AN EXPLORATION OF THE NEURO-PROTECTIVE AND ANTI INFLAMMATORY EFFECTS OF ROLIPRAM: A THERAPEUTIC TARGET FOR ALZHEIMER'S DISEASE

by

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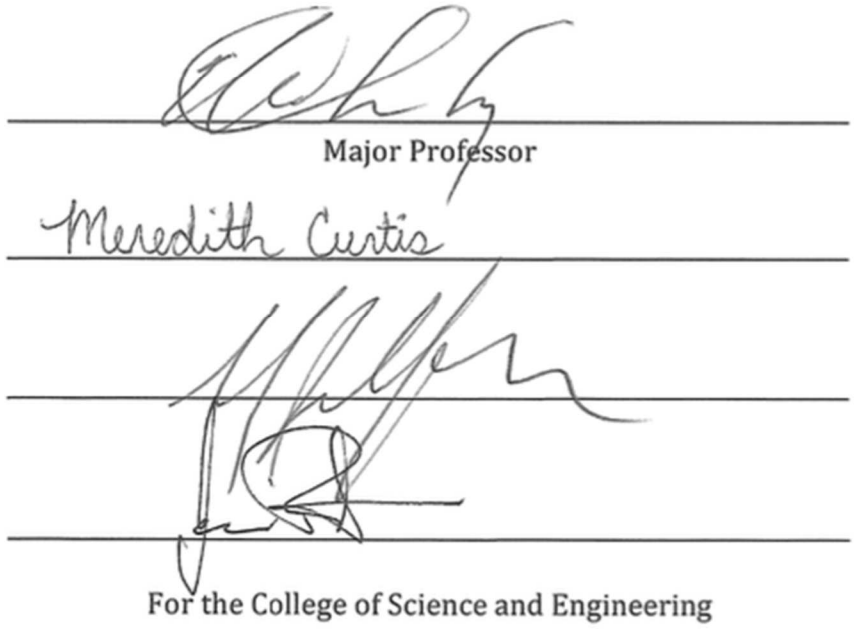

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INTRODUCTION

In 1906, at the South-West German Psychiatrists Meeting in Tubingen, Germany, Dr. Alois Alzheimer first presented the findings from his long-term case study of Auguste Deter (Hippius, 1998). Deter first came to Alzheimer at the age of fifty-one exhibiting erratic behavior, disorientation, and severe memory loss. After her death, at the age of fifty-five, Alzheimer's autopsy of Deter's brain revealed the common pathologies of Alzheimer's Disease (AD): shrinkage of the cerebral cortex, abnormal protein clumping, and fiber tangles. The term "Alzheimer's Disease" was coined in the Eighth Edition of the *Handbook of Psychiatry* in 1910 (National Institute on Aging 2015).

According to the 2015 Alzheimer's Disease Facts and Figures Report, AD is the sixth leading cause of death in the United States and is the only leading cause of death that cannot be prevented, cured, or slowed. From the years 2000-2013, deaths attributed to AD have increased by seventy-one percent, while the other five leading causes of death have decreased in prevalence (Alzheimer's Disease Facts and Figures 2015). In 2012, 5.4 million people were suffering from AD and in 2050 it is projected that 13.8 million people will be diagnosed with this disease (Hebert et al. 2013). Strikingly, one in every three people will die of AD or another form of dementia (Alzheimer's Disease Facts and Figures 2015).

Not only is AD extremely prevalent, and likely to increase in upcoming years, but the cost of care to treat AD is also incredibly daunting. The total annual cost associated with AD is currently between $157 billion and $215 billion dollars, $11

billion dollars of which is paid by Medicare on a yearly basis (Hurd et al. 2013). With the rising numbers of people being diagnosed with this disease, the financial burdens associated with AD are only projected to rise. Therefore, it is extremely important to understand the mechanisms that lead to AD, in order to find a cure. The pathologies first observed by Alzheimer in 1906 are what separate AD from other forms of dementia. The "abnormal protein clumping" is now identified as Amyloid-Beta (Aβ) senile plaques (Dubac 2013). Aβ is a peptide that is cleaved from a larger transmembrane protein called Amyloid Precursor Protein (APP) (Yonkin 1998). Once cleaved, Aβ begins to aggregate, eventually forming senile plaques. These plaques are neuro-toxic and themselves act as inflammatory agents, leading to neuronal cell death (Choi et al. 2013). The "fiber tangles" refers to neurofibrillary tangles (NFTs), which are aggregates of microtubule-associated hyper-phosphorylated tau proteins (Selkoe, 2001). These tangles occur within the neurons and interfere with cell signaling. Alzheimer also noticed a decrease in brain volume in Deter's brain. This shrinkage of the cerebral cortex is due to the significant death of neurons and loss of synapses that are a result of the Aβ plaques and the NFTs (Mattson, 2004). The senile plaques and NFTs are thought to be the cause of the cognitive deficits that are displayed in AD. These pathologies initially begin in the hippocampi of individuals, an area of the brain that is associated with certain types of learning and memory. The disease first begins with mild impairments, such as minor short-term memory loss (Facts and Figures 2015). Patients with AD progressively get worse as the pathologies spread to other parts of the brain, leading to severe cognitive decline. AD eventually leads to death when the parts of

the brain that control involuntary functions, such as breathing and heart rate, are affected, resulting in loss of function (Heneka & O'Banion, 2007).

There are two distinct forms of AD: Early On-set and Late-Onset AD. Early Onset AD, also referred to as familial AD, generally occurs in people between the ages of 40 and 50 (Facts and Figures 2015). This type affects only 5% of all AD patients and seems to be attributed to genetics (Dubuc, 2013). Patients suffering from Familial AD typically express mutations in three particular genes--APP, PSEN1, PSEN2—which all play a role in the production of Aβ (Tanzi & Bertram, 2001). Late-Onset, or Sporadic, AD occurs in people 65 years of age or older. This is the most prevalent form of AD and its cause is not currently known (Dubuc, 2013). However, inflammation, both in the periphery and the CNS, has been shown to play a large role in both types of AD. It is well known that chronic inflammatory diseases, such as Type II diabetes, cardiovascular disease, and hypertension, increase one's risk of developing AD (Schmidt et al., 2002). This is explained by several studies that have shown that neuro-inflammation can be stimulated by repeated bouts of peripheral inflammation (Cunnigham et al., 2013). Our lab has demonstrated that systemic inflammation in non-transgenic mice generated in response to 7 days of intraperitoneal (i.p.) lipopolysaccharide (LPS) injections leads to an elevation in Aβ-deposition in the hippocampus and cognitive decline, two common symptom of AD (Kahn et al., 2012).

Contributing to this inflammation are microglial cells, the immune cells of the brain. Generally microglial cells are important in mounting an acute pro-

inflammatory response to substances that the immune cell deems foreign, or a threat to the brain.

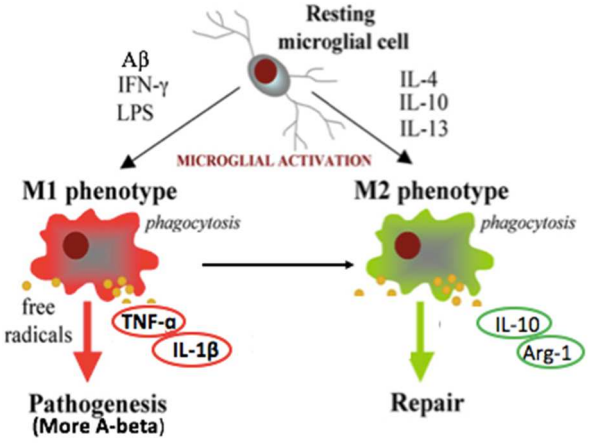

Figure 1. Microglial activation and polarization from the M1 to the M2 phenotype. (Modified from Fumagalli et al., 2011)

Once stimulated, the microglia start secreting pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Copeland, Warren, Lowry, & Calvano, 2005). These cytokines can lead to the activation and recruitment of other immune cells, and result in cell-signaling pathways that are designed to clear the infection (Jack et al., 2017). However, chronic secretion of pro-inflammatory cytokines can be extremely damaging to surrounding tissues. Fortunately, microglia have the ability to transition from a "classically" activated pro-inflammatory state, the M1 phenotype, to an "alternative" neuro-protective anti-inflammatory state, the M2 phenotype, during the progression of an inflammatory response (Figure 1); this act of transitioning from one phenotype to another is often referred to as polarization. M2 microglia secrete anti-inflammatory cytokines, such as IL-10 and IL-4, and neurotrophic factors, all of which tell surrounding cells to promote healing, tissue

regeneration, and debris clearance (Cherry et al., 2014). It is not well understood how this transition occurs, but it is closely linked to that particular cell's environment. Interestingly, the microglia in Alzheimer's patients favor the pro-inflammatory, M1, phenotype which leads to more cell death, more tissue damage, and more amyloid-beta deposition (Saijo et al., 2011). This is likely due to the fact that Aβ peptides bind to several receptors on microglial cells—TLR receptors, RAGE, F_c receptors, complement receptors—which activate them towards a pro-inflammatory state (Chitre & Sathaye, 2016). Microglia cultured from autopsied AD brains also show a heightened pro-inflammatory response to Aβ compared to non-demented controls, suggesting that these M1 microglia contribute to the chronic neuro-inflammation observed in AD (Lue et al., 2001). Using a transgenic model of AD, Jimenez et al., highlighted an age-dependent phenotypic change in AD microglia activation: young mice favored the M2 state, while aged mice demonstrate a heightened M1 cytotoxic phenotype (2008). Furthermore, chronic inflammation and exposure to M1-activators has been linked to a reduction in microglial phagocytosis of Aβ (Koenigsknecht-Talboo, 2005). These findings support the notion that chronic neuron-inflammation, which can be partially attributed to M1-activated microglia, is exacerbating AD pathology.

Interestingly, recent studies have also highlighted the importance of certain members of the NR4A subfamily of nuclear orphan receptor proteins in the polarization of macrophages. The NR4A subfamily of nuclear orphan receptor proteins are transcription factors that do not bind to a known ligand but are activated by other stimuli such as inflammation, growth factors, and certain

hormones. Because macrophages and microglia arise from the same hematopoietic origin, they share similar functional characteristics. Enhanced expression of the nuclear orphan receptors Nurr1 and Nur77 have been shown to play a major role in attenuating the production of pro-inflammatory cytokines by interfering with the NFκB immune signaling pathway (Saijo et al., 2009; Ipseiz et al., 2014). Additionally, increased expression of Nurr1 in macrophages leads to an up-regulation of M2-markers and anti-inflammatory cytokines, such as IL-10nand Arg1 (Mahajan et al. 2015). Not only do Nurr1 and Nur77 play a role in regulating inflammation, these transcription factors are also critical in inducing the expression of proteins that are essential for learning and memory consolidation (McNulty et al., 2012). While acute inflammation induces Nurr1 and Nur77 expression, chronic inflammation, like that present in AD, reduces adequate expression of these nuclear orphan receptors. (Pei et al., 2005). In a study performed by Dickey et al., Aβ suppresses Nur77 in a transgenic AD mouse model (2004). Another study measured the expression of Nurr1 in 5XFAD mice, an animal model of AD, and found that Nurr1 is up-regulated early in the disease, as the pathologies begin to develop—supporting the notion that inflammation leads to increased expression of the nuclear orphan receptor proteins. However, Nurr1 expression declines in an age-dependent manner as Aβ plaques become more prevalent. The suppression of these nuclear orphan receptors may contribute to the bias of microglia in AD patients towards an M1 phenotype, as well as the cognitive decline that is typically associated with AD.

This proposal investigates the efficacy of the drug rolipram in limiting inflammation and cognitive decline often associated with AD. Rolipram, a

phosphodiesterase IV inhibitor originally used to treat depression, has been shown to increase the expression of Nurr1 and Nur77 in primary neuron cultures, resulting in a neuro-protective response (Volakis et al., 2013). Rolipram, and other phosphodiesterase-inhibitors, have been shown to rescue cognition long-term in transgenic AD mouse models, to activate the 26S proteasome to aid in the clearance of hyperphosphorylated tau, and to lessen Aβ burden in transgenic mice (Cuadrado-Tejedor et al., 2011; Myeku et al., 2015; Zhang et al., 2013). In addition, rolipram also acts as an anti-inflammatory agent. In one study, a murine microglial cell line co-treated with rolipram and LPS showed between a 30-70% reduction in the secretion of pro-inflammatory cytokines –TNFα, IL-1, IL-6 –compared to cells treated with vehicle and LPS (Yoshikawa, Suzumura, Tamaru, Takayanagi, & Sawada, 1999). Macrophages have also been shown to demonstrate characteristics of the anti-inflammatory, "alternatively activated," M2 phenotype when treated with rolipram (Erdely et al., 2006).

Rolipram has been used in animal models of inflammation as well. Ariga et al. utilized rolipram as a possible treatment option for chronic obstructive pulmonary disease (2004). Mice pre-treated with rolipram (3mg/kg i.p.), showed a significant reduction in neutrophil recruitment to the lungs after LPS inhalation (Ariga et al., 2004). Treatment with rolipram also led to a decrease in cytokine production, neutrophil recruitment, and neuronal apoptosis in a murine model of ischemic stroke (Kraft et al., 2013). Rolipram is a promising therapeutic agent because it is known to cross the blood brain barrier, a very important aspect when it comes to treating AD. Its neuro-protective and anti-inflammatory properties may help

mitigate or slow the progression of the pathologies and symptoms associated with AD.

The overarching goal of this proposal seeks to determine a possible mechanism by which rolipram treatment induces its anti-inflammatory effects *in vitro*. We hypothesize that if rolipram leads to the up-regulation of Nurr1 in primary neurons (Volakakis et al., 2010), then rolipram may lead to the up-regulation of NR4A proteins in microglia and initiate the M1 to M2 phenotype transition. Within this proposal we investigate how rolipram administration affects nuclear orphan receptor expression on LPS-treated BV2 cells. We also explored rolipram's potential as a therapeutic agent *in vivo*, testing its anti-inflammatory and cognitive-enhancing effects within our inflammation-induced AD model.

METHODS AND MATERIALS

Reagents

(R,S) – Rolipram was purchased from LC Laboratories, Woburn, MA, and was dissolved in 3% DMSO and sterile saline to make a 100μM stock solution. This stock was further diluted to 10μM and 0.3% DMSO using complete Dulbecco's Modified Eagles Medium (DMEM): 10% fetal bovine serum, 1% penicillin streptomycin, and 1% L-glutamine. LPS (Escherichia coli serotype: 055:B5) was purchased from Sigma-Aldrich, St Louis, MO. LPS was reconstituted in sterile saline to form a stock solution at 37.5 μg/ml. This solution was diluted to a working concentration of 1 μg/ml with complete media. All treatments—rolipram, LPS, and media—contained 0.3% DMSO.

Cell Culture

BV2 cells, a microglial cell line, was graciously donated by Dr. Sonny Singh. Prior to experimentation, the BV2 cells were harvested in complete DMEM. These cultures were maintained in a humidified incubator at 37ºC and 5% CO_2 until they reached a passage range between 27 and 29.

Cell Culture Experimental Design

At the appropriate passage, BV2 cells were plated into 60.8cm^2 dishes (Olympus Plastics) at a total density of 2 x 10^6 cells. The cells were allowed to incubate overnight in the presence of complete media, and on the following day, the cell plates were divided into five treatment groups: No treatment (media only), ROL (10μM), LPS (1 μg/ml), Pre-ROL (1 hr pre-exposure to ROL (10μM) prior to LPS (1 μg/ml) exposure), and Pre-LPS (1 hr pre- exposure to LPS (1 μg/ml) prior to ROL (10μM) exposure). Once the designated treatment was added at a final volume of 10ml, the time began. The time for the pre-treatment groups started once the second reagent was added to the plates. The supernatants were collected and cells were lysed at the six different time points: 30min, 1hr, 2hr, 4hr, 12hr, and 24hr. At the designated time point, all five treatment plates were placed on ice and supernatants were collected and kept at -20ºC awaiting future analysis. Following supernatant removal, the cells were then washed with ice cold PBS and lysed using 1X RIPA lysis buffer (RPI, Mt. Prospect, IL) containing 1% protease inhibitors (RPI, Mt. Prospect, IL).

Subjects

C57BL/6J mice, 6-9 months old, were utilized for all studies. These mice were bred in the Texas Christian University (TCU) vivarium from a breeding stock acquired from Jackson Laboratory. All mice were housed in polycarbonate mouse cages in a room that maintained a constant 12-hour light/dark cycle. All mice had access to food and water. They were cared for in accordance with the procedures put forth by the Institutional Animal Care and Use Committee of TCU.

Experimental Design Pilot # 1

Mice were randomly divided into four groups where they were pretreated with one daily intraperitoneal (i.p.) injection of rolipram (ROL) (0.03mg/kg) or saline (SAL) for 7 days, followed by 7 days of LPS (250 μg/kg) or SAL injections: ROL/LPS (n=7), ROL/SAL (n=6), SAL/LPS (n=5), SAL/SAL (n=6). (See Figure 2). (R,S) – Rolipram was purchased from LC Laboratories, Woburn, MA, and was dissolved in 4.5% DMSO and sterile saline; the pre-treatment saline control also contained 4.5% DMSO. The LPS (Escherichia coli serotype: 055:B5) was purchased from Sigma-Aldrich, St Louis, MO.

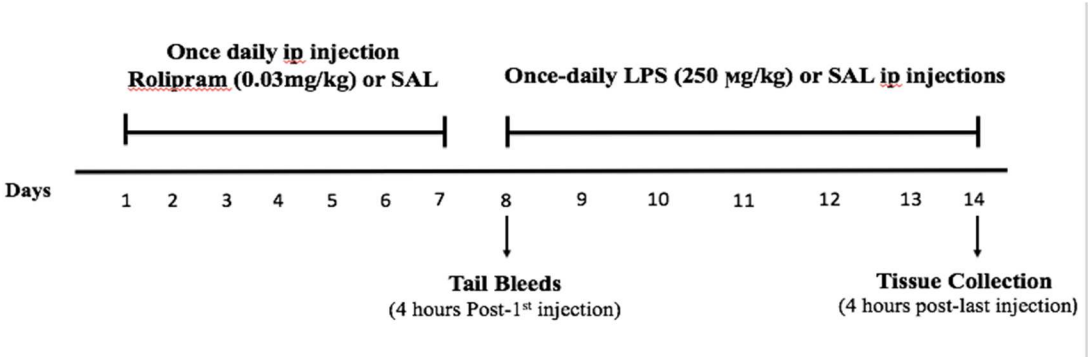

Figure 2. Experiment schematic for pilot study # 1. Pre-treatment for 7 days with rolipram or saline i.p. injections followed by 7 days of LPS or saline i.p. injections. On day 8, four hours post the first LPS or SAL injection, blood was collected from the subject's tail vein. Hippocampal tissue was collected 4 hours after the last injection on day 14.

Experimental Design Pilot #2

Mice were first administered 7 days of LPS (250 μg/kg; i.p.) or SAL injections (i.p.), followed by a single injection of either ROL (0.03mg/kg; i.p.) or SAL (i.p.), received on Day 8, 30 min prior to the training session of contextual fear conditioning (CFC): LPS + SAL (n= 5); LPS + ROL (n=5); SAL + ROL (n= 5); SAL + SAL (n=4); (Figure 3). (R,S) –Rolipram was purchased from LC Laboratories, Woburn, MA, and was dissolved in 4.5% DMSO and sterile saline; the post-treatment saline control also contained 4.5% DMSO. The LPS (Escherichia coli serotype: 055:B5) was purchased from Sigma-Aldrich, St Louis, MO.

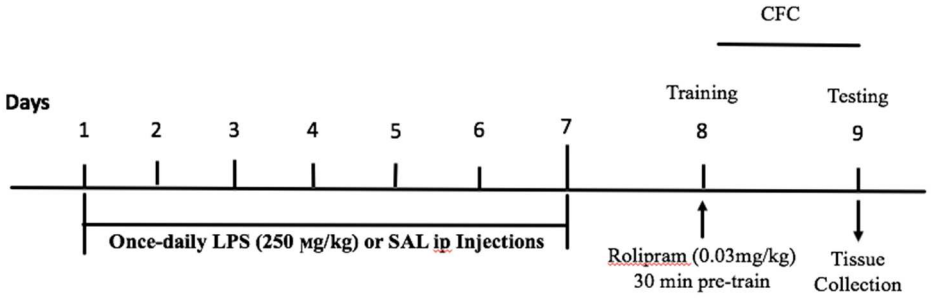

Figure 3. Experiment timeline for pilot study # 2. Mice were administered daily LPS (250 μg/kg, i.p.) or saline injections for 7 days. 24 hours after the last injection, mice were administered 1 injection of ROL (0.03mg/kg; i.p.) or SAL 30min before training in a CFC paradigm. 24 hours-post training, the mice were tested and immediately sacrificed for tissue collection.

Experimental Design Pilot #3

Female mice were administered either 7 days of LPS (250 μg/kg; i.p.) or SAL injections (i.p.), followed by 3 ROL (3mg/kg) or SAL injections: one administered 4 hours after the 7^{th,} , and last, LPS or SAL injection, one administered 24 hours after the 7th injection, and the last administered 48 hours after the 7th injection. Following the final injection, mice were sacrificed and tissue was collected. Male mice also received the same injection series, except males underwent behavioral

testing; therefore, the last two ROL or SAL injections occurred 30min prior to training and 30min prior to testing in a CFC behavioral paradigm (Figure 4).

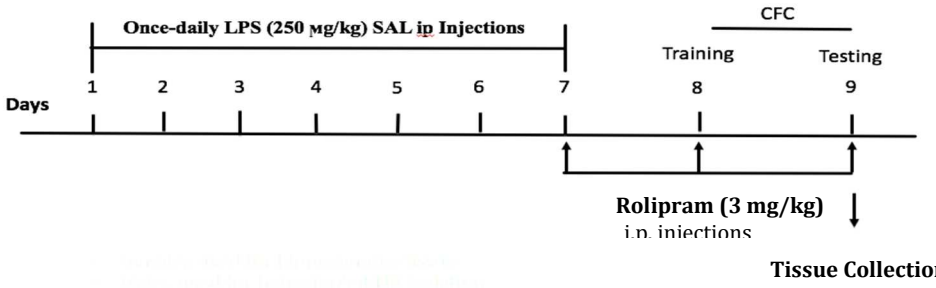

Figure 4. Experimental timeline for pilot study # 3. Mice were administered daily LPS (250 μg/kg, i.p.) or saline injections for 7 days. 4 hours after the last injection, mice were administered either a ROL (3mg/kg, i.p.) or a SAL injection. 24 hours after the last LPS-injection, male mice were subjected to a CFC paradigm. Mice were administered 1 ROL (3mg/kg, i.p.) or SAL injection prior to both training and testing phases. 30 min after testing, mice were sacrificed for tissue collection.

Male mice were utilized for behavioral testing and RT-PCR, while the female tissues were isolated for ELISAs and Western blots. (R,S) –Rolipram was purchased from LC Laboratories, Woburn, MA, and was dissolved in 4.5% DMSO and sterile saline; the post-treatment saline control also contained 4.5% DMSO. The LPS (Escherichia coli serotype: 055:B5) was purchased from Sigma-Aldrich, St Louis, MO.

Experimental Design Pilot #4

Mice were randomly divided into two groups; those receiving 1 injection of rolipram (3mg/kg) and those receiving 1 injection of saline (i.p). (R,S) –Rolipram was purchased from LC Laboratories, Woburn, MA, and was dissolved in 4.5% DMSO and sterile saline; the saline control also contained 4.5% DMSO. Burrowing, a hippocampus-dependent, murine typical task, was used to measure sickness behavior in this pilot study. The experimental design closely followed the protocol described by McLinden et al., in 2012. This burrowing paradigm consists of 3

phases: Group facilitation, Individual baseline, and Testing (Figure 5). Group facilitation lasted for three days, allowing the mice to learn the burrowing task together. All cage-mates were placed in a cage containing a burrowing tube (a plastic cylinder: 20.3cm long, 5.7cm in diameter, and raised 2.5 cm at one end) filled with 200g of food. The mice were allowed to burrow overnight; all of the food was removed by the following morning and the mice were returned to their home cages. Individual baseline accounts for innate differences in burrowing ability. Mice were individually placed in a cage with a burrowing tube containing 200g of food. The amount of food burrowed was measured using a digital scale 2hr after initial placement into the cage with the burrowing tube. Individual mice remained in their burrowing cages until the following morning where they were returned to their home cages. This lasted for 3 days, after which the animals were rank ordered by the average amount of food burrowed during individual baseline days and pseudo-randomly assigned treatment groups. The Testing phase is similar to individual baseline, except the mice received 1 injection of either rolipram (3mg/kg; i.p.) or saline (i.p.) prior to placement in individual cages containing a burrowing tube. Food burrowed was measured 1hr, 2hrs, 24hrs, and 48hrs after the injection was administered.

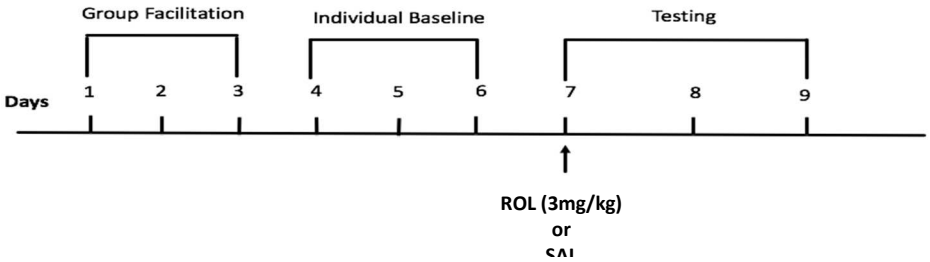

Figure 5. Burrowing study timeline.

Blood Collection

For Pilot #1, blood from the tail vein of each subject was collected into heparin-coated serum tubes (BD Vacutainer,®Franklin Lakes, NJ) four hours after the first LPS injection on Day 8. The blood samples were centrifuged at 10,000 rpm for 5 min, and serum was collected and stored at -20 ºC.

Contextual Fear Conditioning

Contextual fear conditioning (CFC) was the behavioral paradigm used to measure hippocampus-dependent cognitive function. Mice innately freeze when frightened, and CFC utilizes this response by measuring freezing behavior 24 hours after an aversivee stimulus is administered, in order to determine if a mouse has learned a shock/context pairing. The protocol used was explicitly described by Kranjac et al., in 2012. Freezing behavior was measured utilizing automated chambers (Coulbourn Instruments, Whitehall, PA, 7WxDx12H), and FreezeFrame™ software (ActiMetrics Software, Wilmette, IL). The electrified grid floor of the chambers delivers the aversive stimulus. The dotted walls of the chamber and an olfactory cue provides contextual cues to the mouse. 24 hours after the last LPS injection, on Day 8, mice were administered 1 injection of rolipram (0.03mg/kg) 30 min before training. The mice were then tested 24 hours later, on Day 9. The training session consisted of 120-s of acclimation time, followed by a 2-s shock at 0.7mA. The animals were removed from the chamber 60 s after the shock. No shocks were administered during the testing day, rather the system recorded freezing time for 120-s.

Tissue Collection

For all experiments, mice were euthanized by CO_2-inhalation, and whole hippocampi were removed. The hippocampal tissue was homogenized in Pro-Prep lysis buffer (Bio-Rad Laboratories, Hercules, CA) containing protease-inhibitors, immediately frozen in dry-ice, and stored at -80 ºC in preparation for analysis. The male mice from Pilot #3 were euthanized 30 min following behavioral testing, and dorsal hippocampi were removed under RNAase free conditions. Dorsal hippocampi were immediately placed in nuclease-free tubes containing RNAlater® (Ambion, Austin, TX) and kept at -20°C awaiting RNA isolation.

DC Protein Assay

For all experiments, the homogenized hippocampal tissue and cell lysate samples were thawed on ice and centrifuged at 4 ºC for 30 min at 16,000 x g. The clear lysate was isolated and used to perform a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) in adherence to manufacturer's instructions.

TNF-α ELISA

The Mouse TNF-α ELISA Deluxe Standard Kit (Biolegend, San Diego, CA) was utilized, in accordance with manufacturer's instructions, to measure both cell supernatant and murine serum TNF-α levels. The supernatants were thawed and centrifuged at 1500 RPM for 5 min at 4 ºC to remove any non-adherent cells that had escaped into the media. An initial plate was run to determine proper dilution factors. All samples were run in duplicate at 100µl per well and the standard was run in triplicate. Thawed mouse serum was also plated in duplicates of 100µl on a separate plate. The plates were read at 450nm and 570 nm (BMG LabTech FLUOstar

Omega, Cary, NC), as per manufacteur's instructions. TNF-α levels were depicted in pg/ml.

Aβ ELISA Procedure

Following protein concentration analysis, the BetaMark $A\beta_{x-42}$ ELISA (Biolegend, San Diego, CA) was utilized in accordance with manufacturer's instructions, as described previously (Kahn et al., 2012; White et al., 2016). Incubation buffer was used to dilute samples, which were plated in duplicates of equal volume. The HRP-labeled detection antibody was applied for overnight incubation at 2-8 ºC. After incubation, the wells were washed before applying TMB substrate. The plate then incubated in the dark for 45 min before the optical density was read at 620nm. (BMG LabTech FLUOstar Omega, Cary, NC).

Western Blot

Western blots were run to determine differences in Nurr1 expression between the LPS group and the Pre-ROL group at all 6 time points. The protocol followed was previously described in detail by White et. al., in 2016. Proteins were separated by size using Mini-Protean® TGX™ precast Any KD gels (BioRad, Hercules, CA) at 200V for 40min. The proteins were then transferred onto polyvinylidene fluoride (PVDF) membrane (Genesee Scientific, Morrisville, NC) using a semi-dry transfer unit (Biorad, Hercules, CA) that applied 0.15 amps per gel for 45min. The membrane was blocked in 5% bovine serum albumin (BSA) in TBST before antibody was applied for overnight incubation at 4ºC. The antibodies used were anti-Nurr1 rabbit polyclonal IgG and anti-CypB goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies were diluted in 5% BSA in TBST 1:650,

and 1:6000 respectively. CypB was used as the loading control. The following day the membranes were washed in TBST before peroxidase-conjugated Affinipure secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied for a 2hr incubation period. The membrane was again washed in preparation for membrane imaging. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Wiltham, MA), the detection reagent, was directly applied to each membrane before being imaged using a Syngene G:Box (Syngene, Fredrick, MD). Densitometry analysis was performed utilizing Genetools software (Syngene, Fredrick, MD).

RNA Extraction

Maxwell® 16 LEV simply RNA Tissue Kits (Promega Corporation, Madison, WI) were used to isolate RNA from our tissue samples, according to manufacturer's instructions. RNAlater® (Ambion, Austin, TX) was first removed for each sample and tissue was then homogenized in kit-provided homogenization solution, 210 µl/sample, and lysis buffer, 200µl/sample, using a Misonix Microson XL-2000 Ultrasonic Cell Disruptor (QSonica, LLC., Newton, CT). Extractions were performed on Promega's Maxwell® 16 Research System. After the extraction step, isolated RNA from each sample was immediately measured using NanoDrop 2000 (Thermo Scientific) to determine overall RNA concentration and the A_{260}: A_{280} ratio.

cDNA synthesis

Isolated RNA was converted into cDNA utilizing the SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), as per

manufacturer's instructions. Every RNA sample was converted to 500ng/µl prior to cDNA synthesis, and 3µl of each standardized sample was added to a mastermix containing 1ul of Random Hexamers, 1ul dNTPs, and 5 ul of nuclease-free water. This mixture was placed on a PCR block (Biorad T100 Thermocycler) for 5min at 65°C and immediately put on ice. A master mix containing 2ul of 10X Reverse transcription buffer, 4ul of 25mM MgCl2, 2ul 0.1 mM DTT, 1ul RNAase out, and 1 ul of SuperScript III per sample were then added to the cooled original mixtures. The tubes were then placed on the PCR block for 10min at 25°C, 60 minutes at 50°C, and 5 minutes at 85°C. 30 ul of nuclease-free water was added to each PCR product and kept at -20°C until qPCR was performed.

qRT-PCR

qRT-PCR was conducted utilizing a StepOnePlus System (Applied Biosystems, Foster City, CA). Three targets were chosen and purchased from Integrated DNA Technologies (Coralville, IA): NR4A2 (Primer 1: 5'GTAACTGTAGCTCTGAGAAGCG-3'; Primer 2: 5'-CACTGTCCACCTTTATTTCCTC-3'), HPRT (Primer 1: 5'-AACAAAGTCTGGCCTGTATCC-3'; Primer 2: 5'-CCCCAAAATGGTTAAGGTTGC-3'), and BDNF (Primer 1: 5'-GCAACCGAAGTATGAAATAACCA-3'; Primer 2: 5'-GTTTATCACCAGGATCTAGCCA-3'). Samples were run in triplicate on the same plate containing a negative control, which did not contain cDNA. HPRT was chosen to act as the housekeeping gene. qPCR mixtures contained 0.36 ul of forward and reverse primers, 5 ul of SYBR green, 3.64 ul of nuclease-free water, and 1 ul of cDNA, except for the negative controls which replaced the cDNA with 1 ul of nuclease-free water. The thermal

profile included: 25min at 95°C, 60min at the target's optimized annealing temperature, and 15min at 95°C. In accordance with the $\Delta\Delta C_T$ method, an optimization step was first performed to determine optimal annealing temperatures for each primer. The PCR efficiency was also measured for every gene by performing a qPCR reaction on five serial dilutions of a stock solution containing cDNA from each sample. A modification of the Pfaffl's method was used to account for differences in PCR efficiencies of each of the target genes and the house-keeping gene (Pfaffl, 2001). The relative quantification for each treatment group was calculated using the following equation provided by Pfaffl:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}}\,(\text{control-sample})}}{(E_{\text{ref}})^{\Delta CT_{\text{ref}}\,(\text{control-sample})}}$$

Statistical Analysis

The data collected from all biological assays was analyzed using SPSS software (IBM Corporation, Armonk, NY). A two-way analysis of variance (ANOVA) test (Statview 5.0, SAS, Cary, NC), was used to measure the two main effects—treatment and time—on the production of TNF-α by BV2 cells. A two-way analysis of variance (ANOVA) (Statview 5.0, SAS, Cary, NC) was also used for all *in vivo* experiments measuring the treatment effect of LPS or SAL and the condition effect of ROL or SAL on peripheral TNF-α production, freezing behavior, Aβ deposition, BDNF and NR4A2 mRNA expression, and NR4A2 protein expression. A Student's t-test was used in the cell culture experiment to measure the difference in Nurr1 expression between the LPS-treated cells and the Pre-ROL-treated cells at all 6 time

points. The alpha level chosen for all statistical tests was 0.05. Significant effects were further analyzed using Bonferroni pairwise comparisons.

RESULTS

Cell Culture Experiment

The Effects of LPS and Rolipram Treatment on TNF-α production in BV2 cells.

A TNF-α ELISA was performed on supernatants of BV2 cells that had been treated with LPS, ROL, Pre-treated with LPS for 1hr before ROL administration, Pre-treated with ROL for 1hr prior to LPS exposure, or media alone. Cells were treated for 30min, 1hr, 2hrs, 4hrs, 12hrs, and 24hrs. A two-way analysis-of-variance (ANOVA) test was conducted to measure the effects of treatment and time on the production of TNF-α by BV2 cells. This test revealed that there was a significant interaction effect of treatment by time ($F_{(20, 53)} = 0.026$). We were able to unpack these significant differences using Bonferoni pairwise comparisons. Only cells administered LPS alone showed significant differences in TNF-α production across time. At 30min and 1hr, the TNF-α levels were not significantly different from one another across all treatment groups; however, by 2hrs TNF-α levels in the LPS-treated group had increased significantly, which was maintained for the remaining time points (Figure 6a).

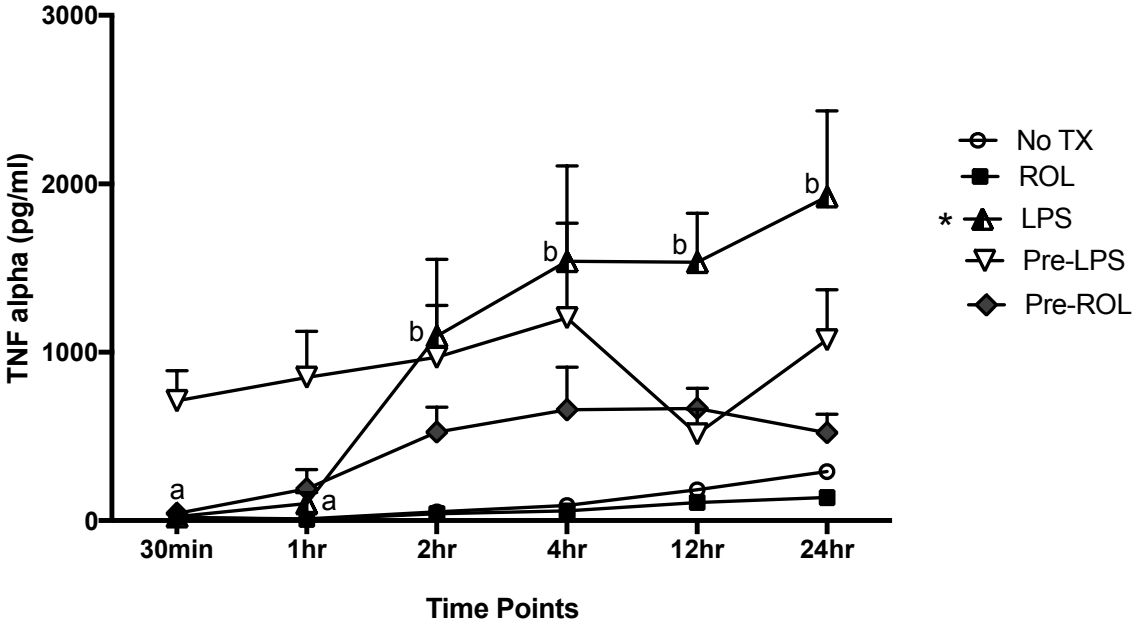

Figure 6a. TNF-α production in five different treatment groups across time. Only the LPS group showed significant differences in TNF-α expression over time, as indicated by * ($p < 0.05$). Means with different letters (a, b) are significantly different from one another ($p < 0.05$). Bars represent the mean $\pm$ SEM.

Bonferoni pairwise comparisons further allowed us to analyze treatment differences in TNF-α production at each individual time point (Figure 6b). At both the 30min and the 1hr time points, there were no significant differences in TNF-α production among the five treatment groups. At the 2hr time point, however, the LPS-treated cells produced significantly more TNF-α, than those treated with ROL and media alone, while they did not differ from the Pre-LPS or the Pre-ROL group. The groups that received rolipram, either before or after LPS exposure, showed diminished TNF-α production not significantly different from either the media only and ROL controls, or the LPS-treated cells. At 4hrs, a similar pattern was maintained except that now both the LPS and Pre-LPS groups expressed significantly higher levels of TNF-α compared to the ROL and No TX controls. The Pre-ROL groups once again showed an increase in TNF-α expression that was neither significantly different from the No TX and ROL controls nor

the LPS and Pre-LPS groups. At 12hrs, most of the differences amongst the treatment groups in TNF-α observed at the 4 hr time point remains the same, except that the Pre-LPS, once again, shows a blunted production of TNF-α that is not significantly different from that observed in any of the other four treatment groups. At the 24hr time point, the Pre-ROL group showed significant reductions in TNF- α compared to the LPS-treated cells. The Pre-ROL group also did not differ from the negative controls. The Pre-LPS group carried forward a similar trend that was observed at the 12hr time point, in which it showed a reduction in TNF- α that was neither significantly different from the LPS-treated cells nor the No TX group. However, the Pre-LPS did show a slight increase in TNF- α compared to the cells receiving ROL alone (p=0.049).

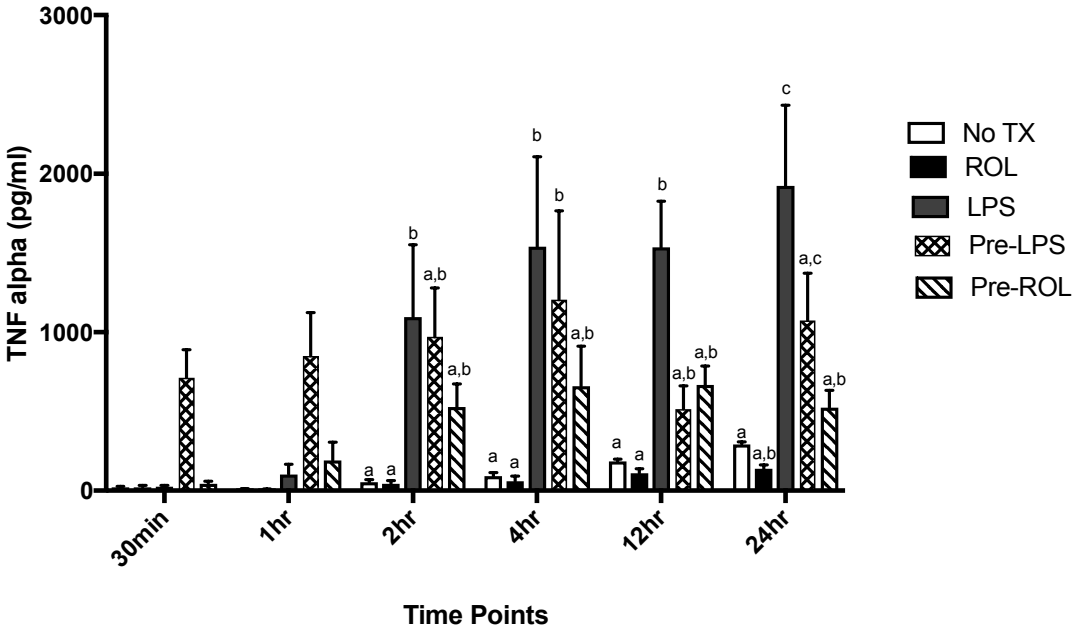

Figure 6b. The effects of rolipram and LPS on TNF-α production in BV2 cells at each time point. Means with different letters (a,b,c) are significantly different from one another ($p < 0.05$). Bars represent the mean ± SEM. (Abbreviations: No TX = no treatment; ROL = rolipram; LPS = lipopolysaccharide; Pre-LPS = LPS for 1hr followed by rolipram; Pre-ROL = rolipram for 1hr followed by LPS).

Western Blot Analysis of Nurr 1 Expression

Next, we sought to determine the mechanism that leads to rolipram's suppression of TNF-α in the Pre-ROL group observed at the 24 hour time point. Western blot analyses were performed to determine if Nurr 1 could be contributing to this protection. Lysates from the LPS and the Pre-ROL groups at all six time points were used to determine if ROL pre-treatment leads to the upregulation of Nurr 1. A Student's T-test was run on normalized densitometry ratios to compare Nurr1 expression between the two treatment groups at each individual time point (Figure 6c.).

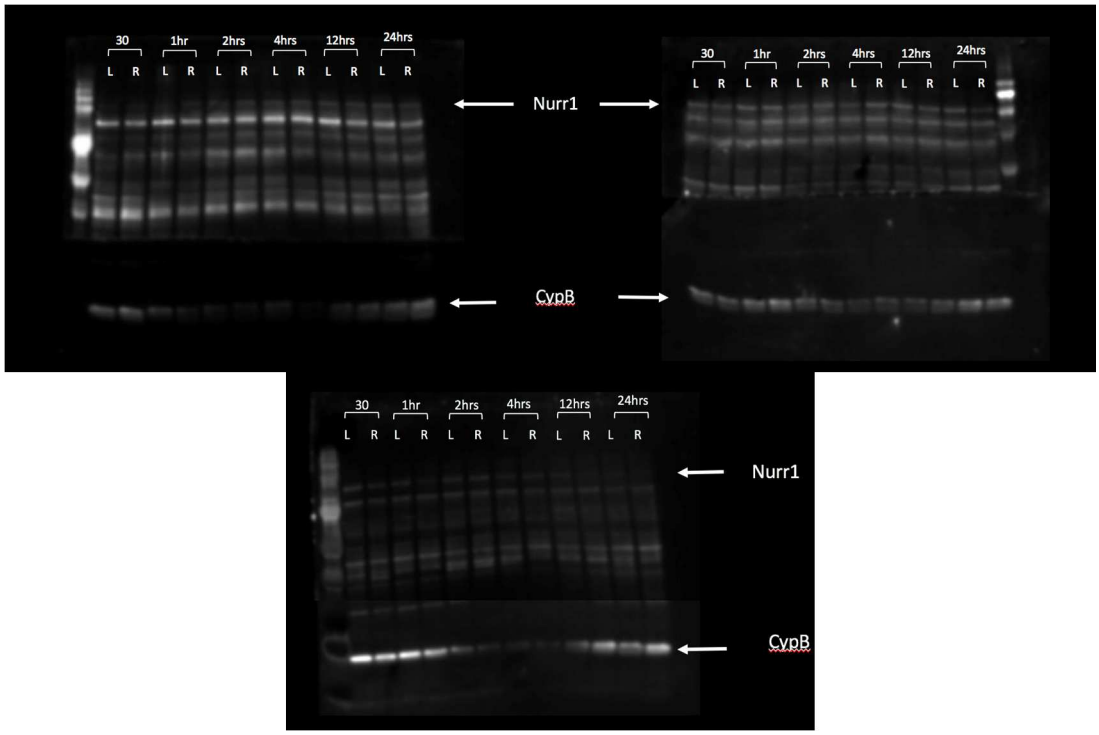

Figure 6c. Western blot images comparing Nurr1 expression in cells administered LPS (1µg/ml) and cells treated with rolipram (10 µM) for 1 hour prior to LPS exposure. These blots show Nurr 1 expression over time in each treatment group. (1, 2, and 3 represent different batches).

There were no significant differences between groups at the 30min and 1hr time points. At 2 hours there is a trending increase in Nurr1 expression in the Pre-ROL group (p=0.094), suggesting that Nurr1 may be contributing to rolipram's anti-inflammatory effects early on, as TNF-α production is blunted at approximately 2 hrs after LPS exposure. This trending increase in Nurr1 by the Pre-ROL group is lost, however, by 4hrs, likely due to the large degree of variability seen between blots. Interestingly, at 12 hours, the Pre-ROL group showed significant reductions in Nurr 1 expression compared to cells treated with LPS alone. Both groups returned to low levels of expression by 24 hours (Figure 6D).

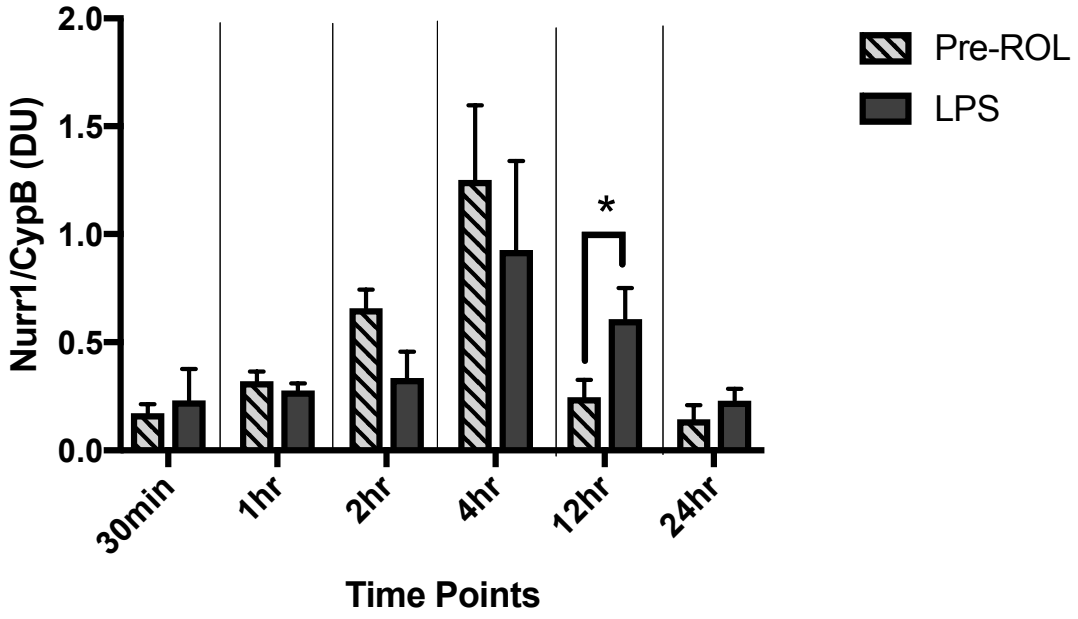

Figure 6d. Western blot analysis of cell lysates from cells treated with either LPS alone (1μg/ml) or those that received an hour of ROL pre-treatment (10 μM) before LPS administration. Antibodies to Nurr1 and CypB were used. A Student's t-test revealed that there is a trending increase in Nurr1 expression in the PreROL group at 2hrs, but there is a significant reduction in Nurr1 expression in the Pre-ROL group at 12hrs. Bars represent the mean $\pm$ SEM.

P***ilot Study #1***

The goal of the first pilot was to determine whether or not rolipram's anti-inflammatory properties could protect against LPS-induced Aβ production. We administered 7 days of rolipram (ROL) or saline (SAL), followed by 7 days of LPS or SAL. We also collected blood 4 hours after the first LPS or SAL injection to measure serum TNF-α levels.

Peripheral TNF-α levels

TNF-α is a common marker used to measure a pro-inflammatory response. To determine if rolipram interferes with LPS-induced inflammation, we plated serum collected 4 hours after the first LPS injection (250 μg/kg) onto a TNF-α-specific ELISA.

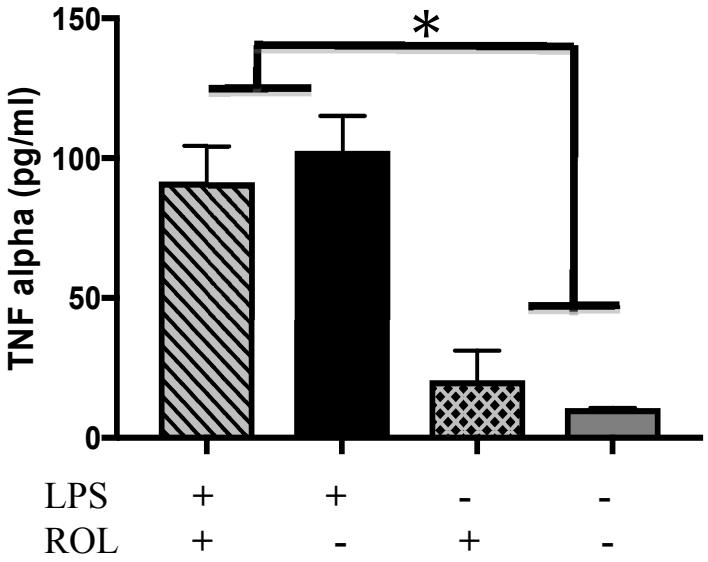

Figure 7a. Peripheral TNF-α expression. Serum TNF-α levels 4 hours post the first LPS (250 μg/kg) or SAL i.p. injection. Mice that were administered LPS showed significantly elevated peripheral TNF-α levels compared to there SAL-treated counterparts, whether or not they had received 7 days of pre-treatment with Rolipram (0.03mg/kg, i.p.). Bars represent the mean ± SEM. (SAL+SAL n=4; SAL+LPS, n = 3; ROL+LPS, n=4; ROL+SAL, n=4).

A two-way analysis of variance (ANOVA) test was conducted to examine the differences between the four groups: ROL + LPS, ROL + SAL, SAL+ LPS, SAL+ SAL. Mice administered LPS (250 μg/kg; i.p.) showed a significant increase in peripheral TNF-α production, despite pretreatment with rolipram (Figure 7a). 7 days of rolipram (0.03mg/kg; i.p.) is not sufficient to reduce inflammation in our AD model. Both rolipram and their saline-treated counterparts showed significant increases in TNF-α production 4 hours following a single i.p. injection of LPS (250 μg/kg) ($F_{(1,11)}$ = 65.036, P-value <0.001). Thus, inflammation was not mitigated by rolipram pre-treatment.

LPS-induced Aβ production

Our lab has repeatedly shown that 7 days of LPS injections (250 μg/kg; i.p.) leads to significant elevations in Aβ deposition in the hippocampi of non-transgenic mice (Kahn et al., 2012). After performing a two-way analysis of variance (ANOVA) to measure the differences in the presence of Aβ amongst the four treatment groups, we found marginally significant ($F_{(1,18)}$ = 4.403, p-value = 0.05) elevations in hippocampal Aβ following 7 days of LPS injections (250 μg/kg), whether or not the mice received previous treatment with rolipram (Figure 2c). Therefore, it appears that 7 days of rolipram treatment (0.03mg/kg; i.p.) prior to 7 days of LPS administration (250 μg/kg) does not protect against inflammation-induced hippocampal Aβ deposition. However, because the LPS-treated animals showed less elevation in Aβ compared to previous experiments performed in our lab (375 pg/ml versus 600 pg/ml, respectively), it is difficult to draw concrete conclusions from this pilot.

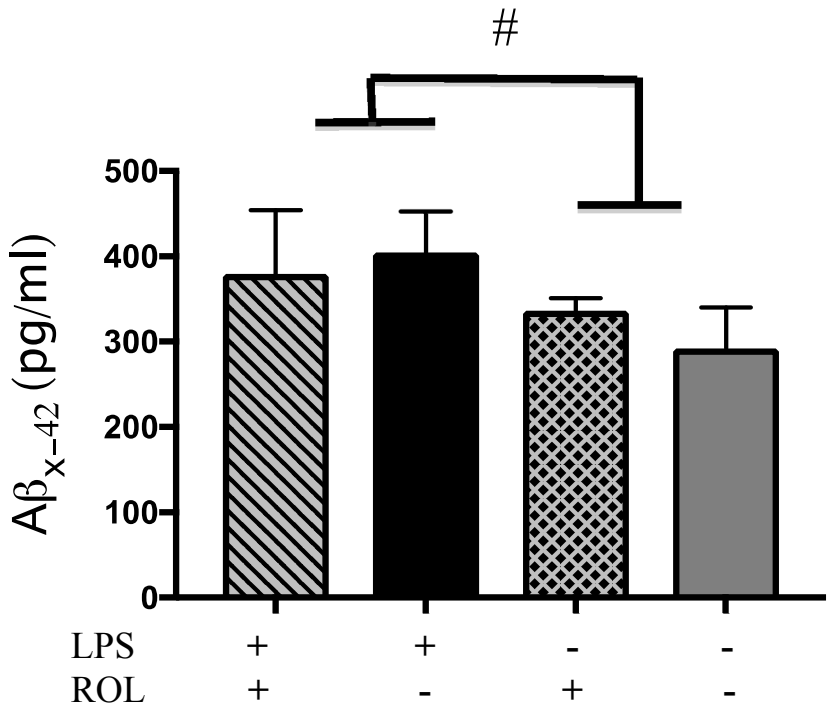

Figure 7b. LPS-elicited Aβ production. Mice administered LPS (250 μg/kg, i.p.) showed marginally significant (#) elevations in Aβ deposition compared to saline controls, no matter the pre-treatment group: 7 days of consecutive ROL (0.03mg/kg, i.p.) or SAL injections. Bars represent the mean ± SEM. (7 SAL + 7 LPS, n = 6; 7 ROL +7 LPS, n=6; 7 ROL + 7 SAL, n=7; 7 SAL+ 7 SAL n=3).

Pilot Study #2

We also wished to explore rolipram's cognitive-enchancing effects in our inflammation-induced AD model. We administered 7 days of either LPS or SAL injections, followed by contextual fear conditioning (CFC), which is used to measure hippocampal-dependent learning. ROL (0.03mg/kg) or SAL was administered 30 min prior to the CFC training period, and tissue was collected immediately after testing.

Hippocampal Aβ deposition

After protein quantification, an Aβ-specific ELISA was run to measure hippocampal Aβ levels. A two-way analysis of variance (ANOVA) test determined that there were no significant differences in Aβ levels amongst the four treatment groups,

although there was a trending treatment effect ($F_{(1,18)} = 4.403$, p-value = 0.07). The LPS groups, on average, showed higher levels or Aβ compared to their saline-treated counterparts, although not significantly (Figure 8a). Because we were unable to replicate our model, no conclusions could be drawn about rolipram's effect on Aβ production.

In this pilot, LPS did not lead to elevated Aβ levels, therefore we hypothesized that there would also be no differences in freezing behavior, as cognitive deficits in our AD model are associated with elevations in Aβ.

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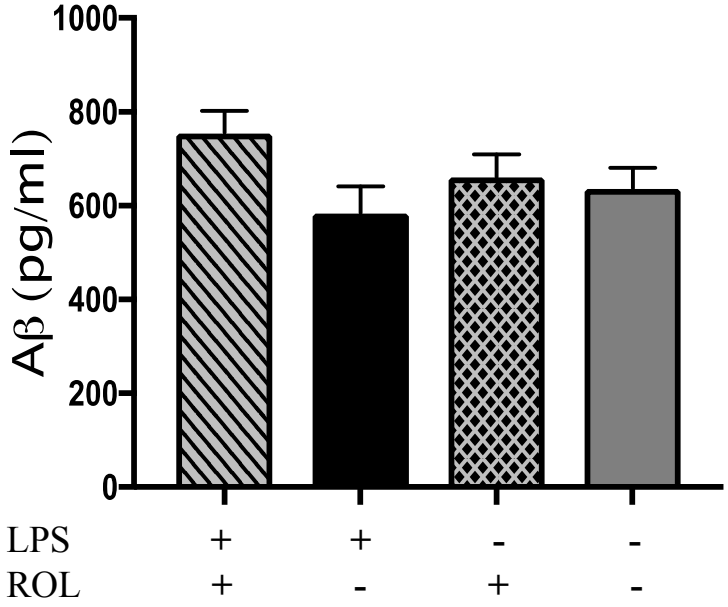

Figure 8a. Central Aβ deposition in response to LPS and ROL administration. Mice administered 7 days of LPS (250 μg/kg, i.p.) showed trending elevations in Aβ production compared to saline controls, no matter which injection they received 30 min prior to training: ROL (0.03mg/kg) or SAL. ($F_{(1,18)} = 4.403$, p-value = 0.07). Bars represent the mean ± SEM.

Contextual Fear Conditioning (CFC)

We utilized CFC, a commonly used behavioral paradigm that assesses cognitive function in a hippocampus-dependent task, to determine if there were cognitive differences amongst the four treatment groups. A two-way analysis of variance

(ANOVA) test revealed that there were no significant differences in freezing time among the four treatment groups: LPS + ROL, LPS + SAL, SAL + ROL, SAL + SAL (Figure 3). No matter which treatment the mice received, they all performed similarly in this behavioral task. This aligns with the fact that there were no significant LPS-induced elevations in Aβ. We were unable to replicate previous work in our lab which demonstrated that 7 injections of LPS leads to an increase in the production of Aβ, as well as Aβ-induced cognitive decline (Kahn et al., 2012). Therefore, we cannot make any conclusions about rolipram's protective and cognitive-enhancing effect in our inflammation-induced AD model.

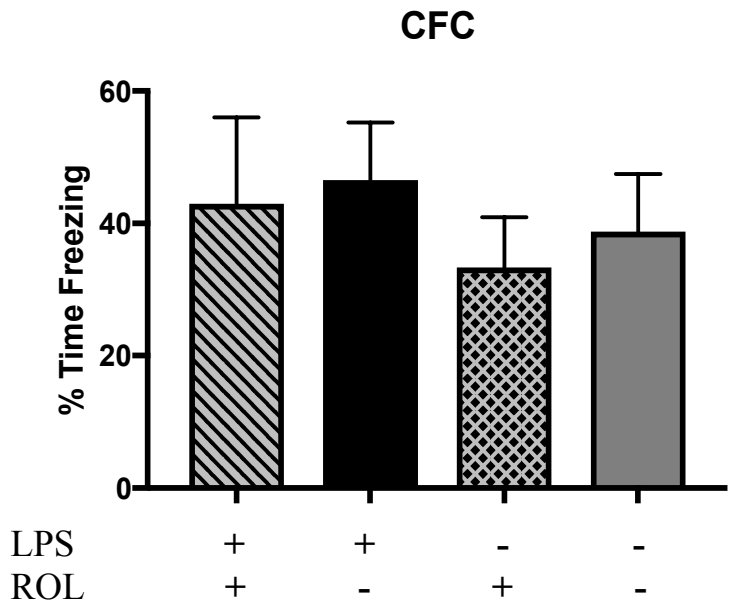

Figure 8b. Percent time freezing in contextual fear conditioning. There were no significant differences in freezing time amongst all four groups: contextual fear conditioning was not significantly impaired by LPS. All mice performed similarly despite being administered different treatments: 7 days of LPS (250 μg/kg; i.p.) +1 ROL (0.03mg/kg; i.p), n= 13; 7 days of SAL (i.p.) +1 ROL (0.03mg/kg; i.p), n=8; 7 days of LPS (250 μg/kg) +1 SAL (i.p.), n=13; 7 days of SAL (i.p.) + 1 SAL, n=8.

Pilot Study #3

Moving forward, we increased the dose and number of rolipram injections in an attempt to elicit the drug's cognition-enhancing properties. Mice were administered 7 days of LPS or SAL injections, followed by one injection of ROL or SAL four hours after the last LPS injection, one injection of ROL 30min before training, and one injection of ROL 30min before testing of our CFC behavioral paradigm.

LPS-induced Aβ production

An ELISA specific for Aβ was performed using lysates from hippocampal tissue in order to measure central Aβ levels. After running a two-way analysis of variance (ANOVA test, we found a significant treatment effect.

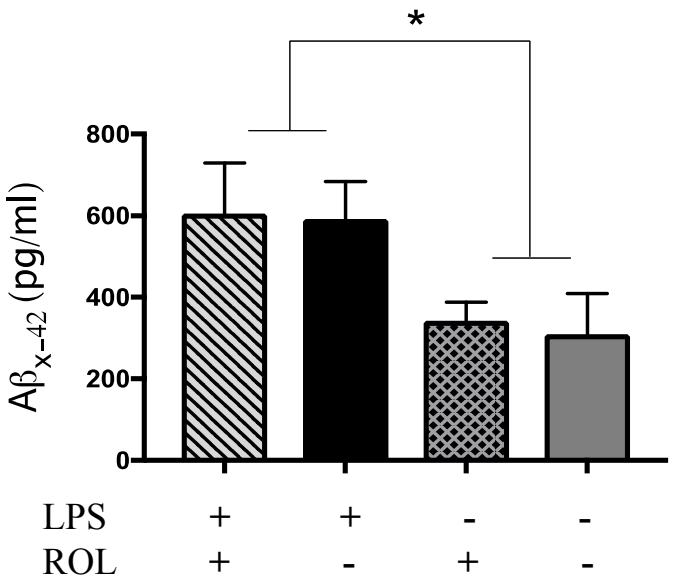

Figure 9a. Aβ-deposition in response to LPS. Mice administered 7 days of LPS (250 μg/kg, i.p.) had significantly elevated Aβ deposition, despite ROL (0.3mg/kg, i.p.) or SAL post-treatment. Bars represent the mean ± SEM. $F_{(1,14)} = 7.375$, p-value = 0.017.

LPS-treated mice had significantly elevated levels of Aβ compared to saline-treated mice ($F_{(1,14)} = 7.375$, p-value = 0.017). 3 injections of rolipram (3mg/kg; i.p.)

administered in this particular time course did not protect against inflammation-induced hippocampal Aβ deposition.

Rolipram treatment had no effect on central Aβ levels (Figure 9a).

Contextual Fear Conditioning (CFC)

Once again we used CFC to determine if there were cognitive differences amongst the four treatment groups. A two-way analysis of variance (ANOVA) test revealed that there was not a significant treatment effect; those receiving LPS did not freeze significantly less than those administered SAL, as our lab has previously demonstrated (Kahn et al., 2012) (Figure 9b). However, there was a significant condition effect: mice administered 3 injections of rolipram (3mg/kg; i.p.) froze significantly more than their saline-treated counterparts ($F_{(1,19)} = 6.195$, p-value = 0.022).

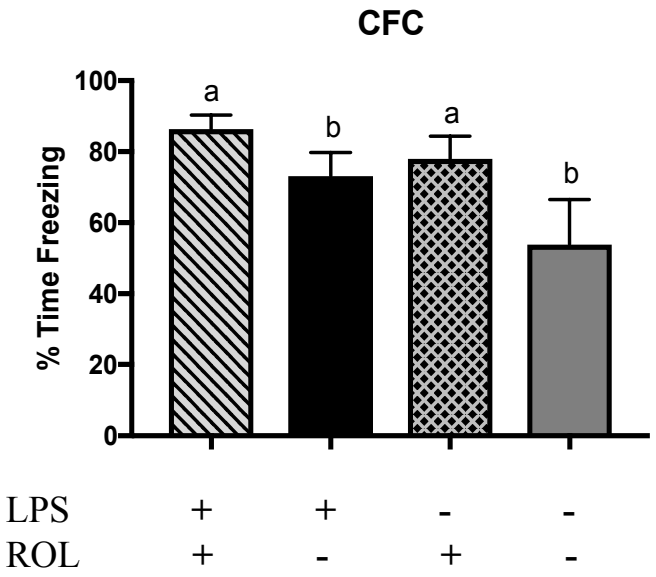

Figure 9b. Percent time freezing in CFC testing. There were no significant differences in percent time freezing between mice that received 7 days of LPS (250 µg/kg, i.p.) and those that were administered SAL. There was a significant condition effect: ROL-treated mice (3mg/kg; i.p.) froze significantly longer than their SAL-treated counterparts. Bars represent the mean ± SEM. Means with different letters (a, b) are significantly different from one another ($p < 0.05$).

We hypothesized that rolipram treatment would rescue cognition in our inflammation-induced AD model, however there was no indication of cognitive decline. Therefore, we cannot conclude whether or not rolipram rescues cognition in our model, as the LPS-treated mice did not exhibit reductions in freezing behavior. However, rolipram-treated mice did show significant elevations in freezing behavior. Treatment with this drug either enhanced cognition, aiding mice in learning the aversive stimulus/context pairing, or induced nausea in these mice, as nausea is a common side effect in humans treated with rolipram, which could also explain the increase in freezing behavior seen within this treatment group.

qRT-PCR

The $\Delta\Delta C_T$ method of qRT-PCR was performed to measure NR4A2 and BDNF hippocampal gene expression. Dorsal hippocampi were removed 30min after CFC testing, which was 1 hour after the last rolipram injection. A two-way analysis of variance (ANOVA) test revealed that there were no significant differences at this time point among the four treatment groups in the expression of either target gene (Figure 9c).

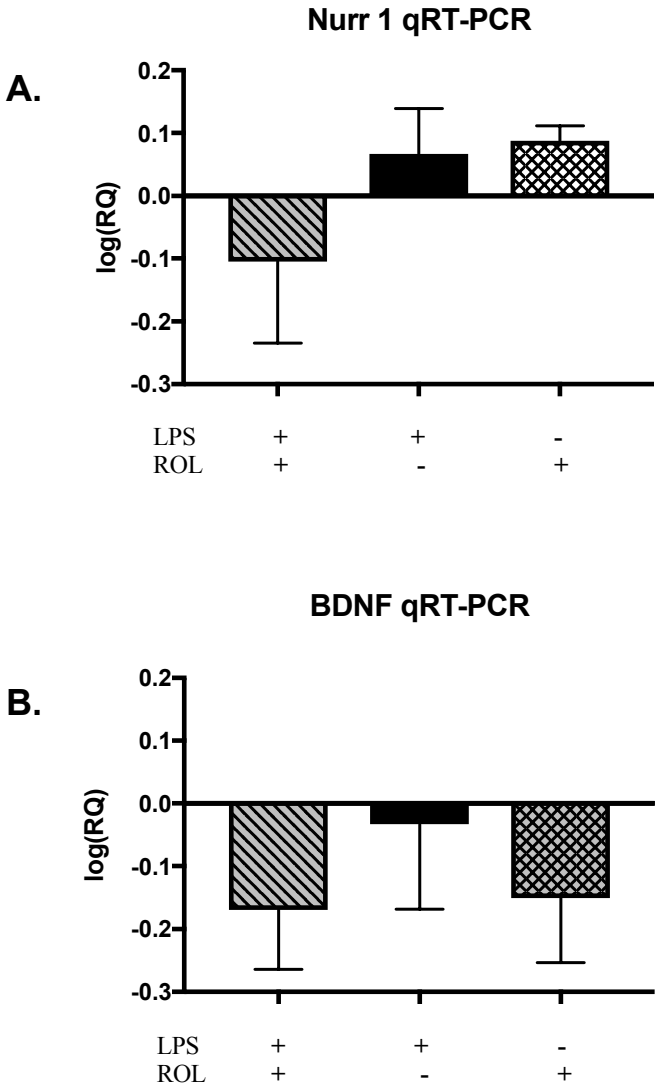

Figure 9c. Three injections of ROL (3mg/kg;i.p.) does not affect Nurr1 or BDNF mRNA expression, as measured by qRT-PCR. The graphs depict the log transformed relative quantitative expression of A] Nurr 1/HPRT and B] BDNF/HPRT for each treatment group compared to the SAL/SAL control: LPS/SAL, LPS/ROL, SAL/ROL ($p > 0.05$). Bars represent the mean $\pm$ SEM.

Pilot Study #4

Rolipram and Burrowing

We performed a burrowing study to determine whether or not one injection of ROL (3mg/kg) elicits sickness behavior in mice. Mice were either administered

ROL or SAL before being placed in separate cages containing burrowing tubes. Grams of food burrowed was measured 1 hr and 2hrs post-injection. A Student's t-test confirmed that mice injected with ROL burrowed significantly less food at the 1hr time-point compared to their saline-treated counterparts (P=0.006). There was also a trending difference between the two groups at the 2hr time-point; on average, the rolipram mice still burrowed less food than the saline-injected mice, although not significantly (p=0.052; Figure 10). These results indicate that rolipram treatment (3mg/kg) results in sickness-like behavior for up to 2 hours after administration.

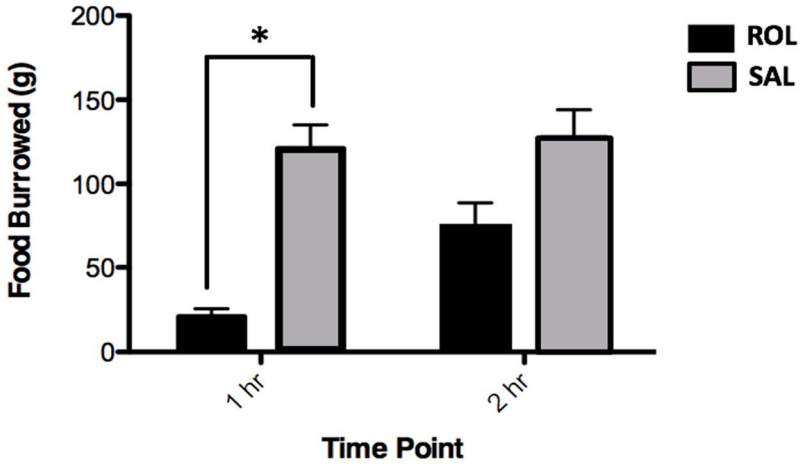

Figure 10. Effect of rolipram (3mg/kg) or SAL on burrowing behavior. Those administered rolipram burrowed significantly less food at the 1 hr time-point compared to those receiving saline (p = 0.006). There is a trending difference between the treatment groups at the 2hr time-point; on average the rolipram group burrowed less food than the saline group, although not significantly (p= 0.52).

DISCUSSION

Rolipram's role as an anti-inflammatory agent has been well documented. Many studies have shown that treating microglia and macrophages with rolipram, either before or after they are stimulated to mount an immune response, drastically reduces pro-inflammatory cytokine production in a dose-dependent manner (Ghosh et al., 2015; Sebastiani et al., 2006; Yoshikawa et al., 1999). Rolipram has also been shown to promote M2 macrophage polarization in cultured bone marrow macrophages through the upregulation of the cAMP-PKA-CREB pathway (Luan et al., 2015). Several studies have illustrated that both macrophages and microglial cells, including BV2 cells, respond to LPS by secreting TNF-α cytokines in a time-dependent manner (Jeohn et al., 2002; Lu et al., 2014). With this in mind, we performed a time-course study that explored rolipram's effect on TNF-α production in LPS-stimulated BV2 cells at six different time points (Figure 6a & 6b). It is well-known that TNF-α levels increase with time after LPS-exposure in microglial cells, eventually reaching a plateau around 2-3 hours (Jeohn et al., 2002). We were able to replicate these findings as cells treated with LPS showed significant elevations in TNF-α levels, which peaked at 2hrs and remained elevated for up to 24 hours. This LPS-induced increase in TNF-α secretion allowed us to determine if treatment with rolipram dampened pro-inflammatory cytokine production when BV2 cells are exposed to LPS. As hypothesized, rolipram treatment protected against this time-dependent increase in LPS-induced production of TNF-α . Cells that were either pre- or post-treated with rolipram did not show significant elevations in TNF-α production across time, as they did not differ from the No TX and ROL-treated negative controls throughout this time-course

experiment. Rolipram-treated cells also expressed significantly lower levels of TNF-α over time compared to LPS-stimulated cells . The LPS group showed time-dependent elevations in TNF-α production, which was blocked by rolipram treatment. Further analysis allowed us to determine differences in TNF-α production between groups at each individual time point. More specifically, pre-treatment with rolipram reduces TNF-α production in 2hr cultures that is sustained at 4, 12 and 24hrs. We first observe rolipram's protective effect in the Pre-LPS group at 2hrs, it is lost at 4hrs, but once again returns to lower levels at 12 and 24 hrs. We found that pre-treatment with rolipram results in better protection than treatment with rolipram following LPS exposure. Overall, pre-treatment with rolipram blocks LPS-induced TNF- production, as observed at 24 hours, while post-treatment with rolipram blunts LPS-induced pro-inflammatory cytokine production. However, the design of this study meant that the experiment had to be run in three separate batches in order to produce an n of 3, and the 1hr time-point only represented an n of 2. There was high variation between batches for each time point, which is represented by the large error bars. This makes it difficult to determine significant differences. The design of this experiment was flawed as well, as the Pre-LPS group was exposed to LPS one hour longer than the other LPS-treated groups. This may skew the comparisons between the Pre-LPS group and the other four treatment groups, which likely makes a difference at the earlier time points, as TNF- levels peak at 2hrs, but is unlikely making a large difference at later time points. More batches must be run in order to minimize error, and further support rolipram's protective effect.

Next, we sought to determine the mechanism by which rolipram induces its anti-inflammatory effect. Western blot analysis was performed on lysates from the LPS-

treated group and the Pre-ROL treated group at all 6 time points to determine if Nurr1 expression is leading to the amelioration of TNF-alpha production observed at 24 hours in the pre-treated Rolipram group. At the 2hr time point, there is a trending increase in Nurr1 expression by the Pre-ROL group, suggesting that rolipram may be contributing to increased Nurr1 expression early on, around the time that this drug is implementing its anti-inflammatory effects. Therefore, Nurr1 may be a key player in this anti-inflammatory process. However, at 12 hours, the Pre-ROL group showed significant reductions in Nurr 1 expression compared to cells treated with LPS alone. Both groups returned to low levels of expression by 24 hours, which could be indicative of a rolipram-induced negative feedback mechanism. Based off of this data, it does not seem that Nurr1 is responsible for rolipram's anti-inflammatory effect seen at the 12 and 24 hour time points, but the trending increase in Nurr1 expression seen at 2hrs in the Pre-ROL group may be contributing to the initial blocking of TNF-alpha production.

Even if Nurr1 does initiate early anti-inflammatory effects, an additional mechanism may exist that maintains low TNF-alpha levels. We hypothesize that a negative feedback loop involving Nurr1, Nur77, which is in the same subfamily as Nurr1 and leads to similar anti-inflammatory effects, and Map Kinase Phosphatase 1 (MKP1), all play a role in rolipram's ability to block LPS-induced pro-inflammatory cytokine production. In its active state, Nur77 is not phosphorylated, and is able to bind NFkB, blocking inflammation (Li et al., 2015). However, when an inflammatory response is initiated, Nur77 gets phosphorylated, releases its hold on NFkB, and pro-inflammatory cytokines are transcribed (Li et al., 2015). Rolipram also leads to enhanced phosphorylation of CREB; phosphorylated CREB is able to translocate into the nucleus

and act as a transcription factor for several target genes (Delghandi, Johannessen, & Moens, 2005). The binding of CREB, NFκB, and other inflammation-stimulated TFs leads to the upregulation of TNF-alpha, IL-1ß, Nurr1, Nur77, and MKP-1 (Choi, Jin, Li, & Yan, 2011; Korhonen et al., 2013). When Nurr1 is transcribed and phosphorylated, it forms the Nurr1/CoREST transrepression complex, blocking pro-inflammatory cytokine production (Murphy & Crean, 2015). Interestingly, rolipram has also been shown to lead to enhanced (MKP-1) expression, which plays a role in mediating the drug's anti-inflammatory effects (Korhonen et al., 2013). MKP-1 may lead to a down-modulation of Nurr-1, but it could also inadvertently activate Nur77, as this nuclear receptor is active when it is not phosphorylated. With this in mind, we hypothesize that rolipram-enhanced MKP-1 expression leads to the down-modulation of the Nurr1/CoREST transrepression complex, but increases Nur77 activity, allowing for Nur77 to take over Nurr1's anti-inflammatory role.

In light of these in-vitro studies, we next sought to explore rolipram's therapeutic effects in our inflammation-induced AD model. At this point, it is well understood in the literature that rolipram acts as an anti-inflammatory agent that curbs the pro-inflammatory effects of activated microglia, both in cell culture and in animal models of inflammation. Buttini et al. demonstrated that three i.p. injections of rolipram (3mg/kg), one administered before and two administered after a single LPS intravenous injection (5mg/kg), is sufficient to reduce microglia production of TNF-α in rat brains (1997). One injection of rolipram (5mg/kg; i.p.) also protects rats from cyclophosphamide-induced haemorrhagic cystitis (Sakura et al., 2008). Diabetic rats administered 23 days of rolipram treatment (0.5mg/kg; i.p) showed a reduction in hippocampal TNF-α levels, an

upregulation in IL-10 expression, and rescued cognition (Miao et al., 2015). In light of these findings, we wished to determine if the anti-inflammatory effects of rolipram interferes with, and thus ameliorates LPS-induced Aβ deposition. Seven days of ROL (0.03mg/kg) treatment prior to 7 days of LPS injections does not protect against LPS-induced inflammation. 1 injection of LPS led to significantly elevated TNF-α levels compared to those that did not receive LPS. Because LPS did not lead to significantly enhanced elevations in Aβ ($p=0.05$), as observed in previous studies, no conclusions could be drawn regarding rolipram's effect on Aβ production.

We initially hypothesized that rolipram's anti-inflammatory effects would protect mice from LPS-induced inflammation, and ultimately inflammation-induced Aβ production. It is possible that the experimental time course, as well as the chosen rolipram dose, may not have been sufficient to prevent an inflammatory response. The rolipram dose (0.03mg/kg; i.p.) was originally chosen because a particular study found long-lasting rescued cognition in transgenic AD mice that were administered 3 weeks of daily i.p. injections of rolipram (0.03mg/kg) at the age of 3 months, and 9-12 weeks later displayed improvements in cognitive function compared to vehicle-treated controls. Therefore, rolipram had long-lasting effects even after it had been cleared from the subject's system, likely due to enhanced CREB phosphorylation (Gong, Vitolo, Trinchese, Liu, & Shelanski, 2004). The cAMP-PKA-CREB pathway, which is perpetuated by rolipram, is essential in blocking the pro-inflammatory response, and our lab sought to discover whether or not this dose (0.03mg/kg; i.p.) administered for one week would sufficiently block inflammation. This was not the case, possibly due to the fact that rolipram was not administered shortly before/in conjunction with LPS or

because 7 injections of rolipram at 0.03mg/kg is too low of a dose. An in depth literature search on rolipram dosing in murine experiments provides a wide-range of dosing options. One inflammation study found that a single dose of rolipram (3mg/kg) administered orally 30min prior to LPS exposure (5μg; i.p.) protects against LPS-induced TNF-α production. Within the same study, one dose of rolipram (30mg/kg) is used to protect against endotoxic shock elicited by a single LPS injection (500 μg) (Sekut, Yarnall, Stimpson, Noel, & Clark, 1995). Pre-treatment with rolipram (20mg/kg; i.p.) 1 hr before LPS inhalation (0.3mg/ml) significantly reduces TNF-α levels and neutrophil recruitment to the lungs, in a model of lung inflammation. However, most of the studies exploring rolipram's use as a therapeutic drug for Alzheimer's Disease utilize much lower doses. One study illustrated rolipram's ability to rescue dendritic spine density in a transgenic AD model: 3 weeks of rolipram injections (0.03mg/kg; i.p.) restored dendritic spine structure (Smith, Pozueta, Gong, Arancio, & Shelanski, 2009). Rolipram (0.1mg/kg) administered 30min before training in contextual fear conditioning, rescued cognitive function in the Tg2576 Mouse Model of AD (Comery et al., 2005). Furthermore, a study measuring rolipram's neuro-protective effects in mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a model of Parkinson's Disease, determined that protective efficacy of rolipram lies upon a bell-shaped dose curve. Rolipram administered at the lower does (1.25mg/kg) was sufficient to protect against MPTP-induced neurotoxicity; however, the high dose of 5mg/kg actually increased animal mortality when co-administered with MPTP (Yang, Calingasan, Lorenzo, & Beal, 2008). In light of these findings, it may be possible that rolipram's beneficial effects are inducible not by a bell-shaped dosing curve, but rather a U-shaped dosing curve: with the

enhanced cognitive effects elicited by low doses of rolipram, and the drugs anti-inflammatory effects produced only by extremely high dosing. In the future, we will test a single injection of rolipram, at a minimum dose of 5mg/kg; i.p., administered 30min prior to each LPS injection (250 μg/ml) over one week.

We also sought to investigate if one injection of rolipram ameliorates the cognitive deficits associated with our inflammation-induced AD model. One study demonstrated a recovery in cognition for traumatic brain-injured (TBI) rats after only one injection of rolipram (0.03mg/kg) 30 minutes before the training segment of contextual fear conditioning, a commonly used behavioral paradigm that tests for hippocampus-dependent cognitive function by measuring freezing responses of mice within an environment in which they previously received a foot shock (Titus et al., 2013). A second study demonstrated that a single injection of rolipram administered 30min before the training session of CFC was sufficient to rescue cognition in 3 month-old APP/PSI transgenic mice. In light of these findings, our lab applied a similar single injection rolipram approach to our LPS-induced AD model, hypothesizing an increase in freezing behavior, and thus a recovery of cognitive function, for those mice treated with rolipram.

No conclusions could be drawn from Pilot #2 because we were unable to replicate our inflammation-induced AD model. LPS did not lead to elevations in hippocampal Aβ, therefore the mice also did not express cognitive deficits. No conclusions could be made about rolipram's therapeutic effects in this model of AD. Therefore, moving forward we increased the dose and number of rolipram injections in an attempt to increase the drug's cognition-enhancing properties in our inflammation-induced AD model.

We continued our investigation of rolipram as a potential therapeutic target to rescue cognition in our unique model of AD. To avoid some of the problems faced by the previous pilot study, pilot # 3 utilized younger mice (5-6mos old), increased the number of rolipram injections, and increased the injection dose (3mg/kg). In so doing, we wished to eliminate the discrepancies seen between the improved cognitive effects following rolipram treatment in unimpaired mice, as well as transgenic models of AD, and our non-transgenic model.

The main purpose of this pilot was to determine if an increased dose of rolipram (3mg/kg; i.p.), and an increase in the number of injections would allow us to replicate the rescued cognition observed in transgenic models of AD.(Gong et al., 2004; Myeku et al., 2015; Titus et al., 2013). The results from the Aβ ELISA revealed significantly elevated levels of hippocampal Aβ in LPS-treated mice compared to those administered saline. Rolipram treatment did not have an effect on central Aβ levels. We hypothesized that elevations in Aβ would lead to cognitive deficits in the LPS-treated groups, which may or may not be rescued by rolipram treatment. The freezing behavior from CFC showed that there was a condition effect in which the rolipram-treated mice froze significantly more than those administered saline, whether or not they were administered LPS. There was not Aβ-induced cognitive decline, therefore no conclusions could be made about rolipram's protective effects. However, mice that received rolipram did show enhanced freezing behavior, indicating that they may have learned the context-stimulus association better than the mice that were not administered rolipram. A possible explanation for enhanced cognition could be that rolipram is leading to the up-regulation of Nurr1 and BDNF, which are both essential and quickly upregulated in response to learning (Colón-

cesario et al., 2006; Yamada, Mizuno, & Nabeshima, 2002). Next we performed qRT-PCR to measure the relative expression of Nurr1 and BDNF in the LPS/SAL, LPS/ROL, and SAL/ROL groups in comparison to the SAL/SAL controls. If the elevated freezing behavior seen in the rolipram-treated groups was attributed to enhanced learning and cognition, rolipram-induced elevations in Nurr1 and BDNF could explain this phenomenon. There were no significant differences in gene expression for Nurr1 or BDNF within any of the treatment groups. None of the treatments varied significantly from the SAL/SAL control (Figure 9c). Gene expression studies are tricky because timing is extremely critical. This time frame, described in Figure 4, was chosen because NR4A receptors have been shown to be up-regulated in response to contextual fear conditioning in and of itself (Malkani & Rosen, 2000). Nurr1 and Nur77 is elevated in the hippocampi of rodents 30min after the foot shock is administered in a CFC paradigm, and again 30min after the testing period 24hours later. I was more interested in observing Nurr1 expression, so the earlier time point was chosen. I also only have n's of 4, 5, and 6, making it difficult to make conclusive statements regarding these results. But based off of this data, it would seem that there must be another explanation for the abnormal freezing behavior of the ROL-treated groups from CFC. It is very possible that our non-transgenic model of AD does not result in NR4A suppression. It is not until the disease has evolved, and the mice begin depositing plaques that Nurr1 and Nur77 are inhibited (Moon et al., 2015). This would explain the success observed with rolipram-treatment in transgenic models of AD, but not in our inflammation-induced AD model. In transgenic models, perhaps rolipram elicits Nur77 and Nurr1 expression, which help to alleviate inflammation as well as improve cognitive function.

There are two explanations as to why, in pilot #3, all of the rolipram-treated mice froze more than saline-treated controls; regardless of whether or not they received 7 days of LPS or SAL treatments. Either rolipram significantly enhanced cognitive function overall through a mechanism independent of up-regulated Nurr1 or BDNF expression, or the dose administered elicited a sickness response. Rolipram's original use as an anti-depressant was short-lived due to adverse side effects in clinical trials, most profoundly nausea, headache, and vomiting (Piaz & Giovannoni, 2000), which indicates that the freezing behavior of the rolipram-treated mice may not be a result of learning. Furthermore, in a study of inflammation, rolipram administered at a dose of 20mg/kg; i.p., produced lethargy in mice for up to 20min (Gonc et al., 1998). Because the dose we administered (3mg/kg) is much less than 20mg/kg, and training and testing occurred after 30min, we were not initially worried that we would encounter the same results. But the unusual behavior displayed in Pilot #3, prompted a burrowing study to determine if the 3mg/kg dose of rolipram is sufficient to produce sickness behavior in mice. Burrowing studies have previously been used in our lab to measure sickness behavior in response to inflammation-inducing agents, such as polyinosinic:polycytidylic acid (poly:IC) (Mclinden et al., 2012). When given the opportunity, mice are highly motivated to burrow and, and will do so unless experiencing sickness or pain (Jirkof et al., 2010). The results of our burrowing study indicate that one injection of rolipram (3mg/kg; i.p.) is indeed sufficient to induce sickness behavior in rodents for up to 2hrs (Figure 10). This finding highlights an extreme flaw in the experimental design of Pilot # 3. Because behavioral testing occurred 30min following a rolipram injection, it is likely that the freezing behavior observed in the rolipram group was not due to learning the context-

shock pairing, but rather that the mice felt ill during the testing phase. All of the mice froze more than what we typically observe in CFC. This may be explained by the fact that rodents are able to empathize with one another. For instance, modulation of pain sensitivity can be altered depending on the physical state of other cage mates. One study found that if two mice within a cage are administered a noxious substance, the mice will exhibit more indications of pain if their cage-mate was also administered the noxious substance than if their cage-mate was healthy (Langford, Crager, Shehzad, & Smith, 2006). If multiple mice are in a cage, and some are administered rolipram, which makes them ill, their cage mates may empathize with them which could result in a higher freezing perentage. Further inspection of the literature revealed that a dose as little as 0.3mg/kg delivered intra-orally to rats induces conditioned-gaping, a selective measure of nausea in rodents (Rock, Benzaquen, Limebeer, & Parker, 2009). Moving forward, it would be necessary to either wait at least two hours before behavioral testing, or carry out the same injection series with a lower dose of 0.03mg/kg. However, a burrowing study would need to be repeated if deciding to utilize the lower dose.

All in all, rolipram's beneficial effects are well documented. Its ability to block inflammation and enhance cognition makes it a viable target for the treatment of AD, which is associated with neuro-inflammation and cognitive decline. Unfortunately, there is a dose-dependent limitation associated with rolipram, in which the higher doses associated with anti-inflammatory effects would be much too toxic, and would induce adverse side effects in humans. But, if rolipram were specifically to be used to enhance cognition then there are much lower effective doses, which are not as likely to elicit nausea or other unpleasant side effects. More research needs to be done to see if rolipram

can provide a protective effect specifically in our model of AD, which could provide further insight into the therapeutic potential of rolipram in inflammation-induced diseases. However, it may be more beneficial to test different phosphodiesterase-inhibitors in the future, such as those structurally related to xanthines and Nitraquazone, which maintain rolipram's beneficial effects—reducing inflammation and enhancing cogntitive function—without the debilitating side effects (Piaz & Giovannoni, 2000).

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VITA

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In August, 2015, Hailey was accepted into the Biology Graduate Program at Texas Christian University. In pursuit of her Masters Degree, Hailey held a Teaching Assistantship for the 2015 and 2016 academic school years. Upon graduation, Hailey will continue her education at the Medical College of Wisconsin as a member of the incoming Class of 2021. Hailey is also a member of the Golden Key Honors Society, as well as a Phi Beta Kappa initiate.

ABSTRACT

AN EXPLORATION OF THE NEURO-PROTECTIVE AND ANTI INFLAMMATORY EFFECTS OF ROLIPRAM: A THERAPEUTIC TARGET FOR ALZHEIMER'S DISEASE

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Alzheimer's Disease (AD) is the sixth leading cause of death in the United States, and it is the only leading cause of death that cannot be prevented, cured, or slowed. This form of dementia is characterized by amyloid-beta (Aβ) senile plaques and neurofibrillary tangles which have been shown to contribute to neuro-inflammation, cell death, and the disruption of cell signaling. These pathologies are likely to blame for the cognitive deficiencies observed in AD patients. Microglial cells, the immune cells of the brain, may be attributing to the exacerbation of some of these pathologies. Microglia are plastic cells that respond accordingly to their environment and can be swayed to become activated toward the M1, pro-inflammatory, or the M2, anti-inflammatory, phenotype. In AD, microglia are heavily polarized toward the M1 phenotype as they are activated by Aβ, dying neurons, and hyper-phosphorylated tau. Chronic inflammation, that is brought about by constantly activated M1 microglia, can lead to an increase in Aβ production and worsening of AD pathology. In this study we explored the use of a drug called rolipram (ROL), a phosphodiesterase inhibitor known to have anti-inflammatory and cognitive-enhancing effects, to induce the transition of microglia from a pro-

inflammatory state towards an anti-inflammatory state. We found that ROL blunts TNF-α production when it is administered to LPS-activated microglia. Furthermore, LPS-induced TNF-α production is blocked when ROL is applied prior to LPS exposure. ROL's protective effect is not due to up-regulation of the anti-inflammatory mediator, Nurr1, but could possibly be attributed to a negative feedback loop with several intermediates playing a role. We also studied ROL's effects in an inflammation-induced AD model. Inability to replicate this model, and difficulty in finding a dose that did not make mice feel ill, prevented us from making conclusions about ROL's neuro-protective, cognitive enhancing, and anti-inflammatory effects in this model. More research must be done to determine a proper dose and injection schedule for ROL in this model. ROL 's use as a cognitive enhancing drug is promising, as several studies have shown enhanced cognitive function in ROL-treated mice with extremely low effective doses. However, it is unlikely that ROL would be used for its anti-inflammatory effects as this requires a much higher dose. Other anti-inflammatory drugs should be investigated that mimic the effects of ROL without the associated adverse side effects.