Lipopolysaccharide (LPS) is a bacterial endotoxin known to activate the innate immune system and trigger increases in both central and peripheral production of pro-inflammatory cytokines, including interleukin-1β (IL-1β) [1]. Moreover, inflammation as a result of peripheral LPS administration has been shown to impair acquisition, consolidation, and reconsolidation of information in learning and memory paradigms [2–4], including contextual fear conditioning, a hippocampus-dependent task that lends itself well to discriminating between sickness behavior and actual memory decrements. Although the exact mechanisms mediating the behavioral effects of LPS administration remains unknown, the clear connection with inflammation is supported by evidence showing that administration of IL-1β to the dorsal hippocampus is sufficient to partially replicate LPS-induced memory impairments [5]. Likewise, administration of IL-1 receptor antagonist (IL-1ra) prior to LPS exposure is capable of preventing similar decrements [4].
A portion of the LPS-induced cognitive deficit may be associated with alterations in brain-derived neurotrophic factor (BDNF) expression, a neurotrophin strongly associated with synaptic plasticity [6]. Specifically, LPS-induced increases in pro-inflammatory cytokines within the hippocampus result in subsequent declines in BDNF and its receptor, TrkB [7]. Inflammation-induced BDNF reductions are also associated with performance decrements during contextual fear conditioning [8], and, conversely, the use of IL-1β can prevent inflammation-induced BDNF declines, and rescue cognition [9]. However, behavioral evidence for protection against LPS-induced cognitive deficit can occur independently of BDNF restoration [8], indicating the potential for other mediating factors. For example, insulin-like growth factor I (IGF-1) is another protein for which expression appears to be inversely correlated with inflammation [10,11], and is also associated with neural development, cognition, [12] and regulation of hippocampal adult neurogenesis [13]. Moreover, IGF-1 has been shown to be neuroprotective, and, therefore, inflammation-induced declines in IGF-1 expression may contribute to neurodegeneration following an immune challenge [11].

Additionally, BDNF, IGF-1, and molecules that regulate inflammation are able to influence long-term potentiation (LTP), an electrophysiological model of learning and memory that associates prolonged synaptic potentiation of excitatory transmission with cellular processes also involved in learning [14]. For example, BDNF is capable of enhancing LTP, [14], while IGF-1 can protect against inflammation-induced inhibition of LTP [15]. Conversely, increases in IL-1β prevent the induction of LTP [16,17], and trigger a corresponding decline in hippocampal glutamate release [18]. This is coupled with evidence that LPS exaggerates the inhibitory properties of GABA, via increased chloride ion conductance through activated GABA_A receptors [19]. Thus, the inflammatory response to LPS or IL-1β appears to inhibit learning and memory, at least in part, by inhibiting excitatory neurotransmitter release and promoting inhibitory transmission, while decreasing the expression of proteins associated with neuroplasticity, cognition, and neurogenesis.

These findings suggest several potential strategies to prevent inflammation-induced cognitive decrements. For example, increasing excitatory transmission through administration of the partial NMDA receptor agonist d-cycloserine rescues contextual memory consolidation after LPS administration [8]. Additionally, utilizing α5-subunit-containing-GABA_A receptor (α5GABA_A) knockout mice, Wang et al. [20] demonstrated that the α5GABA_A is critical for both inflammation-induced memory errors, and disruptions of LTP. Wang et al. [20] then utilized MRK-016 (MRK), an inverse benzodiazepine agonist, to attenuate the flow of chloride ions across the membrane, thus preventing IL-1β-induced acquisition deficits. These findings suggest that the enhanced inhibitory component of inflammation is somehow dependent on α5GABA_A, and that disinhibition may protect against inflammation-based cognitive deficits. To reinforce and extend this hypothesis, we conducted two experiments to evaluate MRK’s ability to protect against LPS-induced acquisition errors and, separately, the potential for MRK to rescue memory consolidation following LPS administration.

We utilized 4–6-month-old, male, experimentally-naive C57BL/6j mice, bred in the Texas Christian University vivarium, from The Jackson Laboratory (Bar Harbor, ME) stock, in all experiments. Animals were housed in standard polycarbonate cages, with food and water available ad libitum, and maintained on a 12-h light/dark schedule. All animals were housed and cared for in accordance, and protocols were approved by the Institutional Animal Care and Use Committee of Texas Christian University.

Experiment One was designed to evaluate MRK’s potential to protect against LPS-induced acquisition errors, using a 2 × 2 (LPS/Saline × MRK/Saline) design. Behavior was assessed with a contextual fear conditioning paradigm, discussed at length in Kranjac et al. [8]. Briefly, 4 h before contextual fear conditioning (CFC) training, animals received either an intraperitoneal (i.p.) injection of saline or LPS (125 µg/kg, serotype 0111:B4; Sigma, St. Louis, MO). A second i.p. injection of either saline or MRK-016 [3-tert-buty1-[(5-methylisoxazol-3-yl)-2-(1-methyl-1H-1,2,4-triazol-5-ylmethoxy)-pyrazolol 1,5-dil[1,2,4]triazine] (3 mg/kg, Tocris, Bristol, UK) was administered 30 min before CFC training. Freezing behavior was monitored using testing chambers (Coulbourn Instruments, Whithall, PA) and FreezeFrame™ software (ActiMetrics Software, Wilmette, IL). Following a 120 s acclimation to the context, that included polka dot wall patterns and a peppermint olfactory cue, a 2 s 0.7 mA un-signalized foot-shock was delivered, after which, animals remained in the chamber for another 60 s. Twenty-four hours later, animals were returned to the chambers, and freezing was measured for 120 s.

To assess the influence of LPS and/or MRK on BDNF and IGF-1 mRNA expression, dorsal hippocampus samples were collected from two separate batches of animals. The first collection occurred immediately after training (4 h after LPS injection), and a second batch was collected immediately after testing. All tissue was collected under RNase-free conditions, and stored in RNAlater™ (Austin, TX) at −20 °C, until processing to isolate the RNA (RNeasy Micro kits, Qiagen, Valencia, CA). RNA yields were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, NanoDrop Products, Wilmington, DE), before being diluted to a uniform concentration for qRT-PCR procedures. We utilized a 7500 Real-Time PCR Thermal Cycling System (Applied Biosystems, Foster City, CA) for the RT and PCR steps, and samples were amplified using Applied Biosystems TaqMan® Gene Expression Assays (Foster City, CA). Target genes were normalized to β-actin prior to analysis, using the DART procedure for quantification of gene expression [2,8].

Experiment Two was designed to evaluate MRK’s potential to prevent LPS-induced disruption of memory consolidation, using the same 2 × 2 design. Animals were trained in CFC with identical contextual cues as described above, but the training protocol consisted of a 120 s acclimation, followed by two 2 s 0.7 mA un-signalized foot-shocks, with a 90 s ITI and a 60 s post-shock interval. Immediately after training, animals received injections per group assignment. Treatment groups were comparable to Experiment One; however, LPS dosage was increased to 250 µg/kg. After 24 h, animals were returned to the chambers and freezing behavior was measured for 120 s. Both studies measured freezing, a behavioral expression of fear, as a dependent variable. The dorsal hippocampus was collected and processed under conditions identical to those previously described; the first collection occurred 4 h after training, and the second immediately after testing (i.e., 24 h after training).

For Experiment One, we used a 2 × 2 ANOVA to evaluate differences in freezing during the training acclimation period, but, as expected, found no significant differences between groups in either experiment; data not shown (DNS). Likewise, consistent with previous studies employing LPS, analysis of animal weights determined that, in both studies, all animals treated with LPS lost a slight but statistically significant amount of weight, in comparison to saline controls (DNS). We used another 2 × 2 ANOVA to assess freezing differences during the 120 s testing period. As hypothesized, we found a significant main effect of LPS treatment [F(1,49) = 6.880, p < 0.05]. However, there was no significant main effect for MRK treatment. Moreover, as hypothesized, the analyses revealed a significant interaction between MRK and LPS treatments, [F(1,49) = 6.830, p < 0.05], in which there were no significant differences in freezing behavior between the MRK/Saline, MRK/LPS, and Saline/Saline treatment groups, but
animals in the LPS/Saline condition froze significantly less (i.e., learned more poorly) than all other conditions ($p < 0.05$) (Fig. 1).

A $2 \times 2$ ANOVA was used to analyze the PCR data for BDNF and IGF-1. Results for BDNF expression at the 4-h collection time-point did not identify any significant main effects or an interaction effect. However, consistent with previous findings [8], between-group comparisons revealed that the Saline/LPS-treated animals expressed significantly less BDNF mRNA than animals treated with Saline/Saline ($p < 0.05$) (Fig. 2). Analysis of IGF-1 failed to show any significant differences between treatment groups (DNS). Further, data from the 24 h time-point did not identify any significant differences for either BDNF (Fig. 2) or IGF-1 (DNS).

Experiment Two was evaluated using the statistical methodology described for Experiment One. No differences in freezing behavior were identified during the training acclimation period, but analysis of the testing data once again revealed a significant main effect of LPS treatment [$F(1,36) = 11.252, p < 0.05$], and a significant interaction between LPS and MRK treatments [$F(1,36) = 4.149, p < 0.05$]. Between-group comparisons from the interaction again showed no significant differences in freezing behavior between the MRK/Saline, MRK/LPS, and Saline/Saline, but animals in the Saline/LPS condition froze significantly less than all other conditions ($p < 0.05$) (Fig. 3).

Analysis of BDNF expression at the 4-h time-point for Experiment Two identified a significant main effect of LPS treatment [$F(1,19) = 11.797, p < 0.05$], but no other significant effects. Between-group comparisons again revealed that Saline/LPS-treated animals expressed significantly less BDNF mRNA than Saline/Saline-treated animals ($p < 0.05$), but no other between-group differences were identified (see Fig. 4). As before, we did not identify any significant effects for IGF-1 at the 4-h time-point (DNS). Analysis of the 24 h time-point failed to identify any significant effects or between-group differences for either BDNF (Fig. 4) or IGF-1 (DNS).

Results from these studies indicate that MRK-016 can prevent LPS-induced deficits in both acquisition and consolidation, and indicate that the use of the inverse benzodiazepine agonist MRK-016 is sufficient to prevent LPS-induced errors in a hippocampus-dependent task. The behavioral data show that animals treated with MRK and LPS perform equally well on CFC when compared to both Saline/Saline and MRK/Saline, but importantly, they perform significantly better than animals that received Saline/LPS. This indicates that MRK-016 can completely prevent LPS-induced acquisition and consolidation decrements, findings that buttress and extend the previous work of Wang et al. [20] beyond the reported protection from acquisition deficits induced by IL-1β. Interestingly, given the short half-life of MRK, 0.3–0.5 h [21], these data suggest that even a brief attenuation of GABAergic transmission is sufficient to protect against LPS-induced cognitive errors assayed 24 h later.

Importantly, interpretation of the behavioral data from the acquisition study (Experiment One) must include recognition of the influence that sickness behaviors may have on the acquisition of contextual information. Administration of LPS may decrease motivation to attend to contextual information in the absence of relevant incentives to shift motivational awareness to the surroundings [22]. Therefore, the immune response to LPS may have diminished the animal’s ability to attend to contextual
information during training. In turn, decreased attention to contextual cues could partially explain the behavioral decrement observed in the Saline/LPS treatment group. However, the use of a foot-shock (0.7 mA shock for 2 s) in our training paradigm should provide sufficient motivation for the animal to attend to available information. Therefore, a more parsimonious explanation of our data from Experiment One would posit that the LPS-induced increases in central pro-inflammatory cytokines, which are undoubtedly maintained throughout both training and memory consolidation, alter these processes, and are the primary cause of the cognitive deficits observed during testing. Additionally, the expected LPS-induced weight loss renders unlikely the possibility that MRK acted as an anti-inflammatory agent or, in some way, mitigated the LPS-induced sickness behaviors.

Our consolidation study results align with those of previous reports of memory consolidation manipulation using bicuculline and muscimol to improve and inhibit consolidation, respectively, when administered immediately after training [23]. However, we found no support for a cognition-enhancing effect of MRK treatment alone, only a prevention of the LPS-induced consolidation deficits. This finding stands in contrast to the improved performance reported after administration of post-training bicuculline in an inhibitory avoidance task [23], but is consistent with those of Wang et al. [20]. Therefore, our results further support an inflammation-specific function of the α5GABA₅ in regard to acquisition decrements, and suggest a role in consolidation decrements as well.

Lastly, qRT-PCR results indicate that animals in the Saline/Saline condition expressed significantly more BDNF mRNA than animals treated with Saline/LPS, however, BDNF expression for both groups treated with MRK was intermediate to, and not significantly different from, that of the Saline/Saline or Saline/LPS groups. This relationship was true in both experiments. These results were unexpected, as blocking GABA₅ receptors with bicuculline has been reported to enhance memory consolidation and BDNF expression [24]. Additionally, our analyses failed to demonstrate any effects of LPS or MRK administration on IGF-1 expression. Although IGF-1 is known to be involved with hippocampal synaptic plasticity [13], previous reports indicate that inflammation can induce IGF-1 resistance without down-regulated expression [25]. Therefore, although we did not test this possibility, it is plausible that IGF-1 activity, rather than IGF-1 expression per se, was modulated following LPS and/or MRK treatment.

The purpose of the current study was to test the hypothesized protective influence of MRK-016 against LPS-induced deficits of acquisition and consolidation, in a hippocampus-dependent paradigm. To the authors’ knowledge, this work represents the first use of MRK to protect against LPS-induced disruptions of both the acquisition and consolidation phases of contextual fear conditioning. These findings suggest that inflammation-induced errors in memory result, at least in part, from an imbalance between excitatory and inhibitory signaling, and that manipulation of these transmitter systems, as done here with MRK, is sufficient to rescue learning and memory processes that would otherwise be disrupted as a result of a peripheral immune challenge. Specifically, manipulation of GABAergic transmission via the inverse benzodiazepine agonist MRK-016 protects against bacterial endotoxin-induced disruptions of learning and memory, and underscores the importance of GABAergic signaling in the induction of inflammation-based cognitive dysfunction.

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