EFFECTS OF MRK-016 ON AMYLOID-BETA INDUCED
LEARNING DEFICITS IN MICE IN CONTEXTUAL
CONDITIONING PARADIGM

By

Jenna Wiles and Samantha Hodges

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Project Approved:

Supervising Professor: Gary Boehm, Ph.D.
Department of Psychology

Karla O’Donal, Ph.D.
Department of Religion

Ellen Broom, Ph.D.
Department of Psychology
ABSTRACT

In the present study, we examined the protective effects of inverse benzodiazepine agonist MRK-016 on the cognitive deficits associated with inflammation-induced accumulation of amyloid-beta (Aβ), to model Alzheimer’s disease-like pathology. We used a 2 x 2 design to test the ability of MRK-016 to protect against cognitive deficits resultant from an increase in Aβ in the hippocampus. We utilized a seven-day injection model of LPS on C57BL/6 mice to increase levels of Aβ. On day 8, we trained animals in a contextual fear conditioning paradigm to associate a conditioned stimulus (CS) with an unconditioned stimulus (US). MRK-016 was administered immediately after training to examine its effects on the consolidation stage of learning. Testing occurred on day 9, 24 hours after training, and the percentage of time the animal spent freezing was evaluated as an indication of learned association. Analysis of the behavioral data revealed a significant interaction between MRK-016 and LPS and between-groups analyses revealed that animals in the LPS/Saline condition froze significantly less during testing when compared to all other conditions. To elucidate the biological mechanism mediating the behavioral data, we collected the dorsal hippocampus from comparable animals 4 hours after training for analysis of Arc and TrkB gene expression using RT-PCR on collected tissue samples. We did not find evidence for any change in Arc expression. However, we did identify that animals in the LPS/Saline condition expressed significantly less TrkB mRNA than all other groups. Overall, these results indicate that MRK-016 has the ability to prevent Aβ-induced cognitive decrements in a hippocampus-dependent memory paradigm.
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<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION ........................................................................................................1</td>
</tr>
<tr>
<td>METHODS ................................................................................................................5</td>
</tr>
<tr>
<td>Experimental Subjects ............................................................5 ...............</td>
</tr>
<tr>
<td>Contextual Fear Conditioning .................................................6 .............</td>
</tr>
<tr>
<td>Tissue Collection, RNA isolation, RT-PCR .........................7 ..................</td>
</tr>
<tr>
<td>Statistical Analysis ..............................................................8 ................</td>
</tr>
<tr>
<td>RESULTS ..........................................................................................................8</td>
</tr>
<tr>
<td>Behavioral Results .................................................................8 ...............</td>
</tr>
<tr>
<td>Biological Results .............................................................10 .................</td>
</tr>
<tr>
<td>DISCUSSION .................................................................................................12</td>
</tr>
<tr>
<td>REFERENCES ............................................................................................15</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

FIGURE 1: CHEMICAL STRUCTURE OF MRK-016 ..................................................3

FIGURE 2: FREEZING BEHAVIOR DURING TESTING..............................................10

FIGURE 3: RT-PCR RESULTS FOR ARC mRNA EXPRESSION.............................11

FIGURE 4: RT-PCR RESULTS FOR TrkB mRNA EXPRESSION............................11
INTRODUCTION

The Alzheimer’s Association estimates that over 5 million Americans are currently living with Alzheimer’s disease (AD) and that by 2050 up to 16 million people will be afflicted by the disease [1]. In addition, AD is the 6th leading cause of death in the United States. The disease is characterized by neuronal death and behavioral deterioration, usually beginning with mild cognitive deficits affecting short-term memory [2]. As the disease progresses, the decline in cognitive abilities corresponds with a reduction in synapses and an increase in senile plaques and neurofibrillary tangles [3,4].

The lack of communication between nerve cells is one of the fundamental causes of memory loss associated with Alzheimer’s disease [5]. Characteristic plaques in the disease contain large amounts of a protein known as β-amyloid. In early stages of the disease, small clusters of β-amyloid peptides are known to block cell-to-cell synaptic signaling [6]. Disruption of this signaling inhibits the ability of neurons to both receive and transmit signals, which can ultimately result in cognitive impairments such as memory formation and information processing. Moreover, aggregation of the β-amyloid peptide results in the formation of Aβ plaques, known to impair learning and memory function partly through decreased synaptic signaling, especially in brain regions such as the hippocampus, entorhinal cortex, and amygdala [7].

A common feature of emerging research in neurodegenerative diseases, such as AD, is the impact of inflammation on the progression of disease pathology [7]. Inflammation alters amyloid peptide processing in people with AD, along with increased Aβ production and plaque formation [8-11]. Our lab has previously shown that initiating a peripheral inflammatory response through administration of the bacterial mimetic,
lipopolysaccharide (LPS), for seven sequential days of a single LPS intraperitoneal (i.p.) injection per day, leads to an increase in central Aβ. LPS is a bacterial endotoxin known to activate the immune system and trigger the release of both central and peripheral pro-inflammatory cytokines, including IL-1β and IL-6 [12]. The elevated levels of Aβ lead to systemic inflammation and cognitive dysfunction in mice, as observed in contextual fear conditioning paradigms and a hippocampus-dependent Morris water maze. Importantly, the observed cognitive deficits could not be attributed to cytokines because cytokine expression measured on day 8, prior to training, was found to be comparable to baseline and saline controls [13]. While mice are known to exhibit sickness behaviors following a single injection of LPS, they did not demonstrate these behaviors at the end of treatment, indicating tolerance to the endotoxin. Additionally, the use of Abl-tyrosine kinase inhibitor, imatinib, blocked the production of Aβ and eliminated the cognitive without blocking the inflammatory response. This confirmed that the accumulation of central Aβ following repeated acute bouts of inflammation is the result of peripheral Aβ that is trafficked into the central nervous system [14]. Moreover, utilizing a 7-day LPS injection model, our lab found that imatinib, an active component of the FDA-approved anti-cancer drug Gleevac, had the ability to decrease hippocampal Aβ elevation and restore normal cognitive function following the systemic inflammation [14].

Many studies have examined potential strategies to prevent inflammation-induced cognitive decrements, as well as investigate the specific mechanisms responsible for the memory loss associated with inflammation. There is currently no treatment that effectively prevents or reverses these memory deficits. Wang et al. found that inflammation produces memory deficits by increasing a tonic inhibitory conductance that
is generated by $\alpha_5\text{GABA}_A$ receptors. The $\alpha_5\text{GABA}_A$ receptor was found to be critical for both the inflammation-induced memory errors and disruptions in long-term potentiation (LTP). The administration of a benzodiazepine inverse agonist, MRK-016, attenuated the inhibitory current by decreasing the flow of chloride ions across the membrane. Wang et al. found that MRK-016 was able to protect against IL-1B-induced memory deficits, suggesting that disinhibition may protect against inflammation-induced cognitive decrements [15].

Our lab extended this hypothesis by demonstrating MRK-016’s ability to protect against LPS-induced memory acquisition and consolidation errors in a hippocampus-dependent task. Results showed that animals treated with MRK-016 and LPS performed significantly better on a contextual fear conditioning paradigm than those treated with saline and LPS [16]. Results from this study indicate that MRK-016 has the potential to overcome inflammation-induced disruptions in consolidation. Additionally, the study was unsuccessful in identifying the molecular mechanism responsible for mediating the behavioral change. The authors evaluated BDNF expression, a neurotrophic factor highly associated with learning and memory, but found that animals treated with MRK-016 and LPS did not express more mRNA than animals treated with saline and LPS.

Exposure to LPS has been shown to result in a decrease in brain-derived neurotrophic factor (BDNF) [17]. Studies have shown that the presence of Aβ can also lead to reduced BDNF expression due to cellular alterations or inflammation [18]. BDNF, along with other neurotrophins, aid in the regulation of synaptic plasticity and have been
shown to influence long-term potentiation (LTP). Additionally, dysregulation of BDNF synthesis is linked to behavioral symptoms in animal models of Alzheimer’s disease. Because the results of Eimerbrink et al. did not show an increase in BDNF expression, it is possible that the behavioral changes could arise from an increase in expression of TrkB, a BDNF receptor, which would increase the efficacy of BDNF without directly influencing BDNF expression [16].

The Arc gene has been shown to play a critical role in synaptic plasticity and memory formation. Arc−/− mice learn poorly in both hippocampus- and amygdala-dependent tasks requiring long-term memory formation [19]. However, short-term memory in these knock-out mice remains intact. Arc has also been shown to induce endocytosis of the glutamate receptor AMPA, which would reduce action potential propagation [20]. Furthermore, in studies which elucidated the selective sorting of Arc mRNA into neuronal dendrites, authors specifically observed Arc expression within the LTP pathway of the dentate gyrus [21]. The CFC task employed in the present study does require LTP because the testing is 24 hours after training, a time period which surpasses that of “working” or short-term memory. Thus, BDNF and Arc, which are both involved in LTP, are pertinent when studying behavior in this paradigm.

Alzheimer’s disease-like pathology can be modeled in mice using an LPS injection series which ultimately causes learning impairment. While it is understood that the inverse benzodiazepine agonist MRK-016 can prevent inflammation-induced cognitive decrements, we investigated whether it could also be effective in protecting against the cognitive dysfunction correspondent with Aβ accumulation. We hypothesize that injection of the therapeutic MRK-016 will protect against cognitive deficits resulting
from Aβ accumulation induced by LPS administration without affecting levels of Aβ. Additionally, we will evaluate the effect of MRK-016 on TrkB and Arc expression to identify possible biological mechanisms that mediate observed behavioral changes. The action of MRK-016 in this murine AD model will contribute to the overall understanding of the effects of AD pathology.

METHODS

Experimental Subjects

The experimental subjects used for this study were male C57BL/6 mice ages 4-6 months, bred in the TCU vivarium from a breeding stock from The Jackson Laboratory (Bar Harbor, ME). All animals were housed and cared for in accordance with NIH standards and guidelines, and in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of TCU. Following a 2x2 experiment paradigm, animals were first assigned an immune condition, then a treatment condition. The immune conditions were either “LPS” or “saline” indicating that the animals, prior to training, received i.p. injections of LPS (250µg/kg, Sigma, St. Louis, MO) or sterile saline, which served as a control. The treatment conditions assigned were either “saline” or “MRK,” indicating the injection each animal received immediately after training. According to the immune conditions, i.p. injections of LPS or saline were administered once per day for 7 consecutive days (days 1-7) within the same hour each day. On day 8, immediately following CFC training, each animal received an injection of either saline or MRK-016 (3mg/kg, Tocris, Bristol, UK). This procedure resulted in four groups: LPS-Sal, Sal-Sal, LPS-MRK, and Sal-MRK. The dose of LPS was chosen based on previous
work which shows that after 7 consecutive injections of LPS, the animal has stopped exhibiting sickness behavior and pro-inflammatory cytokine levels have lowered back to baseline. The dose of MRK-016 was chosen based on existing literature which indicates this dose to be within therapeutic range for mice [22].

**Contextual Fear Conditioning**

Contextual Fear Conditioning (CFC) was the test used to measure learning outcomes. During CFC, freezing behavior was measured in automated fear conditioning chambers (Coulbourn Instruments, Whitehall, PA), and monitored using FreezeFrame™ software (ActiMetrics Software, Wilmette, IL). The chambers contain an electrified grid floor through which an aversive stimulus was delivered. Dotted wall patterns and a peppermint olfactory cue were also included to supply a memorable context, as has been used previously in the lab [17]. Animals were trained on day 8, 24 hours after their last injection of either LPS or saline. The training session began with a 120-second acclimation period followed by a single 2-second 0.5mA shock delivered to the feet through the grid floor. Animals remained in the apparatus for 60 seconds following the shock to conclude the training. Immediately after training, animals received an i.p. injection of either MRK-016 (3mg/kg) or saline. Animals were tested 24 hours after training, on day 9. During CFC testing, the animals were returned to the apparatus with contextual cues identical to those present in training for a total of 120 seconds, but no shock was delivered. During testing, the animal’s movement was recorded and evaluated for freezing behavior, an innate response to fear in rodents. Total freezing time (sec) was collected and expressed as a percentile to function as the dependent variable whereby a
greater percent freezing indicates a stronger context/aversive stimulus pairing and is understood as better learning.

Tissue Collection, RNA isolation, RT-PCR

All behavioral subjects were humanely converted to biological subjects by CO₂ inhalation. Tissue was then collected for biological investigation in two different ways. In one cohort, dorsal hippocampal tissue was removed 4 hours after CFC training and isolated for storage in RNAlater™ (Austin, TX). Tissue was frozen at -20⁰C until extraction of RNA was performed. In a separate cohort, hippocampal tissue was collected immediately after testing on day 9 and prepared for protein extraction and ELISA procedures. For the ELISA procedure, tissues were homogenized with protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL) containing protease inhibitors and left on ice for an additional 30 minutes, followed by overnight storage at -80⁰C. This crude lysate was next centrifuged at 16,000g for 30 minutes, and the purified lysate was removed and stored.

RNA isolation was performed according to manufacturer instructions (RNeasy Micro kits, Qiagen, Valencia, CA) and RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, NanoDrop products, Wilmington, DE) before being diluted to a uniform concentration for RT-PCR. Samples were processed with PrimePCR Expression Probe Assay (BioRad Laboratories Inc., Hercules, CA) for both TrkB, Arc, and β-actin using a CFX Connect (BioRad Laboratories Inc., Hercules, CA) thermocycler. Target genes were normalized to β-actin prior to analysis using BioRad CFX Manager 3.1 software (Biorad Laboratories Inc., Hercules, CA).
Statistical Analyses

Statistical analyses for behavioral data were conducted with IBM SPSS version 22, using a 2 x 2 ANOVA to determine any significant main effects and interactions. To evaluate RT-PCR data we used Bio-Rad CFX Manager 3.1 Gene Study analysis to identify between-group differences in relative β-actin normalized expression levels for each target gene. All data in figures are shown as mean ±SEM. The alpha level used for all statistical analyses was 0.05, followed by Fisher’s PLSD post hoc tests for significant omnibus effects.

RESULTS

Behavioral Results

To test the hypothesis that the injection of MRK-016 would protect against cognitive deficits resulting from Aβ accumulation induced by LPS administration, we utilized a hippocampus-dependent contextual fear conditioning paradigm. We used a two-way between-subjects analysis of variance (ANOVA) to assess differences between treatment groups on freezing behavior during the first 120s of CFC training. As expected, there were no significant differences between groups during this period, $F(1, 38) = .14, p = .71$, partial $\eta^2 = .004$ (Data not shown). This indicates that there is no evidence of LPS-induced disruptions of freezing behavior during training.

We used another 2x2 ANOVA to examine freezing behavior during the 120s of the testing period, which occurred 24 hours post-training. Results did not identify a main effect for the LPS treatment, $F(1,38) = .58, p = .45$, partial $\eta^2 = .02$, or for MRK-016 treatment, $F(1,38) = 2.54, p = .12$, partial $\eta^2 = .06$. However, results did reveal a
significant two-way interaction between the two treatments, $F(1,38) = 7.79, p = .01$, partial $\eta^2 = .17$.

Simple main effect analyses showed that for animals who received saline injections following training, LPS-treated animals froze significantly less than controls, $F(1,38) = 6.31, p = .02$, partial $\eta^2 = .14$, indicating a significant deficit in contextual fear conditioning. For animals who received injections of MRK-016, there was no difference in freezing behavior between the LPS-treated and control animals, $F(1,38) = 2.06, p = .16$, partial $\eta^2 = .05$. These findings illustrate the ability of MRK-016 to protect against LPS-induced memory decrements. Furthermore, for control animals, there was no difference in freezing behavior following MRK-016 or saline injections post-training, $F(1,38) = .72, p = .40$, partial $\eta^2 = .02$. LPS-treated animals that received an injection of MRK-016 froze significantly more than those who received saline, $F(1,38) = 9.61, p = .004$, partial $\eta^2 = .20$; further exemplifying MRK-016’s ability to restore cognition.

Analysis of the interaction revealed that there were no differences in freezing behavior between animals in the MRK/Saline, MRK/LPS, and Saline/Saline conditions; however, those in the LPS/Saline condition froze significantly less than the other animals. These results support the hypothesis that MRK-016 has the ability to overcome Aβ-induced memory decrements in a hippocampus-dependent task. See Figure 2.
Biological Results

To assess the biological mechanism mediating the behavioral findings, dorsal hippocampus tissue was collected 4 hours after CFC training. Results from comparisons done with Bio-Rad CFX Manager 3.1 Gene Study software identified that the MRK/LPS group expressed significantly less mRNA for TrkB compared to all other groups (p<.05). Results from RT-PCR also revealed no significant differences in Arc mRNA expression. See Figure 3. Further, we failed to support the hypothesis that MRK-016 employs an Arc-dependent rescue mechanism for learning. Analyses showed TrkB mRNA expression to be significantly lower in the LPS/MRK condition compared to the LPS/Saline, Saline/Saline, and Saline/MRK conditions. See Figure 4. These results were unexpected, as TrkB is known to be involved in learning and memory formation and its expression was lowest in the condition in which cognition was restored. However, the results still
suggest some relationship exists between the mechanism of MRK-016 and regulation of BDNF and TrkB.

Figure 3. RT-PCR mean (±SEM) for Arc mRNA expression; *p < .05. Treatments had no effect on Arc mRNA expression, with no statistically significant differences between groups.

Figure 4. RT-PCR mean (±SEM) for TrkB mRNA expression; *p < .05. TrkB expression was significantly decreased in the LPS/MRK condition compared to all other conditions.
DISCUSSION

The results presented here show clear cognitive decrements associated with the administration of the 7-day LPS injection series. An Aβ ELISA assay, which is pending, will confirm that the cognitive decrements co-occur with elevated levels of β-amyloid oligomers. However, based on previous similar studies, we are confident even in the absence of the ELISA that the injection series used induces the desired Alzheimer’s disease-like pathology [13]. Results also clearly show that MRK-016 has a protective effect against the consolidation errors experienced by mice with elevated levels of β-amyloid in a hippocampus-dependent task. MRK-016 binds to the α5GABA_A receptor, which is specifically localized in the hippocampus. It is therefore reasonable to assume that hippocampus-dependent tasks will expose the greatest effect in mice behavior following MRK-016 administration. Future investigations should continue to utilize behavioral tasks of this nature.

These findings are congruent with existing literature which shows that Aβ oligomers are more neurotoxic than Aβ plaques, as the LPS-treated mice, which exhibit Alzheimer’s disease-like pathology and learning deficits, have previously been shown to have an abundance of accumulated Aβ oligomers but not plaques [6]. However, one limitation of the study is the induction of Aβ oligomer accumulation. While these oligomers do represent a toxic element of Alzheimer’s disease pathology, they will dissipate with time as opposed to forming plaques, and are an incomplete physiological picture of the condition.

Another limitation to this study pertains to the translational potential of MRK-016 as a therapeutic to an AD population. While the compound does confer cognitive
protection to mice with no apparent immediate side-effects, other studies have shown it is not well tolerated in elderly human subjects [22]. Thus, the greatest application of this study is in augmenting the theoretical understanding of cognitive decline which occurs in Alzheimer’s disease progression, and supports the hypothesis that Aβ-induced disruption of cognition can be overcome through attenuation of inhibitory signaling.

Our data provides evidence that modulation of central GABAergic transmission can protect against learning deficits induced by β-amyloid accumulation. The only documented action of MRK-016 is attenuation of chloride ion flow through the GABA_A receptor. Interestingly, as noted previously by Eimerbrink et al., the half-life of MRK-016 is relatively short, .3-.5 hours, yet this brief period of attenuated GABAergic transmission is sufficient to alter the outcome of a learning experience 24 hours later. Shankar et al, 2007 provide evidence of an NMDA receptor-based theory of AD cognitive decrements which suggests that aggregation of Aβ oligomers around the NMDA receptor are one cause of synapse loss in AD, which would lead to impaired cognition [23]. Our results support this theoretical approach by showing that decreasing inhibitory transmission by attenuation of GABA_A receptor activity can rescue cognitive impairment. The net positive effect on cell potential by the action of MRK-016 at the GABA_A receptor could be balancing the decrease in excitatory transmission caused by Aβ aggregation on the NMDA receptor.

Looking further into the action and effects of agents like MRK-016 will continue to clarify theoretical mechanisms behind the cognitive decline seen in AD. Detailed effects of MRK-016 administration during learning subsequent to α5GABA_A attenuation are currently unclear. Results presented here offer a preliminary investigation into the
mechanism responsible for MRK-dependent cognitive rescue, but further investigation is needed. MRK-016 does not employ an Arc-dependent rescue mechanism for learning, as shown by the uniform expression of Arc mRNA across all experimental conditions. Results suggest some relationship between the action of MRK-016 and regulation of the BDNF/TrkB pathway, due to a significant difference in TrkB mRNA expression in the LPS-MRK condition. Previous studies using MRK-016 showed no significant increase in BDNF expression following MRK-016 administration, but this has not yet been measured in this AD model. Because our results suggest regulation of the BDNF pathway is involved in MRK-016 activity, it could be worthwhile to investigate Nur-77 alongside further investigation of TrkB involvement. Nur-77 is the transcription factor for BDNF production.

Overall, results from this study demonstrate that manipulation of GABAergic transmission via the inverse benzodiazepine agonist MRK-016 can protect against Aβ-induced disruptions of learning and memory. Moreover, these results indicate that Aβ-related cognitive dysfunction is, at least partially, facilitated through a disruption in signal transmission between neurons. Taken together, results from this study indicate accumulation of Aβ within the hippocampus is sufficient to disrupt cognitive performance in a hippocampus-dependent task, and that decreasing the inhibitory efficacy of GABAergic signaling is a sufficient intervention to protect against Aβ-induced cognitive deficits in this model of Alzheimer’s disease-like pathology.
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