MEMANTINE PROTECTS AGAINST LPS-INDUCED NEUROINFLAMMATION, RESTORES BEHAVIORALLY-INDUCED GENE EXPRESSION AND SPATIAL LEARNING IN THE RAT

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Abstract—Neuroinflammation is reliably associated with the pathogenesis of a number of neurodegenerative diseases, and can be detected by the presence of activated microglia. Neuroinflammation can be induced by chronic lipopolysaccharide (LPS) infusion into the 4th ventricle of the rat resulting in region-selective microglia activation and impaired hippocampal-dependent memory. Furthermore, this treatment results in altered behaviorally-induced expression of the immediate early gene Arc, indicating altered network activity. LPS is known to activate microglia directly, leading to increased glutamate release, and in enhanced N-methyl-D-aspartate (NMDA)-dependent signaling. Taken together, the foregoing suggests that decreasing NMDA receptor activation during early stages of chronic neuroinflammation should reduce a) microglia activation, b) overexpression of Arc, and c) spatial memory deficits. Memantine, a low to moderate affinity open channel uncompetitive NMDA receptor antagonist, at low doses was used here to test these hypotheses. Rats were chronically infused into the 4th ventricle for 28 days with LPS alone, vehicle alone (via osmotic minipump) or LPS and memantine (10 mg/kg/day memantine s.c.). The results reported here demonstrate that memantine reduces OX6-immunolabeling for activated microglia, spares resident microglia, and activates Arc (activity-regulated cytoskeletal associated protein, protein) -expressing neuronal populations to control levels (as revealed by Arc immunolabeling and fluorescence in situ hybridization), and ameliorates the spatial memory impairments produced by LPS alone. These data indicate that memantine therapy at low doses, recreating plasma levels similar to those of therapeutic doses in human, acts in part through its ability to reduce the effects of neuroinflammation, resulting in normal gene expression patterns and spatial learning.

Combined, these findings suggest that low, therapeutically relevant doses of memantine delivered early in the development of neuroinflammation-influenced diseases may confer neural and cognitive protection. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: activated microglia, immediate early gene, hippocampal function, cognitive impairment, spatial memory consolidation, NMDA receptor.

Chronic neuroinflammation is implicated in several neurodegenerative diseases, such as Alzheimer’s disease (AD), traumatic brain injury, autism, Down syndrome, HIV dementia, and demyelinating diseases, such as multiple sclerosis and amyotrophic later sclerosis and may contribute to learning and memory deficits associated with these disorders (McGeer and McGeer, 1998; Akiyama et al., 2000; Morganti-Kossmann et al., 2001; Vargas et al., 2005; Fischer-Smith et al., 2004; Banati et al., 2000; Mhatre et al., 2004). During the early stages of AD, the greatest degree of neuroinflammation is found within temporal lobe regions involved in learning and memory (Cagnin et al., 2001). Activated microglia cells and their products are the key mediators of the neuroinflammatory process and contribute to neuronal damage (Barger and Harmon, 1997; Barger and Basile, 2001).

Chronic neuroinflammation can be produced by infusing lipopolysaccharide (LPS) into the fourth ventricle of young rats resulting in activation of microglia within the hippocampus, piriform and entorhinal cortices (Hauss-Wegrzyniak et al., 1998b). LPS selectively binds to a signal-transduction receptor complex (CD14/toll-like receptor 4) that is expressed only by microglia (Lehnardt et al., 2003). By activating microglia through LPS infusion, young rats can show pathological, biochemical and behavioral changes that are similar to those observed in several neurodegenerative diseases associated with neuroinflammation. These include impaired spatial memory, reduction of N-methyl-D-aspartate receptor type 1 (NMDAR1), astrogliosis, elevated cytokines and proinflammatory transcript-
In addition, chronic neuroinflammation has been shown to alter the behaviorally-induced expression of the immediately early gene (IEG) Arc (Rosi et al., 2005a). Arc is induced in hippocampal neurons as a result of neural activity associated with spatial exploration (Guzowski et al., 1999) and Arc protein plays a critical role in the maintenance of long-term potentiation and spatial memory consolidation (Guzowski et al., 2000). Importantly, behaviorally-induced overexpression of Arc occurs selectively in hippocampal pyramidal and granule neurons, which are cell fields containing the highest numbers of activated microglia induced by the LPS infusion (Rosi et al., 2005a).

Considering that Arc mRNA transcription is induced by N-methyl-D-aspartate (NMDA) receptor activation (Steward and Worley, 2001) and calcium-mediated activation of cAMP (Waltereit et al., 2001), one possible explanation for the observation of IEG overexpression could be excess calcium influx through postsynaptic NMDARs. Because chronic neuroinflammation results in increased levels of glutamate (Fine et al., 1996; Espey et al., 1998), a significant proportion of NMDA channels may exist without Mg$^{2+}$ block, thus allowing elevated calcium entry in postsynaptic neurons (Brown and Bal-Price, 2003; Robinson et al., 1993). Therefore, further increases in glutamatergic activity induced by behavior could lead to excessive influx of calcium ions (Albin and Greenamyre, 1992). Because full-blow neuroinflammation appears to involve a positive feedback loop between activated microglia and endangered neurons (de Jong et al., 2005), we hypothesized that preventing NMDAR overactivation that results in excess calcium influx into neurons will stop this positive loop and consequently decrease the number of activated microglia. The current study investigated a direct prediction of this hypothesis: decreasing calcium entry through NMDARs during neuroinflammation will decrease numbers of activated microglia and return normal patterns of Arc expression within the hippocampus. To test this hypothesis we used low doses of memantine, a low-to-moderate affinity open-channel noncompetitive NMDAR antagonist, currently in use for treatment of cognitive impairments as a result of AD.

**EXPERIMENTAL PROCEDURES**

Subjects and surgical procedures

The subjects were 46 male F-344 (Harlan Sprague–Dawley, Indianapolis, IN, USA) rats, 3 months old, individually caged with food and water freely available. Artificial cerebrospinal fluid (aCSF, n=13) or LPS (Sigma, St. Louis, MO, USA E. coli, serotype O55:B5, TCA extraction, 1.0 μg/μl dissolved in aCSF, n=33) was chronically infused for 28 days through a cannula implanted into the 4th ventricle of the brain that was attached (via Tygon tubing, 0.06 O.D.) to an osmotic minipump (Alzet model #2004, to deliver 2.5 μl/h) releasing, s.c., memantine at a dose of 10 mg/kg/day. Memantine was purchased from Tocris Bioscience (Ellisville, MO, USA). Body weights were determined daily and general behavior was monitored for seizures. This research was conducted under the supervision, and with the approval, of the University of Arizona Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used to a reasonable minimum regarding statistical constraints.

Behavioral testing

The rats were handled daily for 10 days before behavioral testing began. To assess the rats’ spatial learning ability, on the third week following the surgeries, all rats were tested in a 185-cm-diameter Morris water maze (Morris, 1984), as described previously (Barnes et al., 1996). During the spatial (hidden-platform) version of the water task, a circular escape platform was present in a constant location, submerged about 1 cm below water surface. The rats were tracked by an overhead video camera connected to a VP114 tracking unit (HVS Image, UK). Custom software (TR, J. Forster: WMAZE, M. Williams) was used to store, reconstruct and analyze the latency to find the hidden platform during each trial. All rats, but two (from the LPS-mem group) who became sick and were excluded from the study, performed three training blocks per day (two training trials per block) for 4 days (24 trials total), with release location varied randomly from trial to trial. Rats were allowed a 60-s intertrial interval within a given training block, and 30–60 min of recovery time between training blocks. To control for possible LPS or memantine-related deficits in visual acuity and swimming, on the fourth day the rats were also trained on a second version of this task, in which a visible platform (2 cm above the water) was moved randomly to one of four locations in the tank. All the animals performed a total of six visible-platform blocks successfully.

To assess the expression of behaviorally induced IEG Arc; three days following water task testing, and 29 days following the implant of the LPS or aCSF osmotic minipump, the rats were separated into two groups: exploration groups (LPS-infused, n=11; LPS-infused+m-memant, n=12 and CSF-infused, n=10) and caged control groups (LPS-infused, n=3; LPS-infused+m-memant, n=5 and aCSF-infused, n=3). The animals from the exploration group were allowed to explore two times the same novel environment for 5 min. The exploration environment was an open square box 61×61 cm with 20-cm high walls divided into nine grids. Each rat was moved to the center of a different grid every 15 s in a pseudo-random schedule during the 5 min exploration session as described in detail previously (Guzowski et al., 1999). The first and second exploration sessions were separated by 30 min. During the rest interval between the two sessions, the animals remained undisturbed in their own cage in the colony room. Immediately after the second 5 min exploration session, the rats were killed by decapitation; the brain was quickly removed (between 160 and 200 s) and frozen in −70 °C isopentane (2-methyl butane, Sigma). Caged control animals that did not explore the novel environment were killed directly from their home cages interspersed with the rats given exploration experience.

Histological procedures

The brains were divided at the midline and half brains from each rat were blocked together and cryosectioned (Vazdarjanova et al., 2002), such that each slide contained brain coronal sections from one rat from each of the groups involved in this experiment (see above). All slides were stored at −70 °C until processed for double immunocytochemistry or in situ hybridization.

Tissue from the contralateral hemisphere was used (~150 mg) to measure memantine concentration by gas chromatography (GC). Samples were homogenized in 2 M HCl containing an...
internal standard (amantadine HCl) and heated to 70 °C for 15 min. After cooling to room temperature, 0.6 ml of hexane and 0.6 ml of 10 M NaOH were added to each sample and mixed for 30 min; the organic phase was transferred into a GC vial. Membrane levels were determined using a GC system coupled to a mass selective detector (Hewlett Packard 5970/5971; Hewlett Packard, San Jose, CA, USA). The analytical column was used with an injection model (splitless 1–3 μl); using helium gas (10 p.s.i. at 1 ml/min); injection temperature was 220 °C and the detection temperature was 240 °C. The ionization mode was positive electrical ionization (Misztal et al., 1996).

Immunofluorescence staining

Three slides were selected from the medial portion of the dorsal hippocampus (anterior–posterior, −− −3.6 mm from Bregma) and were double stained for Arc protein and activated microglia as previously described (Rosi et al., 2005a). Three additional slides from the same medial portion of the dorsal hippocampus were double stained for NMDAR1 and activated microglia (Fig. 4). The tissues were processed as previously described (Rosi et al., 2005a), and left in primary mouse monoclonal NMDAR1 antibody (Chemicon International, Temecula, CA, USA) (dil 1:250) for 48 h, incubated at 4 °C. The sections were then incubated for 2 h at room temperature with the secondary antibody, anti-mouse (Vector, Burlingame, CA, USA) and followed by incubation with the avidin-biotin amplification system (Vector) for 45 min as previously described (Rosi et al., 2004). The staining was visualized using the TSA fluorescence system cyanine 3 (CY3, PerkinElmer Life Sciences, Emeryville, CA, USA). After washing in TBS solution, endogenous peroxidase activity was quenched, followed by a blocking step as previously described (Rosi et al., 2005b) and incubation with monoclonal antibody OX-6 (PharMigen, San Diego, CA, USA) (dil 1:400) for 24 h. The staining was then visualized with TSA fluorescence system cyanine five (CY5, PerkinElmer Life Sciences) and the nuclei were counterstained with Sytox-Green (Molecular Probes, Eugene, OR, USA). Single immunostaining for the resident microglial cells was performed as described above using the mouse anti-rat OX-42 (PharMigen) at the final dilution of 1/400, followed by anti-mouse 1/200 (Vector, Fig. 3). No staining was detected in the absence of the primary or secondary antibodies.

Fluorescence in situ hybridization (FISH)

Three slides were selected from the same medial portion of the dorsal hippocampus adjacent to the ones chosen for immunohistochemistry (described above) and were processed for in situ hybridization to detect Arc mRNA (Guzowski et al., 1999; Vazdarjanova et al., 2002; Figs. 5A–F, 6A–F). Briefly, the tissue was fixed in 4% paraformaldehyde, washed in saline sodium citrate buffer (SSC), and placed in an acetic-anhydride solution, followed by an acetone-methanol solution. After a prehybridization step, the tissue was hybridized with an Arc mRNA probe (100 ng/slide) tagged with digoxigenin and diluted in hybridization buffer (Sigma) for 16–18 h at 56 °C. After a series of washes, including an RNase A step, the slides were incubated overnight in an anti-digoxigenin peroxidase-conjugated antibody (Roche Products, Hertfordshire, UK) at 4 °C, and the stain was visualized using the CY3 TSA fluorescence system. The nuclei were counterstained with Sytox Green (Molecular Probes).

Image acquisition (confocal microscopy)

In order to define the anatomic boundaries and degree of microglial activation and resident microglia within the hippocampus: three 20 μm coronal sections of the dorsal hippocampus at −− −3.6 mm from Bregma, were reconstructed for each animal from each treatment group. The collage was constructed by overlapping 15–20 flat images taken at 25× magnification (1.0 μm optical thickness/­plane). The 2-stacks were collected with a Zeiss (Thornwood, NY, USA) LSM 510 NLO-meta multiphoton/confocal microscope equipped with a 488 nm argon laser and a 543 nm/633 nm helium/neon laser. Contrast and intensity parameters were set using tissue sections taken from an aCSF-infused rat. The parameters were kept constant across sections on a given slide using the 488 nm (for Sytox Green) and the 543 nm (for CY3) laser.

To quantify the resting microglia OX-42 positive (Fig. 3) and activated microglia OX-6 positive (Fig. 2), the reconstructed hippocampal coronal sections were analyzed with MetaMorph imaging software (Universal Image Corporation, West Chester, PA, USA). The hippocampus was divided into two major areas of interest and the CA3 and granule cell layer and hilar region of the DG were drawn as shown in Fig. 1, Rosi et al. (2005). After drawing these regions in each reconstructed image, a threshold tool was used to detect all of the OX-6 or OX-42 staining, and the area of each object was measured. All images were analyzed using the same threshold settings. The detected objects ranged from 5 to 2000 μm². After creating a distribution curve, only those objects of sizes >30 μm² were included in the analysis (the average size was 100 μm²). This object size was chosen to match more accurately the size of a microglial cell and therefore signif-

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Fig. 1. (A) Latency to find the hidden platform in the spatial version of the Morris swim task for three groups of rats: aCSF-, LPS-, and LPS-infused animals treated with memantine (10 mg/kg/day s.c.). Six trials are averaged for each of the 4 days of training. Chronic LPS-infusion into the fourth ventricle impaired the performance of the young rats; however, LPS infusion combined with memantine attenuated this deficit. Overall ANOVA for all groups revealed a significant main effect for LPS vs. aCSF * P<0.0001 and LPS+mem vs. LPS, † P<0.0002. (B) Latency to reach the platform on the first trial of day 4 (overall ANOVA, F2,34=7.50, P<0.005, Bonferroni post hoc tests: aCSF vs. LPS, P<0.001 and LPS vs. LPS+M, P<0.016). The LPS+M group was not statistically different from the aCSF group.
significantly reduce sampling errors. The resulting number of objects was then normalized using the total area of each region of interest, and the number of objects per region (in square millimeters) was reported (see Figs. 2 and 3).

Using the same criteria described above, the entire DG from three coronal sections per rat was reconstructed for the quantification of the cytoplasmic Arc mRNA and Arc protein. According to established nomenclature (Paxinos and Watson, 1997) the part of the granule cell layer that is adjacent to CA1 is called the enclosed blade; and the remaining portion of the granule cell layer is called the free blade. The area of the granule cell layer and the total number of Arc-positive neurons were assessed in each reconstructed flat image and this information, along with an estimate of the total number of neurons, was used to estimate the percent of total Arc-positive neurons. The total number of neurons per square micron in the DG was estimated using a correction factor: the total number of neurons/stack was counted and the area of the granule cell layer (in μm²) from the middle plane was calculated. Utilizing this factor, we calculated the percentage of neurons with Arc protein and/or Arc mRNA in the DG of each rat according to the following formula: 100/(p/(N/A)), where: p=the number of Arc(+) neurons in a given reconstructed flat image; A=the area (in μm²) of the DG, as measured from the reconstructed flat image; N=the total number of cells from all 40 X Z-stacks; A=the total area (in μm²) of the DG from the middle planes of all 40 X Z-stacks.

Fig. 2. (A–D) Representative flat images of reconstructed dentate gyri (DG) and CA3 area of rats infused with LPS alone (A, B) or LPS and memantine (C, D). Activated microglia are shown in red and nuclei in green. Scale bars =100 μm (A, C), 10 μm (B, D). (E) Animals that received memantine treatment showed a significant reduction in the number of activated microglial cells/mm² within the DG and CA3 areas compared with LPS alone [*P<0.0001, significant versus aCSF, LPS +M groups].
Cells were classified as either positive or negative for Arc protein according to the following criteria. Positive neurons had perinuclear/cytoplasmic staining, surrounding at least 60% of the cell, visible in at least three plains together with the cell nucleus

Fig. 3. (A) Representative flat images of reconstructed hippocampi (20 μm coronal section) of rats chronically infused with aCSF, LPS or LPS+memantine. Resting microglia are shown in red and nuclei in green. (B) Histograms show the number of microglia cells/mm² within the DG and CA3 areas. The total number of microglial cells was higher in LPS-infused animals compared with the control aCSF-infused animals and the LPS-infused animals that received memantine. [* P<0.01, significant versus the aCSF and LPS+M groups]. Scale bar=100 μm.
across the Z-stack. To avoid classification errors, we carefully verified that the staining belonged to the cell of interest and not to a dendrite or the cell body of an adjacent cell. The results for both Arc mRNA and Arc protein analysis were expressed as a percentage of total neuronal nuclei analyzed per stack. For each rat, coronal sections of the reconstructed DG were analyzed, for Arc mRNA, for Arc protein, for activated microglia (OX-6 immunopositive) and resting microglia (OX-42 immunopositive).

For the CA3 area, three Z-stacks for each slide (1 μm optical thickness/plane) were imaged with a 250× water immersion lens. Image analysis was done as described earlier, using the dissector method (Guzowski et al., 1999).

Statistical analysis

StatView software was used to perform all the statistical analyses. The latency to find the hidden platform across the 4 days of the water maze test and any interaction between treatments (aCSF, LPS and LPS+memantine) were assessed with ANOVA test with repeated measures and appropriate post hoc tests. One-way ANOVA test for the region of interest was used to analyze the cell numbers: the control (aCSF) and experimental groups (LPS, LPS+memantine) were the independent variables and the percentage of total neurons from the various categories, described above, was the dependent variable. When an overall ANOVA was significant (P<0.05), individual comparisons between groups were performed using Bonferroni post hoc tests to correct for multiple comparisons.

RESULTS

Overall, chronic infusion of LPS and memantine 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.

Memantine levels in the brain

Memantine treatment was well tolerated and did not modify the mobility and the gross behavior in the animals. In order to determine the levels of memantine in the brain, brain tissues from each of the 17 rats with memantine osmotic minipumps, and a subset of animals with no memantine treatment (n=10) were analyzed by GC. The measurements revealed that 10 animals had detectable memantine levels (ranging from one to 6 μg/g); while seven of the animals with the “memantine minipumps” showed levels below limit detection (0.2 μg/g), not different from non-memantine-treated rats. Memantine was infused using osmotic minipumps; this method takes into account the fact that memantine is accumulated in intracellular compartments (primarily by lysosomes). Due to this characteristics of memantine, chronic administration under the conditions used in the current study typically produces a serum concentration of ~1 μM within three days, an extracellular fluid concentration of 0.4–0.7 μM and a brain tissue concentration of 12–15 μM; free drug levels are 20–50% lower due to protein binding (see Rogawski and Wenk, 2003) similar to this reached in humans at therapeutic doses. Consequently, the subset of animals with no memantine detected in the brain (n=7), was taken out for all the analysis performed in the present study, because of an evident dysfunction of the minipump.

Memantine treatment attenuates LPS-induced spatial learning and memory impairments

Chronic LPS-infusion into the fourth ventricle impaired the performance of young rats on the spatial version of the Morris swim task, as compared with control animals infused with aCSF, while memantine treatment ameliorated this deficit. Repeated measures ANOVA of the average latency to find the hidden platform, in the water pool for each daily session, revealed significant differences between groups (F_{2,34}=9.26, P<0.001), significant differences across sessions (F_{3,6}=17.67, P<0.0001) and significant interaction (F_{6,102}=2.22, P<0.05). Repeated measures ANOVA for each group revealed that both the aCSF (F_{3,36}=31.20, P<0.0001) and LPS+M (F_{3,27}=9.28, P<0.001) groups had a significant difference across sessions, indicating that they learned the location of the platform (Fig. 1A). No such difference was observed for the LPS group, confirming previous reports that chronic LPS-infusion impairs spatial learning in rats (Hausa-Wegrzyniak et al., 1999a). Furthermore, memantine ameliorated the LPS-induced spatial memory deficit as revealed by the significantly higher latencies of the LPS group to reach the platform on the first trial of day 4 compared with the aCSF and LPS+M groups (overall ANOVA, F_{2,34}=7.50, P<0.005, Bonferroni post hoc tests: aCSF vs. LPS, P<0.001 and LPS vs. LPS+M, P<0.016). The LPS+M group was not statistically different from the aCSF group (Fig. 1B).

Memantine treatment significantly reduced the number of activated microglia in the hippocampus during LPS-infusion

To evaluate microglial activation within the hippocampus during LPS infusion alone and LPS infusion plus memantine, fluorescence immunostaining for OX-6 was performed and the number of activated microglia was counted. In LPS-infused animals immunofluorescence staining for OX-6 confirmed numerous highly activated microglia selectively distributed throughout the DG and CA3 areas of the hippocampus (Fig. 2A). Activated microglia showed a characteristic bushy morphology with increased cell body size, contracted, and ramified processes (Fig. 2B). Whereas, rats infused with aCSF alone had few mildly activated microglia, evenly scattered throughout the hippocampus, as previously reported in detail (Rosi et al., 2003, 2004, 2005a,b). LPS-infused animals that received memantine treatment showed a significant reduction in the number of activated microglial cells within the DG and CA3 areas (ANOVA: F_{2,34}=20.69, P<0.0001 for the DG area; F_{2,34}=7.23, P<0.004 for the CA3 area) (Fig. 2E). The microglia that remained in the LPS-infused animals treated with memantine presented a morphology characteristic of a less advanced activation state (Fig. 2D).

Memantine treatment did not affect the resident microglia

It has been reported that the treatment with the selective NMDAR antagonist, MK-801, is cytotoxic to microglia in...
vitro (Hirayama and Kuriyama, 2001). Thus, the possibility that memantine may have reduced the number of activated microglia by reducing the total number of resident microglia was examined. When the total number of microglia within the hippocampus was quantified, OX-42 immunoreactive cells were distributed throughout the hippocampus of aCSF, LPS, and LPS-infused animals treated with memantine (Fig. 3A). There was no significant difference between aCSF- and LPS-infused animals treated with memantine; whereas, in LPS-infused animals the total number of microglia was significantly higher as compared with the other two groups (Fig. 3B) (ANOVA: \(F_{2,34}=6.07, P<0.008\) for the DG; \(F_{2,34}=4.41, P<0.01\) for the CA3). Therefore, memantine treatment does not alter the number of resting microglia, but prevents their activation and/or migration from the nearby regions.

**NMDAR1 did not co-localize with hippocampal glial cells**

There is no direct evidence suggesting the presence of NMDAR in microglia; however, metabotropic glutamate receptors have been found in microglial cells (Biber et al., 1999). To examine whether the effect of memantine on microglia activation state was dependent on NMDARs expressed by microglia, brain sections were double-immunolabeled for the constitutively expressed NMDAR1 subunit (red) and OX-6 for microglia (blue) (Fig. 4A, B). Examination of the tissues by high resolution confocal microscopy revealed that activated microglia did not colocalize with NMDAR1. These observations indicate that memantine does not directly act on microglia through a NMDA receptor mechanism; however it is still possible that other membrane receptors in microglial cells may respond to memantine.

**LPS-infusion combined with memantine treatment protected the sparsity of neuronal ensembles expressing Arc mRNA and protein following spatial exploration in the dentate gyrus**

Novel environment exploration resulted in a significant increase of the percentage of neurons expressing Arc mRNA and Arc protein in the DG-enclosed blade, above that of caged control animals in all of the three groups examined (Fig. 5) (ANOVA: \(F_{3-34}=23.59\) cytoplasmic Arc mRNA, in the enclosed blade of the DG; \(F_{3-34}=32.55\) for Arc protein DG in the enclosed blade; \(P<0.0001\) in all cases). In aCSF-infused animals the percentage of cells expressing Arc mRNA and Arc protein in the DG granule cells was comparable to that previously reported (Rosi et al., 2005a; Ramirez-Amaya et al., 2005; Chawla et al., 2005; Vazdarjanova et al., 2006): Arc mRNA, induced by the first exploration session was evident as cytoplasmic mRNA surrounding the cell nuclei of 1.5% (S.E. = 0.2) of the enclosed blade granule cells (Fig. 5A), which is consistent with electrophysiological recordings showing sparse activity in the DG during exploration (Jung and McNaughton, 1993). LPS-infused animals showed a significant increase in the percentage of behaviorally-induced Arc mRNA- and Arc protein-positive neurons in the DG enclosed blade above those of the aCSF-exploration rats (Fig. 5G, H) (Rosi et al., 2005a) (Bonferroni was used as a post hoc to compare LPS and aCSF animals \(P<0.001\)). In LPS-infused animals treated with memantine, the percentage of granule cells expressing behaviorally induced Arc mRNA and Arc protein in the enclosed blade was significantly lower than that of LPS-infused animals \(P<0.001\). There was no statistically significant difference between the proportion of Arc expressing cells in aCSF versus LPS+memantine groups (Fig. 5G, H).

The percentage of neurons expressing Arc mRNA and Arc protein remained unchanged in the free blade of the DG, in the exploration group given aCSF, compared with the caged control animals. The LPS-exploration group, however, showed a significant increase in the percentage of neurons with Arc mRNA and protein, consistent with previous findings (Rosi et al., 2005a). Notably, LPS-infused animals treated with memantine did not show a significantly elevated increase compared with aCSF-infused animals. (ANOVA: \(F_{3-33}=16.15\), Arc mRNA in the DG free blade; \(F_{3-33}=22.52\) for Arc protein; \(P<0.0001\) in all cases) (Fig. 5G, H).
Memantine treatment maintained the sparsity of neuronal ensembles expressing Arc mRNA and protein following spatial exploration in CA3 pyramidal neurons of LPS-infused animals.

Following exploration of a novel environment, the percentage of pyramidal neurons expressing Arc mRNA and Arc protein in aCSF-infused animals, was similar to that previously reported (e.g. ~20%) (Vazdarjanova et al., 2002, 2006; Vazdarjanova and Guzowski, 2004; Ramirez-Amaya et al., 2005; Rosi et al., 2005a). In LPS-infused animals the percentage of neurons expressing Arc mRNA and Arc protein was significantly higher than aCSF control animals.
exploring the same environment (ANOVA; $F_{3,34} = 19.78$, $P < 0.0001$ for Arc mRNA, $F_{3,34} = 12.89$, $P < 0.0001$ for Arc protein) (Fig. 6 G, H; Rosi et al., 2005a). During the treatment with memantine, the percentage of neurons expressing Arc mRNA and Arc protein was not significantly different from aCSF-infused animals and significantly lower than the animals given LPS alone that explored the same novel environment (Fig. 6). Furthermore chronic i.c.v. LPS infusion results in microglial activation within the entorhinal and piriform cortices areas (Hauss-Wegrzyniak et al., 1998b).
DISCUSSION

The current study demonstrates that selective activation of microglia within the hippocampus can be significantly reduced if low doses of memantine, a moderate-affinity uncompetitive open NMDAR channel antagonist, is infused concurrently with LPS. Furthermore, memantine treatment prevents the previously observed (Rosi et al., 2005a) LPS-induced overexpression of the behaviorally-induced IEG Arc. Importantly, treatment with this NMDAR antagonist ameliorates the memory deficit produced by chronic LPS infusion. Because memantine provides a partial block of the NMDAR and restricts calcium entry in the face of high levels of synaptic glutamate, this treatment might in part reduce cognitive decline by attenuating neuroinflammation, which may be particularly important in preventing neuronal dysfunction. Finally, the data support the hypothesis that maintaining the neuroinflammatory response requires a cross-talk between glia and neurons.

Memantine attenuates memory deficits associated with LPS infusion

Memantine treatment did not affect spontaneous locomotor activity or other motor behaviors such as swimming in the water maze. It has been shown previously that memantine has no effect on learning and memory in young male rats at neuroprotective doses (Misztal and Danysz, 1995; Barnes et al., 1996), such as that used in this study. By contrast, memantine treatment resulted in a partial but significant improvement in acquisition and retention of the spatial version of the water maze during LPS infusion. This suggests that NMDA receptor-mediated events are important but not solely responsible for the cognitive deficits observed in chronic neuroinflammatory processes. The behavioral effects observed for memantine, however, are clearly dose-dependent. Ataxia, myorelaxation and stereotypy were observed when, high doses of memantine (20 mg/kg) are given acutely (Misztal and Danysz, 1995). In a recent report, Creeley et al. (2006) using adult female rats, showed that such acute administration of memantine (20 mg/kg) by i.p. injection produces behavioral deficits. In humans, steady state therapeutic doses (typically 20–30 mg/day), result in plasma levels of memantine in a range of 0.4–0.9 μM without detrimental behavioral effects. The dose and route of administration used in the current study were previously shown to produce steady state plasma levels similar to those in humans at therapeutic doses (Rogawski and Wenk, 2003). The explanation of the behavioral differences in the rat experiments is likely due to the fact that memantine is sequestered and accumulated into lysosomal or other intracellular compartments with chronic infusion, reducing plasma levels of memantine compared with those achieved after acute i.p. injection. While some NMDAR antagonists at high doses block synaptic plasticity, and learning in the Morris swim task, memantine, at low doses, has low to moderate affinity for the NMDAR channel, strong voltage-dependent channel blocking characteristics, and fast channel unblocking kinetics (Parsons et al., 1995). Because of these unique biophysical and pharmacological properties, memantine given at low doses can selectively block the pathological influx of calcium through NMDARs without affecting the voltage-dependence of NMDAR transmission, which is critical for learning and memory (Zajaczkowski et al., 1996) and durability of synaptic plasticity (Barnes et al., 1996).

Memantine counteracts LPS-induced changes in the pattern of Arc expression in DG and CA3 hippocampal subregions

During chronic neuroinflammation, the expression of the IEG Arc is altered in hippocampal regions showing activated microglia (Rosi et al., 2005a). The present results show that treatment with memantine protects against LPS-induced nonspecific increases in the proportion of hippocampal neurons activated by spatial exploration. That is, there is a reduction in the sparsity of hippocampal ensembles following exploratory behavior in LPS-infused animals. In fact, the size of DG and CA3 neuronal ensembles expressing Arc in LPS+memantine-treated rats was similar to those of control-infused rats and significantly lower than those of LPS-infused rats (Figs. 5–6). The observation that both Arc mRNA and Arc protein expression returned to normal levels suggests that memantine affects Arc transcription. The mechanism underlying memantine’s effect on Arc transcription may result from its ability to reduce post-synaptic calcium levels in the presence of neuroinflammatory factors, such as LPS. Consistent with this hypothesis is the finding that Arc expression is NMDAR-dependent (Steward and Worley, 2001). Arc transcription requires calcium-mediated cAMP activation (Walter et al., 2001), and that memantine is known to act only on the open NMDAR channel inhibiting inward depolarizing currents (Danysz et al., 1997). During neuroinflammation astrocytes and activated microglia release glutamate and proinflammatory cytokines (Bezzi et al., 2001), likely leading to excessive calcium entry in postsynaptic neurons. This excessive calcium influx can stimulate gene expression in a higher proportion of neurons, as observed previously and in the present study (Rosi et al., 2005a; Figs. 5–6). Memantine, by restoring the normal calcium influx through the NMDARs, may help prevent this altered behaviorally-induced IEG expression within the DG and CA3 hippocampal areas. In addition to the hippocampal areas, chronic LPS infusion results also in an activation of microglia within the piriform and entorhinal cortices (Hauss-Wegrzyniak et al., 1998b). The percent of cells expressing Arc in entorhinal and piriform areas following exploratory behavior has not yet been thoroughly examined, and therefore numbers of Arc-positive cells expected to be behaviorally activated are not known. Current studies are under way to characterize these patterns and determine whether there is correlation between activated microglia and expression of Arc in these brain regions, as well.

Possible mechanisms of memantine’s effect on microglial activation

The present study shows that treatment with memantine was able to reduce dramatically the number of activated
microglia during chronic neuroinflammation, without affecting the number of resident microglia. Recently, it has been reported that memantine treatment enhanced functional recovery and anti-inflammatory effects in a rat model of intracerebral hemorrhage (Lee et al., 2006); however, it was not clear from these data what mediates the anti-inflammatory action of memantine. The observed reduction in microglial cells may result from a direct effect of memantine upon microglia or from an indirect effect through neuronal NMDARs. The lack of NMDARs in activated microglia in the hippocampus at 28 days after the beginning of LPS-infusion in the present study (Fig. 4), suggests that if memantine acts directly on microglia it does so either by affecting NMDARs expressed only during an early stage of neuroinflammation, or by affecting alpha-7 nicotinic receptors, recently shown in cultured microglia (Shytle et al., 2004), for which memantine shows low affinity (Buisson and Bertrand, 1998). The potency of memantine at nicotine alpha7 receptors varies considerably from 0.33–1.68 µM in rat up to 5 µM in human. This makes it unlikely that alpha-7 receptor activation contributes to the therapeutic effects of memantine in rats or AD patients (who typically only achieve a tissue level of 1 µM). Memantine may also affect 5HT-3 receptors indirectly; however, this effect is only seen following acute administration of high doses (Rogawski and Wenk, 2003). Our studies avoided potential complications due to non-specific effects of memantine associated with high doses by administering this drug at considerably lower doses per day using an Alzet osmotic minipump. A more likely possibility is that memantine affects microglial cells by leading to increases in extracellular glutamate and consequently higher degree of neuronal depolarization. Consistent with this possibility are findings that microglial cells have unique potassium channels that make them very sensitive to changes in extracellular potassium and the degree of neuronal depolarization (Kettenmann et al., 1990).

The present data suggest that memantine can block LPS-induced alterations in hippocampal Arc expression. Because Arc is selectively expressed in neurons in the hippocampus (Vazdarjanova et al., 2006) which contain NMDARs, it follows that memantine acts directly on hippocampal neurons to counteract LPS-induced increases in neuronal depolarization, consequently decreasing the probability of microglia activation. Furthermore, it is known that neurons can release proinflammatory molecules that can exacerbate activation of microglia (de Jong et al., 2005). High levels of glutamate, such as those present during neuroinflammation, induce neuronal release of chemokines that activate nearby and remote microglia (de Jong et al., 2005). In addition, during LPS infusions elevated levels of glutamate activate the phospholipase A2 (PLA2) with the consequent release of arachidonic acid (AA) from neurons. This results in the formation of prostaglandins that act on microglia to increment the cycle of inflammation (Lee et al., 2004). By restoring normal calcium entry through the NMDARs, memantine treatment may prevent the release of AA and the consequent elevation of prostaglandins, resulting in lowered activation of microglial cells. The possibility of a reduction in portioned synthesis by blocking NMDAR has also been recently suggested by Munhoz et al., 2006. Additionally, in vitro studies (Viviani et al., 2003) report that neurons exposed to IL-1beta, a proinflammatory cytokine produced by both glia and neurons during neuroinflammation, may increase postsynaptic entry of calcium ions. Importantly, a similar anti-inflammatory effect of an NMDAR antagonist has been shown for MK-801 by Thomas and Kuhn (2005), where MK801 protected against methamphetamine-induced neurotoxicity, further suggesting the importance of NMDAR involvement in the neuroinflammatory process.

The finding that memantine infused concurrently with LPS results in a normalization of gene expression and a reduction in the number of activated microglia, without affecting the total number of microglia (Figs. 2 and 3), suggests that modulation or partial blockage at the NMDAR, is critical to the maintenance of the full-blown neuroinflammatory response. Taken together, the present results can be explained by postulating that in the presence of LPS, memantine, by stochastically blocking only open NMDA channels, returns gene expression mechanisms to normal and breaks the self-propagating cycle of neuroinflammation. During neuroinflammation various stimuli can selectively affect glial or neuronal function; the final outcome may be an alteration in the interaction between these cell types. Neuroinflammation appears to drive a positive feedback toxic cycle in which activated microglia release inflammatory mediators, such as cytokines (IL-1beta and tumor necrosis factor-alpha) and AA. These factors can clearly exacerbate neuronal injury (Griffin et al., 1998; Akiyama et al., 2000; Barger and Basile, 2001; Mrak and Griffin, 2005). Thus, memantine may decrease the probability that neurons would release proinflammatory factors that further activate microglia. In this sense, memantine may be said to have an indirect anti-inflammatory effect. The present results underscore the importance of a balanced cross-talk between microglia and neurons that is necessary to lessen the adverse consequences of neuroinflammation and neuronal damage (Polazzi and Contestabile, 2002).

The current findings suggest that memantine by promoting normal levels of calcium entry through NMDARs decreases the probability of microglial activation, and results in decreased release of proinflammatory factors from neurons. It should be noted, however, that memantine was infused concurrently with LPS; therefore, it was able to counteract the effects of LPS at the earliest stages of neuroinflammation. It remains to be determined whether memantine would be effective in reversing these effects at later stages of neuroinflammation. In addition, current studies in our laboratory aim to determine the extent to which memantine affects critical input pathways to the hippocampus (e.g. entorhinal cortex), that may also participate in LPS-induced inflammatory interactions present within internal hippocampal circuitry.

Overall, memantine treatment may confer some advantages over traditional anti-inflammatory drugs; unlike these traditional treatments, which suppress all microglia,
memantine may indirectly target the activation state without affecting the resident microglia. The present results suggest that pharmacological approaches directly targeting restoration of the normal neuron–glia dialogue may be useful in brain inflammatory conditions, which are neuro-pathogenic.

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APPENDIX

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2006.08.017.