATCTTGATC	Cytoskeletal protein (housekeeping gene)
CD163	TCATTCAACCCTCACTGCAC	CTTGAGGAAACTGTAAGCCG	Macrophage antigen not expressed by microglia
GFAP	AGATCCGAGAAACCAGCCTG	CCTTAATGACCTCGCCATCC	Astrocyte antigen
IL-1β	CCTTGTGCAAGTGTCTGAAG	GGGCTTGGAAGCAATCCTTA	Proinflammatory cytokine
IL-6	AGAAAAGAGTTGTGCAATGGCA	GGCAAATTTCCTGGTTATATCC	Proinflammatory cytokine
MHCII	AGCACTGGGAGTTTGAAGAG	AAGCCATCACCTCCTGGTAT	Microglia/macrophage antigen
IκBα	CACCAACTACAACGGCCACA	GCTCCTGAGCGTTGACATCA	Induced by NF-κB to inhibit NF-κB function
TNFα	CAAGGAGGAGAAGTTCCCA	TTGGTGGTTTGCTACGACG	Proinflammatory cytokine

and expressed as a ratio to their housekeeping protein. HMGB-1 protein was measured in cell culture supernatants using identical Western conditions except that measurement of GAPDH was excluded. Primary antibodies included rabbit anti-rat HMGB-1 monoclonal antibody (1:4000; Abcam), rabbit anti-rat NLRP3 monoclonal antibody (1:1000; Abcam), mouse anti-rat active NF-κB monoclonal antibody (1:1000; Millipore), rabbit anti-rat GAPDH monoclonal antibody (1:200,000 Abcam), and mouse anti-rat β-actin (1:200,000; Sigma-Aldrich).

Microglia isolation and culture conditions. Hippocampal microglia were isolated using a Percoll density gradient as described previously (Frank et al., 2006). We have shown previously (Frank et al., 2006) that this microglia isolation procedure yields highly pure (>95%) microglia [ionized calcium-binding adapter molecule 1-positive/major histocompatibility complex II (MHCII)-positive/cluster of differentiation 163 (CD163)-negative/glial fibrillary acidic protein (GFAP)-negative]. Immunophenotype and purity of microglia was assessed and verified using real-time RT-PCR of MHCII, CD163, and GFAP. Microglia were routinely found to be MHCII-positive/CD163-negative/GFAP-negative (data not shown). 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% CO_2 under the experimental conditions described above.

MTT assay of cell viability. Microglia were incubated with 12 mM MTT (Life Technologies) at 37°C for 4 h. Fifty microliters of DMSO were added to each well and incubated at 37°C for 10 min. Absorbance was measured at 540 nm, and cell viability was determined according to the protocol of the manufacturer (Life Technologies).

Real-time RT-PCR measurement of gene expression. A detailed description of the PCR amplification protocol has been published previously (Frank et al., 2006). cDNA sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Primer sequences were designed using the Eurofins MWG Operon Oligo Analysis and Plotting Tool (http://www.operon.com/technical/toolkit.aspx) and tested for sequence specificity using the Basic Local Alignment Search Tool at the NCBI (Altschul et al., 1997). Primers were obtained from Invitrogen. Primer specificity was verified by melt curve analysis. All primers were designed to span exon/exon boundaries and thus exclude amplification of genomic DNA (for primer description and sequences, see Table 1). PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR kit (Qiagen). Formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Relative gene expression was determined by taking the expression ratio of the gene of interest to β-actin.

Statistical analysis and data presentation. All data are presented as mean ± standard error of the mean (SEM). Statistical analysis consisted of Student's *t* test or ANOVA using Prism 5 (GraphPad Software). Omnibus *F* values are reported for each ANOVA and serve as a criterion for *post hoc* analysis (Newman–Keuls test). Threshold for statistical significance was set at $\alpha = 0.05$.

Results

Stress exposure induces hippocampal microglia to release HMGB-1

The first goal was to determine whether HMGB-1 is increased in the hippocampus after stress exposure. The hippocampus was targeted because it is an area that shows robust IS-induced priming of neuroinflammatory processes *in vivo* (Johnson et al., 2002) and *ex vivo* (Frank et al., 2007) and yields a sufficient number of microglia to perform *ex vivo* experiments. There was a significant interaction between IS vs HCC and 0 vs 24 h post-stress treatment ($F_{(1,23)} = 11.97$, $p < 0.01$). *Post hoc* analysis revealed that HMGB-1 protein was significantly increased 0 h after IS compared with HCC rats ($p < 0.001$) and remained significantly elevated 24 h after IS compared with HCC rats ($p < 0.01$; Fig. 1*A*). This result provided initial evidence that HMGB-1 is modulated by stress. However, HMGB-1 is located primarily in the nucleus and is not considered to be an alarmin unless released into the extracellular space (Lotze and Tracey, 2005). Simply measuring HMGB-1 protein or mRNA in brain tissue cannot indicate whether the increased HMGB-1 was secreted. Microglia were targeted for such an analysis because they are the predominant innate immune cell in the brain. To measure released HMGB-1, rats were exposed to IS or served as HCCs, and hippocampal microglia were isolated immediately after stress treatment. HMGB-1 was measured in the supernatant after a 24 h incubation period, which allowed sufficient time for detectable levels of protein to accumulate. Previous exposure to IS led microglia to release an increased amount of HMGB-1 compared with microglia isolated from HCC rats ($t_{(13)} = 5.429$, $p < 0.0001$; Fig. 1*B*). Although we have demonstrated previously that the isolation procedure does not change the immunophenotype of microglia (Frank et al., 2006), the presence of HMGB-1 in the supernatant of HCC microglia suggests that the culture conditions contribute to the release of HMBG-1 *ex vivo*, and this should be noted. Nonetheless, HMGB-1 was significantly elevated in the supernatant from microglia exposed to IS. Of course, the IS-induced increase of HMGB-1 could be attributable to cell death. Therefore, an MTT assay was performed to measure cell viability of microglia after the supernatant was removed. We found no difference in cell viability ($t = 0.933$, $p = 0.93$; Fig. 1*C*), demonstrating that the elevated levels of HMGB-1 are likely not attributable to microglia death, thus suggesting that IS-induced active release of HMGB-1 from microglia into supernatant.

Stress induces NLRP3 and NF-κB protein in hippocampus

Next, we examined NLRP3 and upstream signaling markers in hippocampal tissue after stress exposure. NLRP3 protein was significantly increased 0 and 24 h ($F_{(2,23)} = 3.907$, $p < 0.05$; Fig. 2*B*) after stress treatment compared with HCC rats. However, NLRP3 mRNA was not significantly changed by stress exposure (Fig. 2*A*). NLRP3 is induced by the transcription factor NF-κB, and so we measured NF-κB as an upstream NLRP3 marker (Bauernfeind et al., 2009). We used an antibody that binds to an epitope of the p65 subunit that is exposed after degradation of the inhibitory chain nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), thus representing active NF-κB. Stress

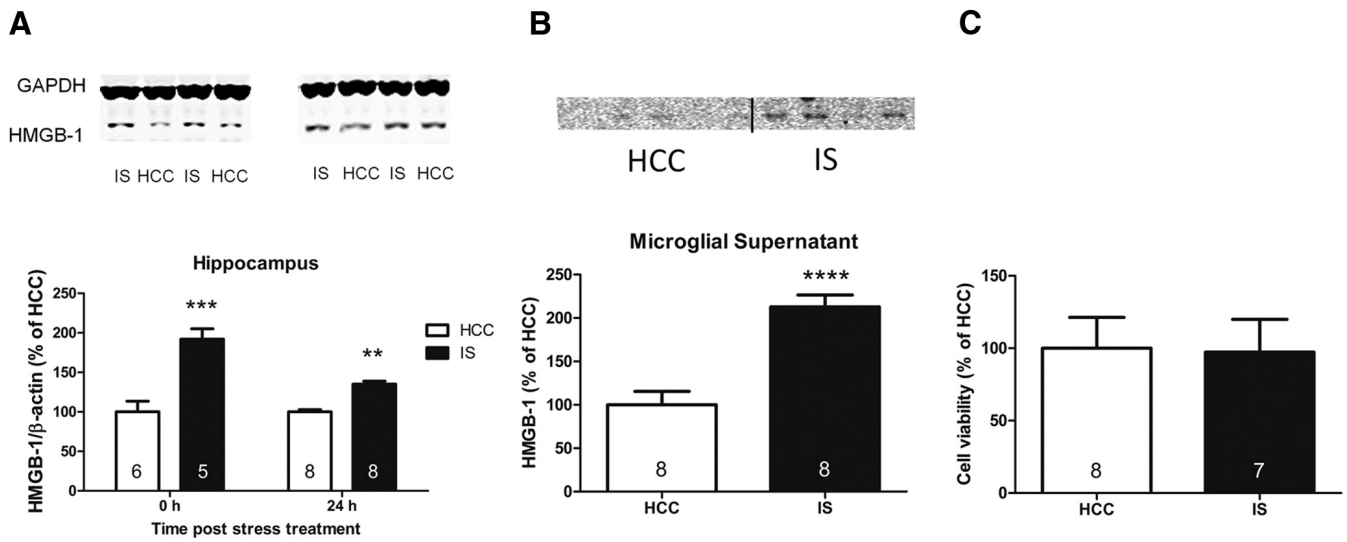

Figure 1. Effect of IS on HMGB-1. ***A***, HMGB-1 is increased in the hippocampus 0 and 24 h after IS compared with HCCs. ***B***, Hippocampal microglia were isolated 0 h after IS or from HCCs and incubated for 24 h. HMGB-1 is increased in the supernatant of microglia isolated from rats exposed to IS. ***C***, There was no difference in cell viability between microglia isolated from IS or HCC treatment. The graphs show mean and SEM. Sample sizes are represented in the bar graph. Representative Western blots are shown above each graph when appropriate. $^{**}p < 0.01$, $^{***}p < 0.001$ versus the HCC group.

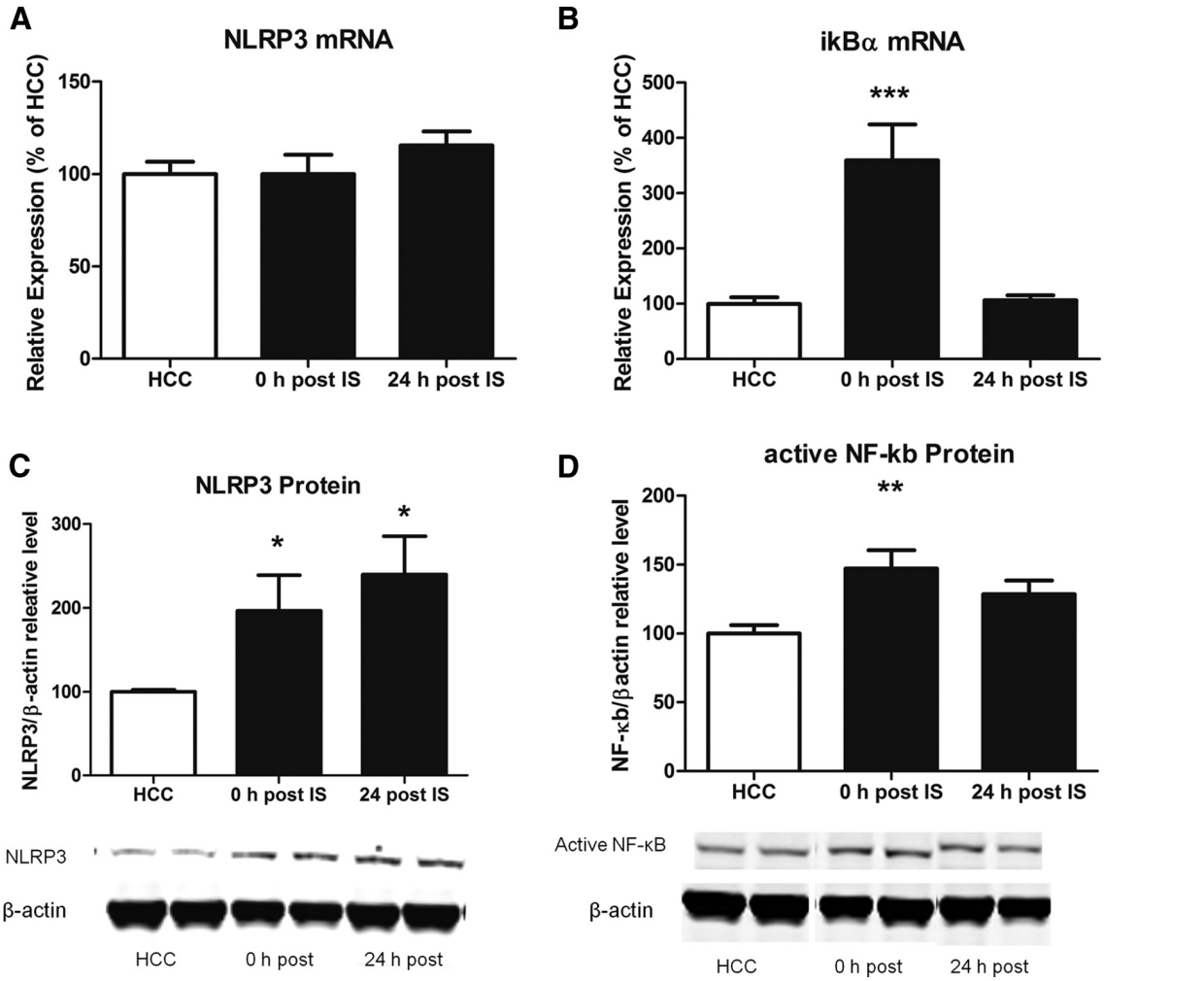

Figure 2. Effect of IS on hippocampal NLRP3 and NF-κB. ***A***, NLRP3 mRNA is not changed 0 and 24 h after IS compared with HCCs. ***B***, IκBα is increased 0 h, but not 24 h, after IS. ***C***, NLRP3 protein is increased 0 and 24 h after IS compared with HCCs. ***D***, Active NF-κB protein is increased 0 h, but not 24 h, after IS compared with HCCs. The graphs show mean and SEM. Representative Western blots are shown below each graph. $n = 8$ per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the HCC group.

exposure increased activated NF-κB at 0 h ($F_{(2,23)} = 5.537$, $p < 0.01$) but not 24 h after stress treatment (Fig. 2*D*). Similarly, IκBα gene expression was increased 0 h ($F_{(2,23)} = 12.79$, $p < 0.001$) but not 24 h after stress exposure (Fig. 2*B*).

The HMGB-1 antagonist BoxA prevents stress-induced sensitization of the microglial proinflammatory response to LPS *ex vivo*

If HMGB-1 signaling is critical to the mediation of IS-induced microglial priming, then blocking HMGB-1 signaling during stress exposure should prevent subsequent stress-induced sensitization of the microglial proinflammatory response. HMGB-1 has three major protein domains consisting of two tandem DNA binding domains (BoxA and BoxB) and an acidic C terminus. The BoxB domain is critical for the proinflammatory actions of HMGB-1 (Li et al., 2003). The BoxA fragment will bind to receptors but does not initiate proinflammatory actions. However, BoxA occupancy of TLRs will keep HMGB-1 from binding and acts as a competitive antagonist to HMGB-1, displacing radiolabeled HMGB-1 from receptors on macrophages without any intrinsic inflammatory activity (Yang et al., 2004). It is highly selective in that it is the binding fragment of HMGB-1 and so binds only where HMGB-1 would bind. BoxA (10 μg) was injected ICM before IS or control treatment. Hippocampal microglia were isolated 24 h later and stimulated with LPS *ex vivo*. To determine whether BoxA blocked stress-induced sensitization of the microglia proinflammatory response, area under the curve (AUC) for LPS concentration was computed for each subject to reflect the cumulative proinflammatory response to LPS. IS significantly potentiated interleukin-1β (IL-1β) gene expression to LPS, which was blocked by BoxA (interaction between IS vs HCC and vehicle vs BoxA, $F_{(1,20)} = 6.142$, $p < 0.05$; Fig. 3*A*). A similar interaction was observed with IκBα ($F_{(1,20)} = 4.839$, $p < 0.05$; Fig. 3*B*). In addition, IS significantly potentiated NLRP3 gene expression to LPS, which was blocked by BoxA treatment (interaction between IS vs HCC and vehicle vs BoxA, $F_{(1,20)} = 4.094$, $p < 0.05$; Fig. 3*C*). To verify that BoxA is not a general TLR2/4 antagonist but acts selectively against HMGB-1, naive hippocampal microglia were incubated with 2 μg/ml BoxA and 100 ng/ml LPS (TLR4 agonist) or 100 ng/ml Pam3CysSerLys4 (TLR2 agonist) for 4 h. IL-1β gene expression was measured as an indicator of the microglial inflammatory response to each TLR agonist. Both LPS and Pam3csk4 increased IL-1β expression, as would be expected, and BoxA did not at all reduce this increase (Fig. 3*D*,*E*).

ICM administration of disulfide HMGB-1, but not fully reduced HMGB-1, primes the proinflammatory response of microglia to LPS *ex vivo*

To further examine whether HMGB-1 primes proinflammatory neuroimmune responses and acts in the brain as it does in the periphery, recombinant disulfide or fully reduced HMGB-1 was injected ICM, and the proinflammatory response of hippocampal microglia to LPS was assessed 24 h after injection. Disulfide HMGB-1 significantly modulated the microglia proinflammatory response to LPS *ex vivo* for IL-1β (interaction between vehicle and disulfide HMGB-1 vs LPS dose, $F_{(4,30)} = 6.933$, $p < 0.001$), IL-6 ($F_{(4,30)} = 4.984$, $p < 0.01$), tumor necrosis factor-α 32 (TNFα; $F_{(4,30)} = 6.970$, $p < 0.001$), IκBα ($F_{(4,30)} = 2.994$, $p < 0.05$), and NLRP3 ($F_{(4,30)} = 7.461$, $p < 0.001$) gene expression (Fig. 4). *Post hoc* analysis showed that disulfide HMGB-1 potentiated the proinflammatory response of IL-1β (10 ng/ml LPS, $p < 0.01$; 100 ng/ml, $p < 0.0001$), IL-6 (100 ng/ml LPS, $p < 0.001$), TNFα (10 ng/ml LPS, $p < 0.05$; 100 ng/ml, $p < 0.0001$), IκBα (100 ng/ml LPS, $p < 0.01$), and NLRP3 (100 ng/ml, $p < 0.0001$) compared with vehicle-treated rats. The AUC was computed for each subject to compare the cumulative LPS proinflammatory response between HMGB-1- and vehicle-treated rats. Administration of disulfide HMGB-1 resulted in a significant increase in AUC for IL-1β ($t_{(6)} = 5.568$, $p < 0.01$), IL-6 ($t_{(6)} = 2.618$, $p < 0.05$), TNFα ($t_{(6)} = 3.032$, $p < 0.05$), IκBα ($t_{(6)} = 2.734$, $p < 0.05$), and NLRP3 ($t_{(6)} = 2.967$, $p < 0.05$). In contrast, fully reduced HMGB-1 failed to significantly affect the proinflammatory response of microglia to LPS for IL-1β ($p = 0.80$), IL-6 ($p = 0.75$), TNFα ($p = 0.87$), IκBα ($p = 0.77$), or NLRP3 ($p = 0.62$) compared with vehicle-treated rats (Fig. 5).

Discussion

The present experiments provide the first evidence that exposure to a stressor modulates the alarmin/DAMP HMGB-1 in the CNS and that HMGB-1 primes microglia and amplifies subsequent neuroinflammatory responses. Here, an acute stressor increased HMGB-1 in the hippocampus, an increase still present 24 h later. HMGB-1 can be released via two distinct mechanisms. The first can yield release from any cell type undergoing non-apoptotic death. The second has been characterized primarily in innate immune cells and involves secretory processes in the absence of cell death (Lotze and Tracey, 2005; Klune et al., 2008). Because microglia are the predominant innate immune cells in the CNS, we examined whether stress induces microglia to actively secrete HMGB-1. Consistent with the effects of stress on hippocampal HMGB-1 *in vivo*, hippocampal microglia were found to release HMGB-1 *ex vivo* after stress exposure. It is important that microglia cell viability did not differ between stress and non-stress groups, suggesting that stress induces microglia to actively secrete HMGB-1, a previously unknown phenomenon. However, the present results do not exclude the possibility that other CNS cells produce HMGB-1 in response to stress (Qiu et al., 2008).

Extracellular HMGB-1 acts via RAGE to signal immune cell migration and TLR2/TLR4 to induce proinflammatory mediators (Park et al., 2004). We demonstrated previously that CNS blockade of TLR2 and TLR4 during IS prevents stress-induced priming of the microglial proinflammatory response (Weber et al., 2013). This finding suggested that stress induces the release of an endogenous ligand in CNS, which primes microglia through TLRs. Therefore, we sought to examine the role of HMGB-1 during and after stress. To do so, we used the HMGB-1 antagonist BoxA. BoxA was administered ICM before stress exposure, and hippocampal microglia were isolated 24 h later to characterize their response to LPS *ex vivo*. Microglia incubated in media alone expressed low levels of inflammatory genes, regardless of stress or BoxA treatment. However, IS potentiated the proinflammatory response to LPS, an effect that was blocked by BoxA. That is, BoxA prevented microglia from becoming sensitized and supports the *ex vivo* finding that IS induces the release of HMGB-1 from microglia. The half-life of BoxA is unknown, so a potential confounding factor is that BoxA could be present at microglia and act as a general TLR2 and TLR4 antagonist, thereby blocking the ability of LPS to stimulate proinflammatory cytokines. However, this possibility is unlikely because BoxA did not have any tendency to reduce the inflammatory effect of the TLR2 agonist Pam3csk4 or the TLR4 agonist LPS on microglia *in vitro*, supporting the contention that BoxA is specifically an HMGB-1 antagonist.

As noted, HMGB-1 in the periphery can have different functions depending on the redox states at three cysteine sites that dictate receptor interaction and proinflammatory activity. Im-

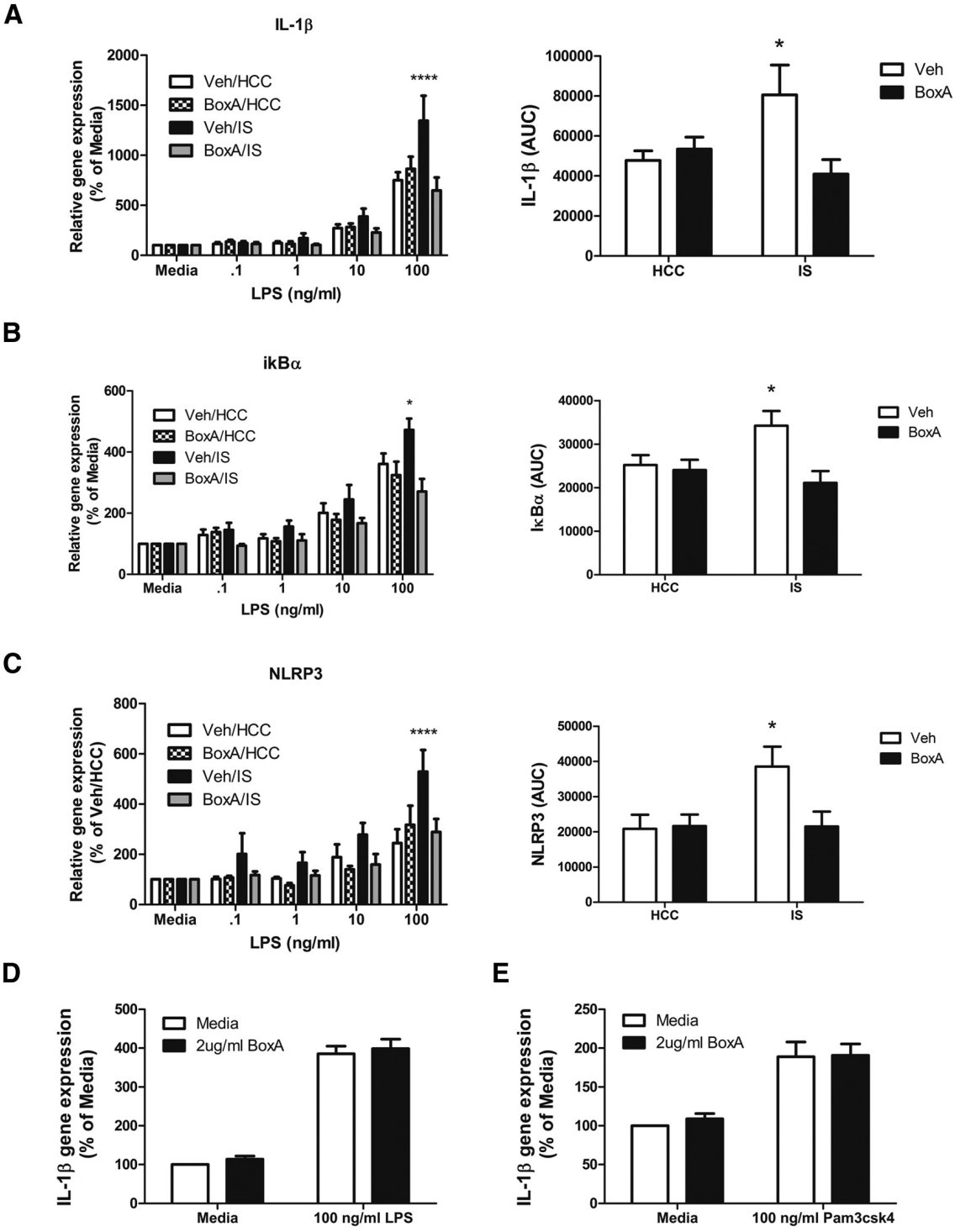

Figure 3. Effect of the HMGB-1 antagonist BoxA on IS-induced priming of microglia to LPS *ex vivo*. ***A–C***, BoxA (10 μg) or vehicle was injected ICM before IS or in HCCs. At 24 h after stress treatment, microglia were isolated from the hippocampus and challenged with LPS (0, 0.1, 1, 10, and 100 ng/ml) for 4 h, and microglial proinflammatory gene expression was measured. To determine whether BoxA blunted stress-induced sensitization of the microglial proinflammatory response, the AUC for LPS concentration was computed for each rat, and the means were compared. $n = 6$ per group. BoxA treatment blocked the IS-induced sensitized microglial IL-1β (***A***), IκBα (***B***), and NLRP3 (***C***) response to LPS. The graphs show mean and SEM. *$p < 0.05$ versus non-asterisk groups. ***D***, ***E***, To determine whether BoxA interferes with the ability of TLR2/4 to recognize other agonists, isolated microglia were treated with BoxA (2 μg/ml) or media, and the TLR4-specific antagonist LPS (100 ng/ml; ***D***) or the TLR2-specific antagonist Pam3csk4 (100 ng/ml; ***E***) for 4 h. IL-1β gene expression was measured as an indicator of the microglial proinflammatory response. $n = 4$ per group.

portantly, neither the HMGB-1 antibody used in the present study, nor most others, distinguishes between these different forms, and therefore it is unknown which form of HMGB-1 is released in the CNS.

Whether these different HMGB-1 forms might have different effects in the CNS has not been explored previously. To determine which form primes microglia, disulfide or fully reduced HMGB-1 was administered ICM, and microglia proinflammatory responses to LPS were measured *ex vivo* 24 h later. Similar to the effects of stress exposure, microglia incubated in media alone expressed the same level of inflammatory cytokines, regardless of HMGB-1 treatment. Previous reports show that disulfide

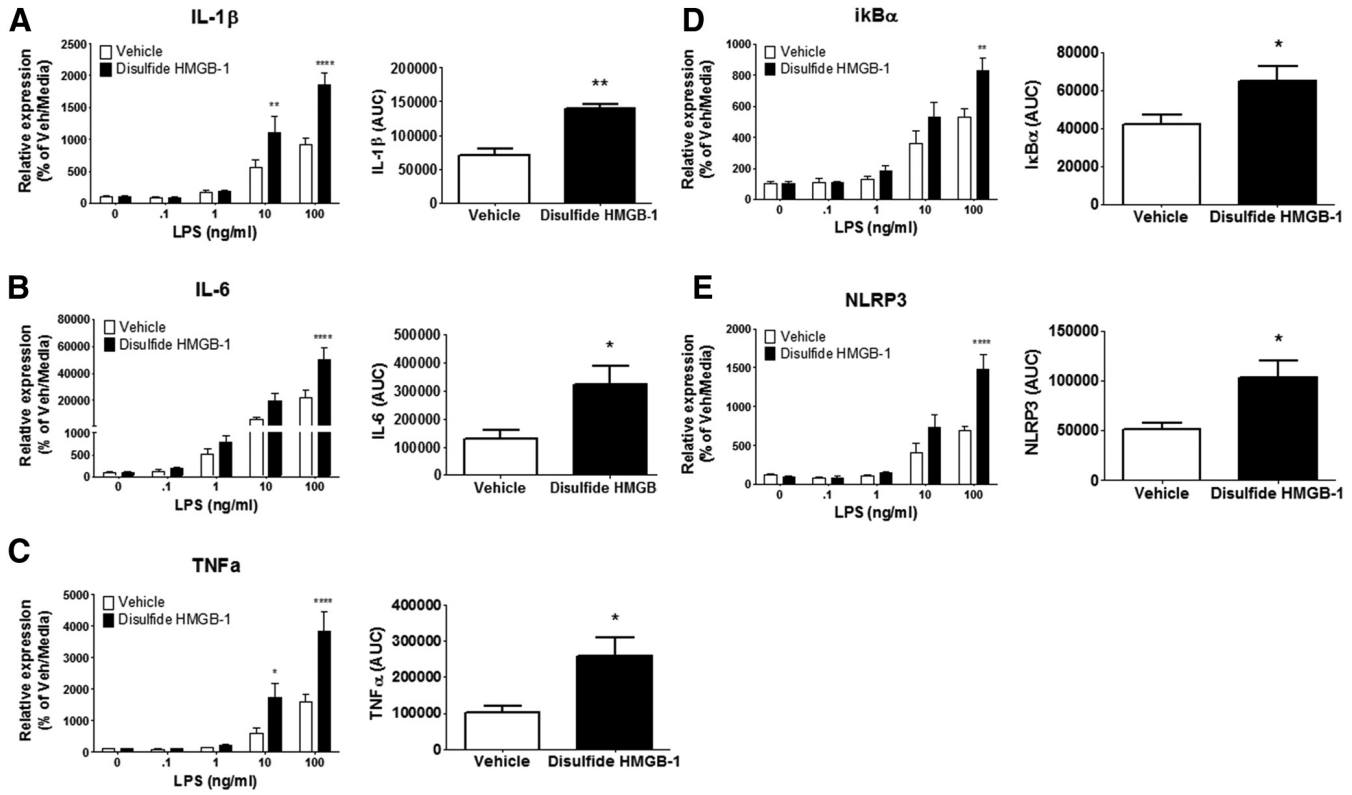

Figure 4. Effect of disulfide HMGB-1 on hippocampal microglia proinflammatory to LPS *ex vivo*. Disulfide HMGB-1 (1 μg/10 μl) or vehicle was injected ICM. Hippocampal microglia were isolated 24 h after treatment and stimulated with LPS (0, 0.1, 1, 10, and 100 ng/ml) for 4 h, and proinflammatory gene expression was measured. Relative mRNA expression to the vehicle/media group is shown for IL-1β (***A***), IL-6 (***B***), TNFα (***C***), IκBα (***D***), and NLRP3 (***E***). Data are presented as mean and SEM, $n = 4$ per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ versus the vehicle group with same LPS concentration; AUC was also calculated and presented as mean and SEM. *$p < 0.05$, **$p < 0.01$ versus the vehicle group.

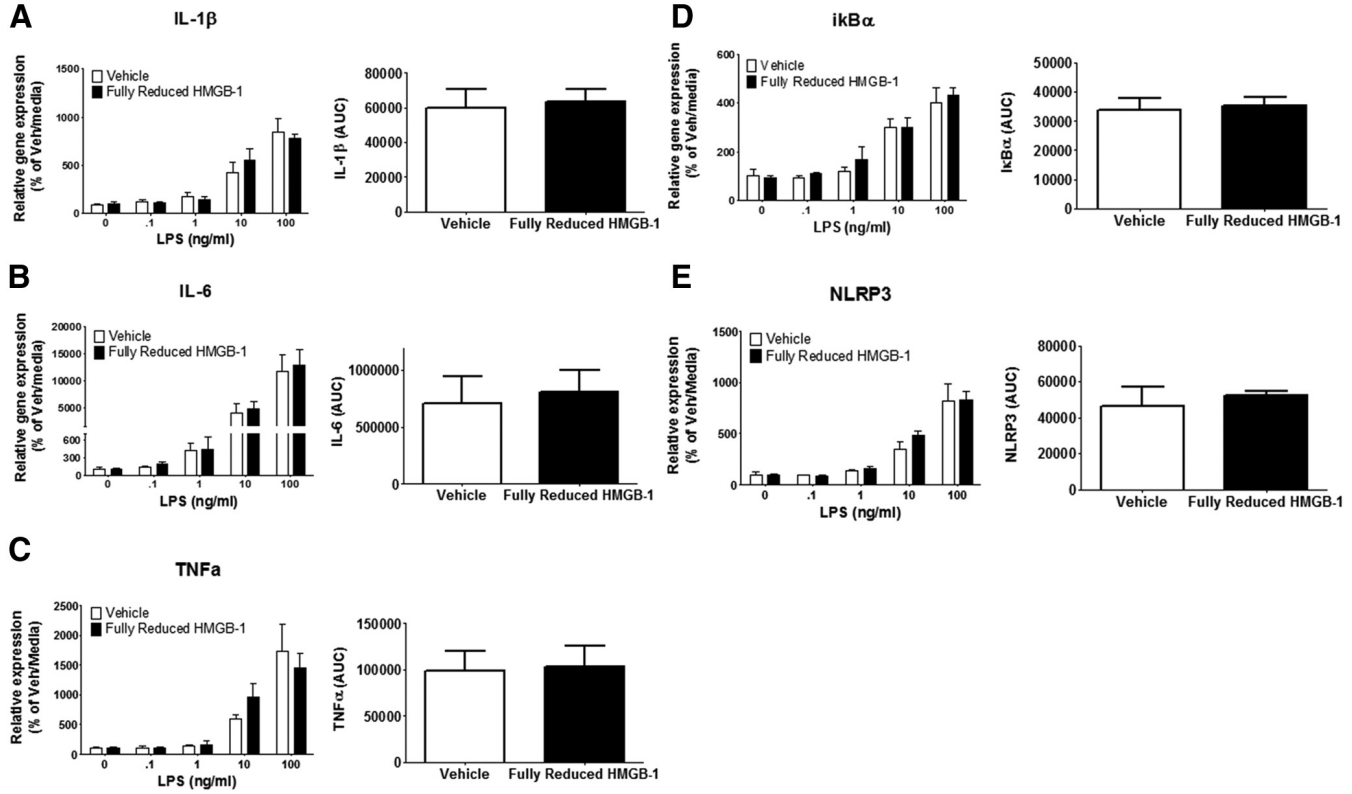

Figure 5. Effect of fully reduced HMGB-1 on hippocampal microglia proinflammatory to LPS *ex vivo*. Fully reduced HMGB-1 (1 μg/10 μl) or vehicle was injected ICM. Hippocampal microglia were isolated 24 h after treatment and stimulated with LPS (0, 0.1, 1, 10, and 100 ng/ml) for 4 h, and proinflammatory gene expression was measured. Relative mRNA expression to the vehicle/media group is shown for IL-1β (***A***), IL-6 (***B***), TNFα (***C***), IκBα (***D***), and NLRP3 (***E***). Data are presented as mean and SEM, $n = 4$ per group. The AUC was also calculated and presented as mean and SEM.

HMGB-1 is sufficient to stimulate proinflammatory cytokines (Scaffidi et al., 2002; Park et al., 2004; Agalave et al., 2014). However, disulfide HMGB-1 failed to increase basal levels of proinflammatory cytokine expression here. One possibility is that the proinflammatory cytokine effect of disulfide HMGB-1 had dissipated by 24 h after treatment. However, other reports suggest that HMGB-1 does not always induce proinflammatory cytokines (Rouhiainen et al., 2007; Sha et al., 2008; Cassetta et al., 2009). Additional studies are needed to measure the inflammatory properties of each redox form closer to the time of injection. Interestingly, microglia isolated from disulfide HMGB-1-treated, but not fully reduced HMGB-1-treated, rats showed a potentiated inflammatory response to LPS. That is, disulfide HMGB-1 was sufficient to prime hippocampal microglia, as does stress. The effects of HMGB-1 on microglial priming are consistent with previous work demonstrating that HMGB-1 is sufficient to prime the inflammatory response of isolated splenocytes (Valdes-Ferrer et al., 2013). It should be noted that stress exposure increases macrophage and monocyte trafficking into the brain (Wohleb et al., 2011, 2013) and microglia density in several stress-reactive brain areas, including the hippocampus (Nair and Bonneau, 2006; Tynan et al., 2010). It is possible that fully reduced HMGB-1 drives this type of trafficking, which could contribute to stress-induced neuroinflammatory responses *in vivo*. However, the present experiments were designed to target microglial priming specifically, and so the number of microglia were controlled for in the *ex vivo* environment.

Although the present studies provide evidence that HMGB-1 mediates stress-induced microglial priming, they do not indicate the cellular mechanism by which HMGB-1 does so. We have shown previously that ICM administration of a TLR2/4 antagonist before the stressor blocks stress-induced microglia priming (Weber et al., 2013). Of particular relevance, TLR2 and TLR4 activation can lead to the formation of the NLRP3 inflammasome, a complex that is key to generating IL-1β protein (Hanamsagar et al., 2012). IL-1β is often referred to as the "master regulator" of inflammation because it is a potent signal that stimulates the production of other inflammatory molecules (Basu et al., 2004). NLRP3 is unique among NLR inflammasomes in that assembly and activation of the NLRP3 inflammasome involves a two-step process. First, a priming signal is required to increase NLRP3 protein levels to a critical threshold. A second signal is then required that induces NLRP3 to form a complex with Apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1. Formation of this complex results in proteolytic cleavage of pro-caspase-1 to active caspase-1, which cleaves pro-IL-1β into the bioactive mature IL-1β (Martinon et al., 2009). Interestingly, HMGB-1 has been found to increase NLRP3 mRNA and protein (Xiang et al., 2011). Indeed, it has been argued that DAMPs, such as HMGB-1, prime the NLRP3 inflammasome and potentiate inflammation (Leemans et al., 2011).

Given the above, it is important that stress exposure increased NLRP3 protein in the hippocampus, an increase still present 24 h after stressor termination. However, the present IS paradigm does not alter IL-1β protein in the hippocampus 24 h after stressor termination (Frank et al., 2007), suggesting that the NLRP3 inflammasome is primed but not active. This result is consistent with other findings in which chronic stress increased NLRP3 in the prefrontal cortex (Pan et al., 2014). Unexpectedly, NLRP3 mRNA was not changed here after the stressor. However, the stress paradigm is ~2 h. Therefore, it is possible that NLRP3 mRNA did increase but had returned to basal levels by the end of the stressor. Activation of the transcription factor NF-κB drives NLRP3 to prime inflammasome formation (Bauernfeind et al., 2009). Importantly, active NF-κB protein and IκBα mRNA, an inhibitor of NF-κB that is induced after NF-κB activation, were increased 0 h, but not 24 h, after stress. The finding that microglial NLRP3 mRNA was sensitized to LPS after stress exposure further implicates this pathway in stress-induced neuroinflammatory priming. Interestingly, treatment with BoxA before stress blocked NLRP3 sensitization, suggesting that stress-induced HMGB-1 drives NLRP3 priming. In addition, administration of disulfide HMGB-1, but not fully reduced HMGB-1, sensitized microglia NLRP3 mRNA to LPS. As a body, these data provide initial evidence that stress exposure may prime neuroinflammatory processes via HMGB-1 activating NF-κB to prime the NLRP3 inflammasome.

The present results encourage the expansion of the role of HMGB-1 into other stress paradigms. Chronic unpredictable stress (Munhoz et al., 2006) and repeated social defeat (Wohleb et al., 2012), for example, increase inflammatory processes to a later immune challenge. Thus, HMGB-1 may function broadly.

Of course, this leaves the question of what CNS signal leads to HMGB-1 increases and release from microglia. Because blocking TLR2 and TLR4 during IS prevented stress-induced microglial priming (Weber et al., 2013), one possibility is that an unknown ligand, signaling at TLR2 and/or TLR4, initiates HMGB-1 release from microglia. Another possibility involves glucocorticoids as critical mediators. Previous studies show that glucocorticoids, and glucocorticoid receptor signaling in particular, are necessary and sufficient to prime microglia (Frank et al., 2010). This is an active topic of investigation.

Although the purpose of the present studies was to explore mechanisms by which stressors sensitize neuroinflammatory responses to subsequent immune challenges, they also comment more generally on the role of DAMPs, such as HMGB-1, at least in the brain. When it was first argued that immune cells respond to "danger signals," the term danger was used quite loosely. However, as a recent review of this concept by Pradeu and Cooper (2012) makes clear, by danger, immunologists meant damage to cells or tissues. Although we cannot dissociate between the physical and psychological effect of the stressor, the present results suggest consideration of the idea that, perhaps, DAMPs respond to external threats as well as cellular damage.

References

Agalave NM, Larsson M, Abdelmoaty S, Su J, Baharpoor A, Lundback P, Palmblad K, Andersson U, Harris H, Svensson CI (2014) Spinal HMGB1 induces TLR4-mediated long-lasting hypersensitivity and glial activation and regulates pain-like behavior in experimental arthritis. Pain 155:1802–1813. CrossRef Medline

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402. CrossRef Medline

Basu A, Krady JK, Levison SW (2004) Interleukin-1: a master regulator of neuroinflammation. J Neurosci Res 78:151–156. CrossRef Medline

Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol 183:787–791. CrossRef Medline

Bianchi ME (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 81:1–5. CrossRef Medline

Cassetta L, Fortunato O, Adduce L, Rizzi C, Hering J, Rovere-Querini P, Bianchi ME, Alfano M, Poli G (2009) Extracellular high mobility group box-1 inhibits R5 and X4 HIV-1 strains replication in mononuclear

phagocytes without induction of chemokines and cytokines. Aids 23:567–577. CrossRef Medline

de Pablos RM, Villaran RF, Arguelles S, Herrera AJ, Venero JL, Ayala A, Cano J, Machado A (2006) Stress increases vulnerability to inflammation in the rat prefrontal cortex. J Neurosci 26:5709–5719. CrossRef Medline

Espinosa-Oliva AM, de Pablos RM, Villaran RF, Arguelles S, Venero JL, Machado A, Cano J (2011) Stress is critical for LPS-induced activation of microglia and damage in the rat hippocampus. Neurobiol Aging 32:85–102. CrossRef Medline

Fillman SG, Sinclair D, Fung SJ, Webster MJ, Shannon Weickert C (2014) Markers of inflammation and stress distinguish subsets of individuals with schizophrenia and bipolar disorder. Transl Psychiatry 4:e365. CrossRef Medline

Frank MG, Wieseler-Frank JL, Watkins LR, Maier SF (2006) Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: Immunophenotypic and functional characteristics. J Neurosci Methods 151:121–130. CrossRef Medline

Frank MG, Baratta MV, Sprunger DB, Watkins LR, Maier SF (2007) Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. Brain Behav Immun 21:47–59. CrossRef Medline

Frank MG, Miguel ZD, Watkins LR, Maier SF (2010) Prior exposure to glucocorticoids sensitizes the neuroinflammatory and peripheral inflammatory responses to *E. coli* lipopolysaccharide. Brain Behav Immun 24:19–30. CrossRef Medline

Gadek-Michalska A, Tadeusz J, Rachwalska P, Bugajski J (2013) Cytokines, prostaglandins and nitric oxide in the regulation of stress-response systems. Pharmacol Rep 65:1655–1662. CrossRef Medline

Hanamsagar R, Hanke ML, Kielian T (2012) Toll-like receptor (TLR) and inflammasome actions in the central nervous system. Trends Immunol 33:333–342. CrossRef Medline

Holguin A, Frank MG, Biedenkapp JC, Nelson K, Lippert D, Watkins LR, Rudy JW, Maier SF (2007) Characterization of the temporo-spatial effects of chronic bilateral intrahippocampal cannulae on interleukin-1beta. J Neurosci Methods 161:265–272. CrossRef Medline

Johnson JD, O'Connor KA, Deak T, Stark M, Watkins LR, Maier SF (2002) Prior stressor exposure sensitizes LPS-induced cytokine production. Brain Behav Immun 16:461–476. CrossRef Medline

Kang R, Chen R, Zhang Q, Hou W, Wu S, Cao L, Huang J, Yu Y, Fan XG, Yan Z, Sun X, Wang H, Wang Q, Tsung A, Billiar TR, Zeh HJ 3rd, Lotze MT, Tang D (2014) HMGB1 in health and disease. Mol Aspects Med. Advance online publication. Retrieved November 12, 2014. doi:10.1016/j.mam.2014.05.001 CrossRef Medline

Klune JR, Dhupar R, Cardinal J, Billiar TR, Tsung A (2008) HMGB1: endogenous danger signaling. Mol Med 14:476–484. CrossRef Medline

Leemans JC, Cassel SL, Sutterwala FS (2011) Sensing damage by the NLRP3 inflammasome. Immunol Rev 243:152–162. CrossRef Medline

Li J, Kokkola R, Tabibzadeh S, Yang R, Ochani M, Qiang X, Harris HE, Czura CJ, Wang H, Ulloa L, Warren HS, Moldawer LL, Fink MP, Andersson U, Tracey KJ, Yang H (2003) Structural basis for the proinflammatory cytokine activity of high mobility group box 1. Mol Med 9:37–45. Medline

Lotze MT, Tracey KJ (2005) High-mobility group box 1 protein (HMGB): nuclear weapon in the immune arsenal. Nat Rev Immunol 5:331–342. CrossRef Medline

Martinon F, Mayor A, Tschopp J (2009) The inflammasomes: guardians of the body. Annu Rev Immunol 27:229–265. CrossRef Medline

Matzinger P (2002) The danger model: a renewed sense of self. Science 296:301–305. CrossRef Medline

Munhoz CD, Lepsch LB, Kawamoto EM, Malta MB, Lima Lde S, Avellar MC, Sapolsky RM, Scavone C (2006) Chronic unpredictable stress exacerbates lipopolysaccharide-induced activation of nuclear factor-κB in the frontal cortex and hippocampus via glucocorticoid secretion. J Neurosci 26:3813–3820. CrossRef Medline

Nair A, Bonneau RH (2006) Stress-induced elevation of glucocorticoids increases microglia proliferation through NMDA receptor activation. J Neuroimmunol 171:72–85. CrossRef Medline

Pan Y, Chen XY, Zhang QY, Kong LD (2014) Microglial NLRP3 inflammasome activation mediates IL-1beta-related inflammation in prefrontal cortex of depressive rats. Brain Behav Immun 41:90–100. CrossRef Medline

Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E (2004) Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 279:7370–7377. CrossRef Medline

Pradeu T, Cooper EL (2012) The danger theory: 20 years later. Front Immunol 3:287. CrossRef Medline

Qiu J, Nishimura M, Wang Y, Sims JR, Qiu S, Savitz SI, Salomone S, Moskowitz MA (2008) Early release of HMGB-1 from neurons after the onset of brain ischemia. J Cereb Blood Flow Metab 28:927–938. CrossRef Medline

Rouhiainen A, Tumova S, Valmu L, Kalkkinen N, Rauvala H (2007) Pivotal advance: analysis of proinflammatory activity of highly purified eukaryotic recombinant HMGB1 (amphoterin). J Leukoc Biol 81:49–58. CrossRef Medline

Scaffidi P, Misteli T, Bianchi ME (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418:191–195. CrossRef Medline

Sha Y, Zmijewski J, Xu Z, Abraham E (2008) HMGB1 develops enhanced proinflammatory activity by binding to cytokines. J Immunol 180:2531–2537. CrossRef Medline

Tynan RJ, Naicker S, Hinwood M, Nalivaiko E, Buller KM, Pow DV, Day TA, Walker FR (2010) Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions. Brain Behav Immun 24:1058–1068. CrossRef Medline

Valdes-Ferrer SI, Rosas-Ballina M, Olofsson PS, Lu B, Dancho ME, Li JH, Yang H, Pavlov VA, Chavan SS, Tracey KJ (2013) High-mobility group box 1 mediates persistent splenocyte priming in sepsis survivors: evidence from a murine model. Shock 40:492–495. CrossRef Medline

van Zoelen MA, Yang H, Florquin S, Meijers JC, Akira S, Arnold B, Nawroth PP, Bierhaus A, Tracey KJ, van der Poll T (2009) Role of toll-like receptors 2 and 4, and the receptor for advanced glycation end products in high-mobility group box 1-induced inflammation in vivo. Shock 31:280–284. CrossRef Medline

Venereau E, Casalgrandi M, Schiraldi M, Antoine DJ, Cattaneo A, De Marchis F, Liu J, Antonelli A, Preti A, Raeli L, Shams SS, Yang H, Varani L, Andersson U, Tracey KJ, Bachi A, Uguccioni M, Bianchi ME (2012) Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release. J Exp Med 209:1519–1528. CrossRef Medline

Venereau E, Schiraldi M, Uguccioni M, Bianchi ME (2013) HMGB1 and leukocyte migration during trauma and sterile inflammation. Mol Immunol 55:76–82. CrossRef Medline

Weber MD, Frank MG, Sobesky JL, Watkins LR, Maier SF (2013) Blocking toll-like receptor 2 and 4 signaling during a stressor prevents stress-induced priming of neuroinflammatory responses to a subsequent immune challenge. Brain Behav Immun 32:112–121. CrossRef Medline

Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, Nelson RJ, Godbout JP, Sheridan JF (2011) β-Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. J Neurosci 31:6277–6288. CrossRef Medline

Wohleb ES, Fenn AM, Pacenta AM, Powell ND, Sheridan JF, Godbout JP (2012) Peripheral innate immune challenge exaggerated microglia activation, increased the number of inflammatory CNS macrophages, and prolonged social withdrawal in socially defeated mice. Psychoneuroendocrinology 37:1491–1505. CrossRef Medline

Wohleb ES, Powell ND, Godbout JP, Sheridan JF (2013) Stress-induced recruitment of bone marrow-derived monocytes to the brain promotes anxiety-like behavior. J Neurosci 33:13820–13833. CrossRef Medline

Xiang M, Shi X, Li Y, Xu J, Yin L, Xiao G, Scott MJ, Billiar TR, Wilson MA, Fan J (2011) Hemorrhagic shock activation of NLRP3 inflammasome in lung endothelial cells. J Immunol 187:4809–4817. CrossRef Medline

Yang H, Tracey KJ (2009) Targeting HMGB1 in inflammation. Biochim Biophys Acta 1799:149–156. CrossRef Medline

Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, Susarla SM, Ulloa L, Wang H, DiRaimo R, Czura CJ, Roth J, Warren HS, Fink MP, Fenton MJ, Andersson U, Tracey KJ (2004) Reversing established sepsis with antagonists of endogenous high-mobility group box 1. Proc Natl Acad Sci U S A 101:296–301. CrossRef Medline

Yang H, Lundback P, Ottosson L, Erlandsson-Harris H, Venereau E, Bianchi ME, Al-Abed Y, Andersson U, Tracey KJ, Antoine DJ (2012) Redox modification of cysteine residues regulates the cytokine activity of high mobility group box-1 (HMGB1). Mol Med 18:250–259. CrossRef Medline