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Chronic brain inflammation leads to a decline in hippocampal NMDA-R1 receptors

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Abstract

Background: Neuroinflammation plays a prominent role in the progression of Alzheimer's disease and may be responsible for degeneration in vulnerable regions such as the hippocampus. Neuroinflammation is associated with elevated levels of extracellular glutamate and potentially an enhanced stimulation of glutamate N-methyl-D-aspartate receptors. This suggests that neurons that express these glutamate receptors might be at increased risk of degeneration in the presence of chronic neuroinflammation.

Methods: We have characterized a novel model of chronic brain inflammation using a slow infusion of lipopolysaccharide into the 4th ventricle of rats. This model reproduces many of the behavioral, electrophysiological, neurochemical and neuropathological changes associated with Alzheimer's disease.

Results: The current study demonstrated that chronic neuroinflammation is associated with the loss of N-methyl-D-aspartate receptors, as determined both qualitatively by immunohistochemistry and quantitatively by in vitro binding studies using [3H]MK-801, within the hippocampus and entorhinal cortex.

Conclusion: The gradual loss of function of this critical receptor within the temporal lobe region may contribute to some of the cognitive deficits observed in patients with Alzheimer's disease.

Background

Neuroinflammation plays a prominent role in the progression of Alzheimer's disease [AD, [1,2]]. Brain regions, particularly those involved in learning and memory, which demonstrate the greatest degree of microglia cell activation early in the disease ultimately show the highest rate of atrophy and pathology [3]. Neurons within the entorhinal cortex (EC) and hippocampus degenerate in AD [4,5] and are particularly vulnerable to the consequences of chronic neuroinflammation and aging [6-9].

Although the mechanism underlying the degeneration of these cells is unknown, excitotoxicity via the stimulation of glutamate receptors may play an important role [10-15]. Glutamate N-methyl-D-aspartate (NMDA) receptors are highly concentrated in the hippocampus and EC and their activation has a dual role in normal neuroplasticity as well as neurodegeneration [12,16,17]. Impaired NMDA receptor function may therefore contribute to the cognitive deficit observed in AD [18,19]. The number of NMDA receptors within the hippocampus, EC and basal

forebrain substantia innominata declined following an acute neuroinflammatory challenge produced by an injection of lipopolysaccharide (LPS) into the cisterna magna [20]. Therefore, neurons that express NMDA receptors within these brain regions might be at increased risk in the presence of chronic neuroinflammation similar to that present in the brains of AD patients [1,3]. Brain inflammation leads to increased extracellular levels of glutamate [21] that may induce increased calcium entry through the NMDA receptors and the degeneration or dysfunction of NMDA receptive neurons [22]. Activated glia may also potentiate NMDA-mediated toxicity via the production and release of nitric oxide [23] or interleukin-1β [24], suggesting that neuroinflammation may exacerbate excitotoxicity in neurons.

We have developed a model of chronic brain inflammation using a slow LPS infusion into the 4th ventricle of rats that reproduces many of the behavioral, electrophysiological, neurochemical and neuropathological changes associated with AD [14,15], including the presence of activated microglia within the hippocampus and EC, impaired long term potentiation in the dentate gyrus, impaired learning and memory, and a significant loss of CA3 hippocampal pyramidal cells and entorhinal pyramidal neurons in layers II & III [6-9,25-27]. Similarly, the long term infusion of LPS into the basal forebrain was associated with the selective degeneration of cholinergic basal forebrain neurons [13,14]. A critical role for stimulation of the NMDA receptors is supported by the finding that the neurodegenerative consequences of chronic neuroinflammation upon basal forebrain cholinergic neurons can be reversed by treatment with the NMDA receptor antagonist memantine [13,14]. The current study demonstrates that chronic neuroinflammation is associated with the loss of NMDA receptors within the hippocampus and EC. Because NMDA receptors contain the obligatory NR1 subunit [28], receptor localization was determined using a monoclonal antibody that recognizes all variants of the NR1 subunit. A quantitative verification of the loss of these receptor sites is also shown using an in vitro binding assay with [3H]-MK-801.

Methods
Subjects
Twenty-two young (3 months old) male F-344 rats (Harlan Sprague-Dawley, Indianapolis, IN) were singly housed in Plexiglas cages with free access to food and water. The rats were maintained on a 12/12-h light-dark cycle in a temperature-controlled room (22°C) with lights off at 0800. All rats were given health checks, handled upon arrival and allowed at least one week to adapt to their new environment prior to surgery.

Materials
LPS (E. coli, serotype 055:B5) was obtained from Sigma Chem. (St. Louis, MO). [3H]MK801 was obtained from New England Nuclear, Boston, MA.

Surgical procedures
Standard procedures were used for the surgery [6,9]. Each rat was anesthetized with isoflurane gas and placed in a stereotaxic instrument with the incisor bar set 3.0 mm below the ear bars. The scalp was incised and retracted and a hole was made at the appropriate location in the skull with a dental drill. A chronic indwelling cannula was inserted into the 4th ventricle. Coordinates for the 4th ventricle infusions were as follows: 2.5 mm posterior to Lambda, on the mid-line, and 7.0 mm ventral to the dura. An osmotic minipump (Alzet, Palo Alto, CA, model 2004, to deliver 250 ηL/h) was attached via a catheter to a chronic indwelling cannula that had been positioned stereotaxically so that the tip extended to the coordinates given above. Each minipump was prepared to inject either the vehicle artificial cerebrospinal fluid (aCSF) or 250 ηg LPS/h (prepared in aCSF). The composition of the aCSF (in mmol/L) was 140 NaCl; 3.0 KCl; 2.5 $CaCl_2$; 1.2 Na_2HPO_4, pH 7.4. The following post-operative care was provided to all rats: betadine was applied to the exposed skull and scalp prior to closure to limit local infection and 5 ml of sterile isotonic saline were injected subcutaneously to prevent dehydration during recovery. The rats were closely monitored during recovery and kept in an incubator (Ohio Medical Products, Madison, WI) at temperatures ranging from 30–33°C. Body weights were determined daily and general behavior was monitored for seizures.

Immunohistochemistry
Twenty-nine days after surgery rats from each group were anesthetized and were either transcardially perfused with cold saline containing 1 U/ml heparin, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, or sacrificed by decapitation, the brains frozen (-70°C) and used for the fluorescence labeling studies. The perfused brains were post-fixed one hour in the same fixative and then stored (4°C) in phosphate buffered saline, pH 7.4. Free-floating, serial coronal sections (40 μm) were taken by vibratome from perfused tissues for staining with standard avidin/biotin peroxidase methods. The frozen brains were arranged into a block of gelatin as a group of three brains representing rats from both groups in order to reduce variability in the immunostaining between slides. The blocks were then sectioned (20 μm) using a cryostat and prepared for fluorescence labeling. The monoclonal antibody OX-6 (final dilution 1:400, Chemicon, San Diego, USA) was used to visualize activated microglia cells [6]. This antibody is directed against Class II major histocompatibility complex (MHC II) antigen. Since NMDA

receptors contain the obligatory NR1 subunit [28], in order to label all NMDA receptors with equal probability, we used a monoclonal antibody anti-NR1 subunit specific, NMDAR1 (Chemicon, final dilution 1:250). After quenching endogenous peroxidase activity and blocking nonspecific binding, the sections were incubated (4°C) either overnight (for OX-6) or 3 days (for NMDAR1) with primary antibodies directed against the specific epitopes (MCH II and R1, respectively). Thereafter, the sections were incubated for 2 h (22°C) with the secondary monoclonal antibody, rat adsorbed biotinylated horse anti-mouse immunoglobulin G (final dilution 1:200, Vector, Burlingame, USA), Sections were than incubated for 1 h (22°C) with avidin-biotinylated horseradish peroxydase (Vectastain, Elite ABC kit, Vector). After washing again in PBS, the sections were incubated with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Vector) as chromogen. The reaction was stopped by washing the sections with buffer. No staining was detected in the absence of the primary or secondary antibodies. Sections were mounted on gelatin-chrome-alum-coated glass slides, air-dried and coverslipped with Cytoseal (Allan Scientific, Kalamazoo, MI) mounting medium. The location of immunohistochemically-defined cells was examined by light microscopy. Immunofluorescence was visualized with fluorescent substrates (FITC, Fluorescein, Perkin-Elmer, Boston, MA) and all nuclei were counterstained with ToPro3 (1:1,000 in TBS, Molecular Probes). A Z-section image series were acquired using a confocal microscope (Carl Zeiss, model 510NLO-META, Thornwood, NY) with a 25 × water immersion objective. Pinhole size and contrast values were kept constant for each area on a slide. No staining was detected in the absence of the primary or secondary antibodies.

[3H]-MK-801 Receptor Binding Assay
The entire left hippocampus from the brain of four rats infused with aCSF and four infused with LPS for four weeks was isolated and stored (-70°C) until assayed for NMDA receptors using [3H]MK-801 according to a modified method previously described [7]. Crude membrane fractions were prepared by initial homogenization in 20 volumes of 0.32 M sucrose containing 1.0 mM EGTA and centrifuged at 1000 × g for 10 min at 4°C. The resulting supernatant was centrifuged at 40,000 × g for 40 min at 4°C. The resulting pellet was resuspended in 20 volumes of 1.0 mM EGTA and centrifuged (40,000 × g, 40 min, 4°C). The pellet was resuspended in 50 mM Tris-acetate buffer (pH 7.4) and centrifuged (47,900 × g, 10 min, 4°C). This final sequence was repeated three times to remove any endogenous components of the tissue that might interfere with binding. The tissues were stored frozen overnight and then centrifuged again (47,900 × g, 10 min, 4°C). The final pellet was resuspended in 15 volumes (to achieve approx. 0.4 mg/ml protein) of 50 mM

Tris acetate buffer. The homogenate was used immediately for binding studies. Due to the small size of the samples and the desire to avoid pooling tissues only single-point determinations were made. The assays were conducted in an incubation volume of 500 μl containing [3H]MK-801 (1.0 ηM) and 100–150 μg of membrane protein at 25°C for 60 min in the presence of 100 μM glycine and 50 μM spermidine. Non-specific binding was defined by the addition of 10 μM MK-801. Incubation was terminated by dilution with 4 ml of ice-cold 50 mM Tris-acetate buffer, pH 7.4, followed immediately by rapid filtration through Whatman GF/B glass fiber filters on a cell harvester (Brandel, model PHD 2000, Gaithersburg, MD). The filters were rinsed three times with 4 ml of buffer. All filters were presoaked in 0.3% polyethylenimine (pH 7.0) for at least 2 h at 25°C. The filter-bound radioactivity was determined by liquid scintillation spectrometry. Membrane protein levels were determined [29] with bovine serum albumin as standard. The results were analyzed by Student's t-test. (SigmaStat software, Jandel Scientific, San Rafael, CA).

Results
Overall, chronic infusion of LPS was well tolerated by all rats. Initially after surgery, all LPS-treated rats lost only a few grams of weight. Within a few days, however, most rats had regained weight and continued to gain weight normally for the duration of the study.

Immunohistochemistry
LPS infused rats had numerous, highly activated microglia cells (OX-6 positive) distributed throughout the hippocampus and EC (see Figure 1). Rats infused with aCSF showed only a few mildly activated microglia scattered throughout the brain (Figure 1A), similar to our previous reports [6,9,10]. Activated microglia were widely scattered throughout the hippocampus (Figure 1B) and were characterized by a contraction of their highly ramified processes that appeared bushy in morphology (Figure 1C,1D).

Rats infused with aCSF showed numerous NMDAR1 immunoreactive large neurons throughout the hippocampus and EC that had intense dark staining within the cytoplasm of the cell bodies that extended into the dendrites. Chronic infusion of LPS for four weeks reduced the number of NMDAR1-immunoreactive cells within the hilar region of the dentate gyrus as well as in area CA3, as compared to the staining in these hippocampal regions of rats infused with aCSF (see Figure 2). Chronic infusion of LPS had a lesser effect upon NMDAR1 immunoreactivity within cells in the EC (Figure 3).

[3H]-MK-801 Receptor Binding Assay
Rats chronically infused with LPS had significantly (t = 10.8, df = 6, p < 0.001) fewer [3H]MK-801 binding sites in
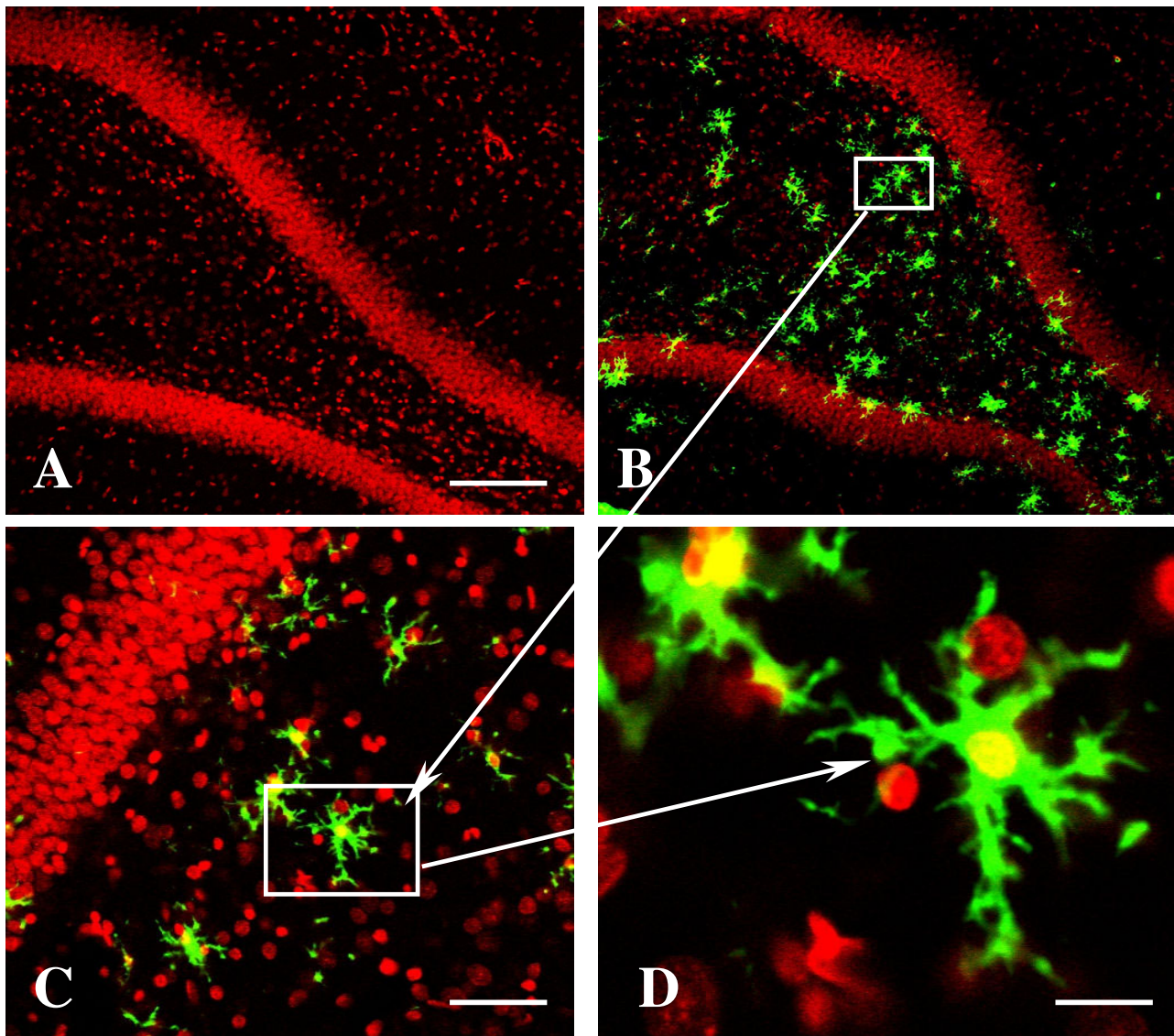
Figure 1
Confocal microscope images of activated microglial cells MHC II (green OX-6 positive) in the Dentate Gyrus. Rats infused with aCSF (A) had only a few activated microglia scattered throughout the brain. Chronic infusion of LPS into the 4th ventricle produced high activated microglia distributed throughout the hippocampus (B). Higher magnifications of an activated microglia (C, D) show the characteristic contracted and ramified processes with bushy morphology. Cell nuclei are stained red (ToPro3). Scale bars: (A-B) 100 μm; (C) 25 μm; (D) 2.5 μm.

the hippocampus compared to the aCSF infused animals (See Figure 4).

Discussion
Chronic neuroinflammation in young rats produced by infusion of LPS into the 4th ventricle for 28 days was associated with an increased number of highly activated microglia cells throughout the temporal lobe and greatly

decreased immunolabelling of NMDAR1 receptors within the pyramidal layer of the CA3 and hilar regions of the dendate gyrus and to a somewhat less degree within the EC. The loss of immunostaining may reflect either diminished receptor protein concentration or an inflammation-induced conformational change in the protein structure such that the antibody no longer recognized its antigenic binding site. We have previously shown using electron
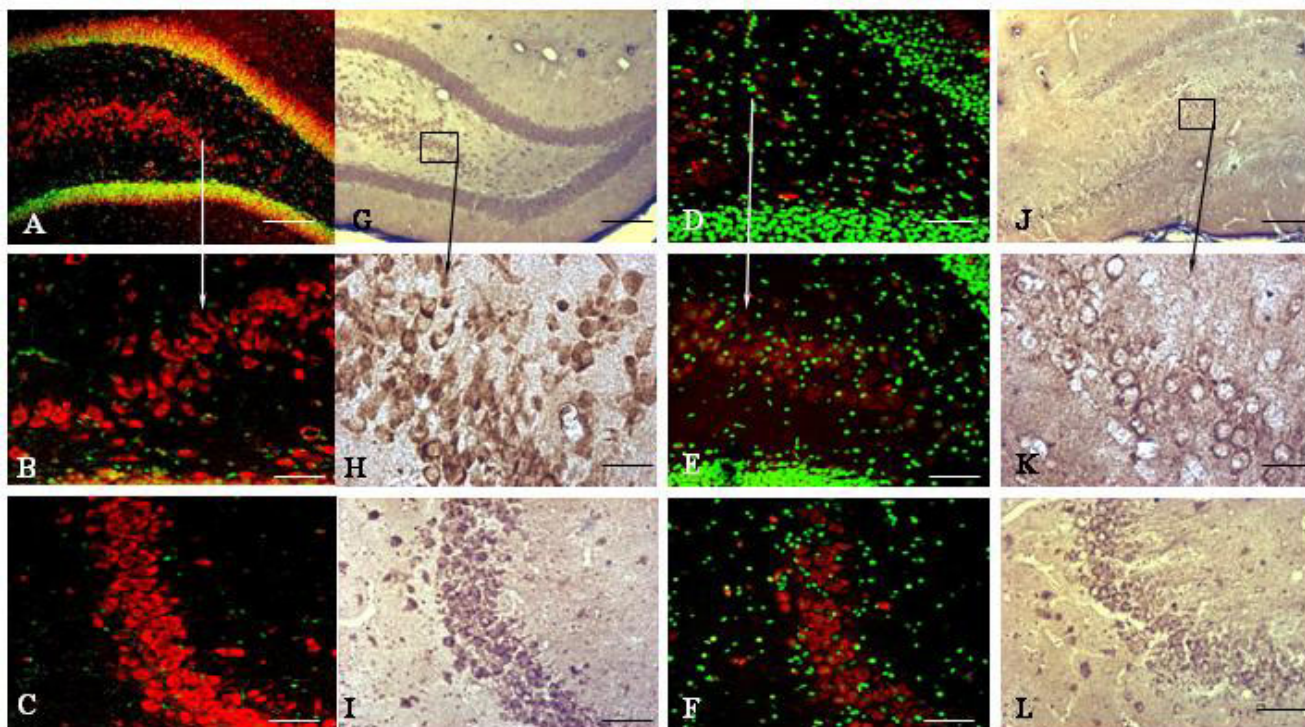
Figure 2
Confocal microscopic images of NMDAR1 receptors within the hippocampus. In rats infused with aCSF (A, B, C), fluorescence labeling showed large NMDAR1-positive neurons (red) in dentate gyrus (A), hilar region (B) and CA3 area (C). All nuclei are stained green (Sytox). Scale bars: (A) 100 μm; (B, C) 25 μm. Immunohistochemistry of NMDAR1-positive neurons revealed dark staining in the cytoplasm that extended along the dendrites in cells within the dentate gyrus (G), hilar region (H), and CA3 (I). Scale bars: (G) 100 μm; (H, I) 25 μm. Confocal microscopic images showed reduced NMDAR1 staining within the hippocampus of LPS infused rats: dentate gyrus (D), hilar region (E) and CA3 area (F). Scale bars: (D, E, F) 25 μm. Immunohistochemistry of NMDAR1-positive neurons revealed fewer cells expressing NMDAR1 receptors with a lower degree of immunoreactivity throughout the dentate gyrus (J), hilar region (K) and CA3 (L). Scale bars: (J) 100 μm, (K, L) 25 μm.

microscopy that chronic neuroinflammation in the hippocampus is associated with numerous changes in the intracellular components involved in the protein synthesis; in contrast, no significant changes were associated with the mitochondria or lysosomes [25]. The decline in immunoreactive receptor sites was paralleled by a decline in the number of [³H]MK-801 binding sites within the hippocampus, which is consistent with a previous report on the effects of acute exposure to LPS upon NMDA receptor density within this brain region [20]. Taken together, these findings are consistent with the hypothesis that selected vulnerable cells degenerated as a consequence of the chronic neuroinflammatory processes. We have previously shown that neurons in the EC degenerated in a model of chronic neuroinflammation similar to that used in the present study [6,8,9]. We speculate that the loss of

entorhinal afferents might underlie a component of the decline in NMDA R1 immunoreactivity within the hippocampus [30] given that the EC provides the main glutamatergic afferents to the hippocampus via the perforant pathway and this is usually the first region to undergo degenerative changes in AD [5,31]. Because so little is known regarding the consequences of long term neuroinflammation produced in this model, it is impossible to be certain whether the loss of NMDA glutamate receptors that we report is selective for this brain region or this particular receptor. We have previously only documented the loss of pyramidal neurons using this model [6] although we are currently pursuing this question.

In the current model of chronic brain inflammation we have hypothesized the following sequence of events
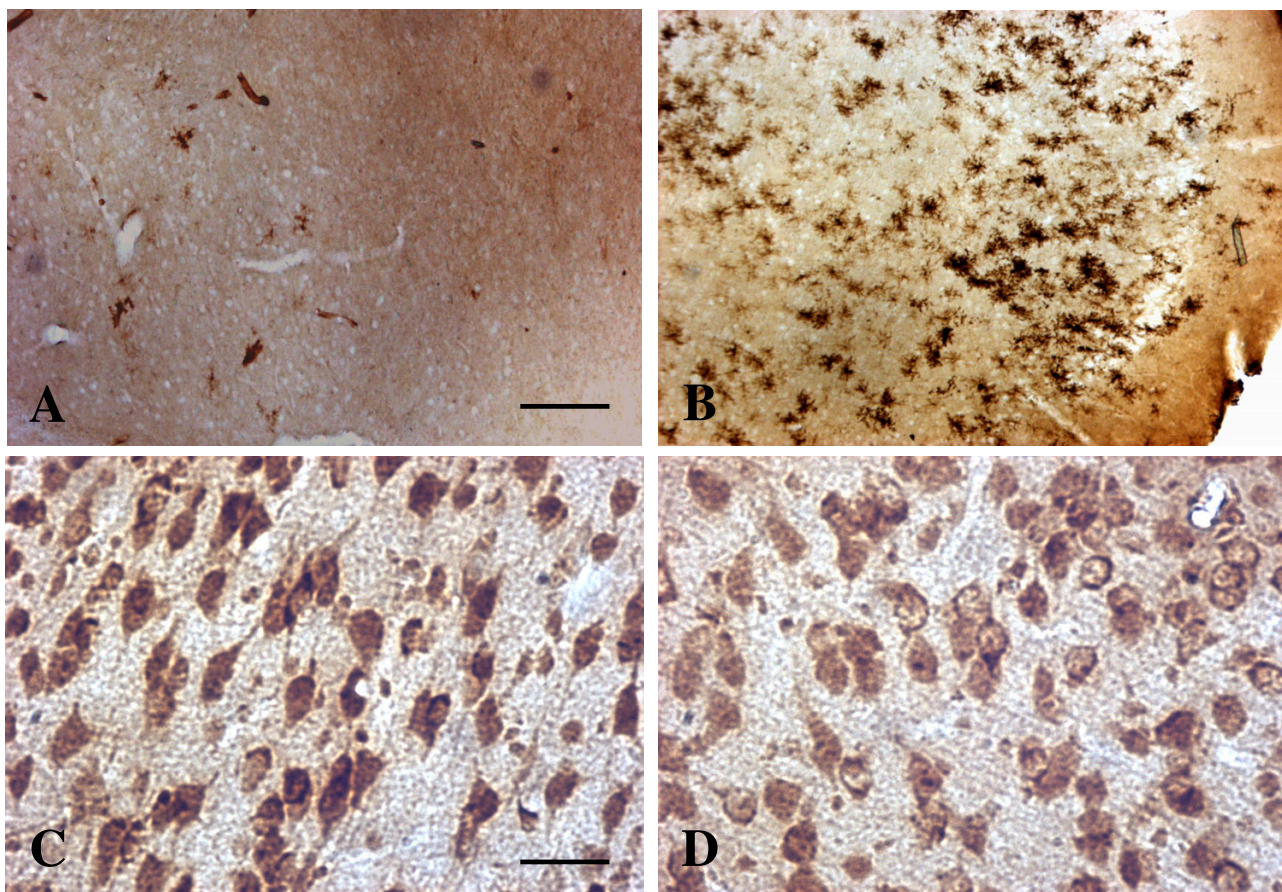
Figure 3
Immunostaining for activated microglia in the entorhinal cortex. Highly activated microglial cells (B) that are typical of LPS infused rats were completely absent in the brains of rats infused with aCSF (A). NMDAR1-immunoreactive cells within the entorhinal cortex of rats infused with aCSF (C) were characterized by darkly stained cell bodies and dendritic arbors. Rats infused with LPS (D) showed reduce level of immunoreactivity. Scale bars: (A, B) 100 μm; (C, D) 25 μm.

leading to the degeneration of NMDA-expressing neurons [14,15]. The infusion of LPS leads to the release of inflammatory cytokines by activated astrocytes and microglia [32]; these cytokines stimulate the production of other inflammatory mediators such as prostaglandins [33]; prostaglandins would induce the release of glutamate from astrocytes [21,36] leading to increased levels of extracellular glutamate and the stimulation of glutamate receptors, the depolarization-dependent unblocking of NMDA receptors by Mg^{2+}, and the entry of toxic amounts of Ca^{2+} into neurons and the subsequent generation of toxic levels of nitric oxide and initiate a cascade of reactive oxygen intermediates [34,35]. Prostaglandins and various cytokines may also indirectly elevate the extracellular concentration of glutamate by inhibiting its reuptake by

astrocytes [37,38]; in addition, blockade of the uptake of glutamate by astrocytes results in significant neurodegeneration [37,38]. We have hypothesized that a similar cascade of biochemical events, possibly initiated by the loss of forebrain norepinephrine [39], may occur associated with normal aging [14,15,26]. Consistent with this hypothesis and the results of the current study is a recent report that chronic administration of an anti-inflammatory drug could attenuate the age-related loss of hippocampal NMDAR1 receptors [40].

Conclusions
Taken together, our hypothesis and the results of our current study suggest that neurons expressing NMDA receptors would be vulnerable to degeneration in the presence
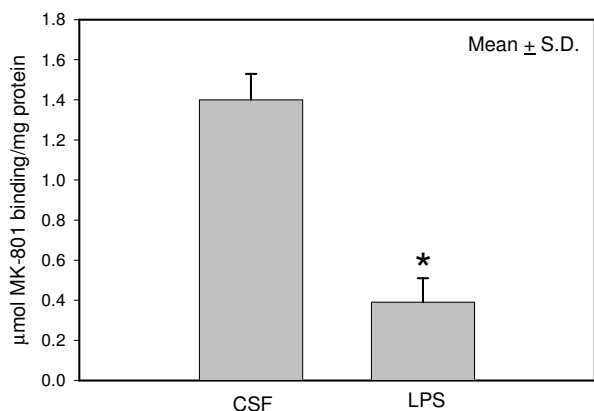
Figure 4
NMDA receptor number declined significantly (p < 0.001 vs. CSF) in the hippocampus following chronic neuroinflammation produced by infusion of LPS into the 4th ventricle.

of chronic neuroinflammation. Due to the widespread presence of inflammation in vulnerable brain regions, a similar series of biochemical processes might contribute to the cognitive deficits observed in patients with AD [1-3] or associated with normal aging [14].

List of Abbreviations
AD: Alzheimer's disease; aCSF: artificial cerebrospinal fluid; EC: entorhinal cortex; NMDA: N-methyl-D-aspartate; LPS: lipopolysaccharide; MHC II: Class II major histocompatibility complex;

Competing Interests
None declared.

Authors' Contributions
SR and GLW participated in the design of the study and preparation of the manuscript. SR performed the surgeries and the histological studies. GLW performed the receptor binding assay. BHW was responsible for the initial characterization of the animal model. VRA assisted with the confocal microscopic analyses. All authors read and approved the final version.

Acknowledgments

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