Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission

Olivier Pascual[a,b,c,1,2], Sarrah Ben Achour[a,b,c,2], Philippe Rostaing[a,b,c], Antoine Triller[a,b,c], and Alain Bessis[a,b,c]

[a]Institut de Biologie de l'Ecole Normale Supérieure, F-75005 Paris, France; [b]Institut National de la Santé et de la Recherche Médicale U1024, F-75005 Paris, France; and [c]Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8197, F-75005 Paris, France

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Fine control of neuronal activity is crucial to rapidly adjust to subtle changes of the environment. This fine tuning was thought to be purely neuronal until the discovery that astrocytes are active players of synaptic transmission. In the adult hippocampus, microglia are the other major glial cell type. Microglia are highly dynamic and closely associated with neurons and astrocytes. They react rapidly to modifications of their environment and are able to release molecules known to control neuronal function and synaptic transmission. Therefore, microglia display functional features of synaptic partners, but their involvement in the regulation of synaptic transmission has not yet been addressed. We have used a combination of pharmacological approaches with electrophysiological analysis on acute hippocampal slices and ATP assays in purified cell cultures to show that activation of microglia induces a rapid increase of spontaneous excitatory postsynaptic currents. We found that this modulation is mediated by binding of ATP to P2Y1R located on astrocytes and is independent of TNFα or NOS2. Our data indicate that, on activation, microglia cells rapidly release small amounts of ATP, and astrocytes, in turn, amplified this release. Finally, P2Y1 stimulation of astrocytes increased excitatory postsynaptic current frequency through a metabotropic glutamate receptor 5-dependent mechanism. These results indicate that microglia are genuine regulators of neurotransmission and place microglia as upstream partners of astrocytes. Because pathological activation of microglia and alteration of neurotransmission are two early symptoms of most brain diseases, our work also provides a basis for understanding synaptic dysfunction in neuronal diseases.

inflammation | lipopolysaccharide | purine | toll-like receptor 4 | epilepsy

Fine control of neuronal activity is crucial to rapidly adjust to subtle changes of the environment. This fine tuning was thought to be purely neuronal until the discovery that glial cells could also modulate neuronal activity (1–3). Indeed, in addition to their supporting role, the function of astrocytes as active players of synaptic transmission is now widely accepted. Astrocytes express glutamate receptors and therefore, respond to glutamate that spills over from synapses. This spillover triggers the release of gliotransmitters (glutamate, D-Serine, or ATP) that can modulate neuronal activity, synaptic transmission, and plasticity (4–7).

Microglia is the other major glial cell type of the brain, and its role has mostly been characterized in pathologies (8, 9). Microglia display features compatible with the regulation of neuronal activity, although the mechanisms of this regulation remain to be determined. Under pathological conditions, activation of microglia is a common early feature of most brain diseases, which is the primary stage of inflammation, followed by synaptic alterations (10–12). It has also been shown that prenatal activation of microglia is sufficient to impact synaptic function in adulthood (13, 14). Under physiological conditions, microglial cells are present in all regions of the adult brain at rather high density (15). The density of microglia is comparable with the density of astrocytes, and both cell types are closely associated morphologically (16). Microglial cells are highly dynamic and react rapidly to the modification of their environment (17). They express membrane receptors for all known neurotransmitters (18) and thus, are putatively able to sense neuronal activity and/or communicate with astrocytes. In response to stimuli, microglia are activated, and they release neurotransmitters (19), which are small molecules such as nitric oxide, trophic factors, or cytokines, all known to control neuronal function and synaptic transmission (20, 21). In addition, changes in plasticity and neuronal activity have been shown to modify the resident time of microglia processes at synapses (22). Although long-term effects of microglial activation and inflammation have been studied (14, 23, 24), early consequences of such activation are still unknown, especially the cell type involved and the consequences on neuronal activity.

Here, we activated microglia with LPS, a proinflammatory molecule (25, 26), and monitored neuronal activity. The combination of pharmacology and electrophysiology in brain slices as well as ATP assays in purified cell cultures revealed that LPS-activated microglia releases ATP. This ATP stimulates astrocytes to release glutamate, which modulates neuronal activity through metabotropic glutamate receptors (mGluRs). Such mechanism may have important physiopathological relevance during most brain diseases.

Results

LPS Induces a Rapid Transient Increase in AMPA Excitatory Postsynaptic Current Frequency. To determine the short-term effect of microglial activation, we bath-applied LPS (500 ng/mL) to acute brain slices and monitored spontaneous activity of CA1 excitatory neurons by patch-clamp recordings in whole-cell configuration. The recording of excitatory neurotransmission was favored by the use of a low-chloride intrapipette solution and holding cells at −70 mV. Under these conditions, LPS application increased the frequency of spontaneous excitatory postsynaptic currents (EPSCs) within the first minutes (Fig. 1 *A* and *B*) by 43.6 ± 13.9% (n = 12 neurons) with no change in their amplitude (Fig. 1*B* and Fig. S1*A*). Kinetics analysis of LPS-induced currents (rise time, decay time, and duration) indicates that the increased EPSC frequency is not due to a new type of current that would account for the direct action of microglia on synapses (Fig. S1*B*). This EPSC frequency increase was transient, lasting for about 10 min, but could be reproduced after a 15-min washout.

To test if the effect of LPS on excitability was local, we blocked the action potentials using the sodium channel blocker tetrodotoxin (TTX; 1 μM). In the presence of TTX, LPS still induced an increase in miniature EPSC (45.8 ± 11.7%; n = 6), indicating that this increase was independent of a network effect (Fig. 1*C*). We next identified the neurotransmitter receptors involved in this response using pharmacology. When AMPA transmission was

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[1]To whom correspondence should be addressed. E-mail: olivier.pascual@biologie.ens.fr.

[2]O.P. and S.B.A. contributed equally to this work.

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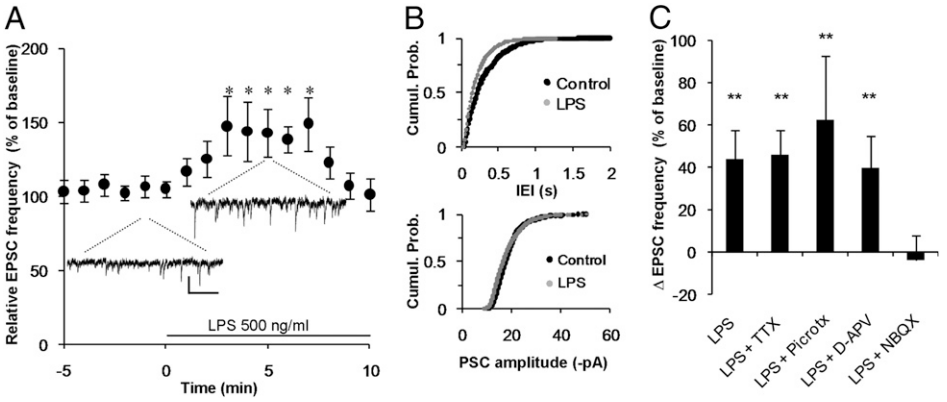

Fig. 1. LPS increases EPSC frequency at AMPAergic synapses. (*A*) Relative EPSC frequency on acute hippocampal slices was immediately and transiently increased after LPS application (500 ng/mL, $n = 12$, mean ± SEM, paired *t* test, $*P < 0.05$). *Insets* show representative traces before and after LPS application. (Scale bar: 100 ms, 10 pA.) (*B*) Representative cumulative probability plots for interevent interval (IEI) and peak amplitude before (black circles) and after (gray circles) application of LPS; 10 of 12 cells responded by a significant increase in frequency (KS test < 0.05). (*C*) The LPS-induced increase of EPSC frequency is not affected by the application of TTX ($n = 6$), picrotoxin (Picrotx; $n = 11$), or D-APV ($n = 14$), but it is prevented by the AMPAR antagonist NBQX ($n = 7$, mean ± SEM, *t* test, $**P < 0.01$).

blocked by 50 μM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), LPS application failed to increase the frequency of the remaining spontaneous PSCs (Fig. 1*C* and Fig. S1*C*). In contrast, neither NMDA receptor antagonist D-2-amino-5-phosphonovalerate (D-APV; 50 μM) nor $GABA_A$ receptor antagonist picrotoxin (100 μM) prevented the LPS-mediated increase in EPSC frequency (Fig. 1*C*). Together, these results suggest that the LPS-mediated increase in EPSC frequency involved AMPAergic transmission.

Microglial Activation Modulates Neuronal Activity. LPS is a widely used proinflammatory agent and is known to activate microglia. However, because LPS could potentially activate other cell types, we determined if microglia are necessary to achieve the LPS-mediated increase in EPSC frequency. To functionally show the involvement of microglia in the regulation of EPSCs, we assessed the effect of LPS application in brain slices from mice deficient in the myeloid-specific transcription factor Pu-1 that lack microglia (27) (Fig. 2*A*). Because Pu-1–deficient mice die at birth from septicemia (27), we cultured organotypic slices from Pu-$1^{-/-}$ or control WT brain neonates. In slices cultured for 10–14 d, neuronal activity recorded from Pu-$1^{-/-}$ slices was not significantly different from WT slices (1.15 ± 0.23 Hz vs. 1.17 ± 0.34 Hz, respectively). However, in brain slices from Pu-$1^{-/-}$ mice, LPS application did not increase EPSC frequency ($n = 8$) compared with WT slices ($n = 9$) (Fig. 2*B*). This finding shows that microglia is necessary to achieve the LPS-mediated modulation of EPSC frequency. Since we cannot rule out that the lack of response to LPS could be due to developmental alterations secondary to the *Pu-1* mutation, we also acutely blocked microglial activation in WT acute juvenile hippocampal slices. We used minocyclin, an antiinflammatory agent that exerts its action by primarily preventing microglial activation (28, 29). We found that minocycline (50 nM) did not significantly change the basal EPSC frequency (Fig. S1*C*) but prevented the neuronal response to LPS application ($n = 5$) (Fig. 2*C*). This finding supports the hypothesis that the LPS-mediated regulation of EPSC occurs through the activation of microglia. LPS is a well-established ligand of toll-like receptor 4 (TLR4) (30), and we found that LPS application induced an EPSC response within minutes. However, in nervous system, TLR4-mediated responses have been reported to occur several hours after ligand application (31). This finding raised the possibility that the LPS-induced modulation of EPSC could be TLR4-independent. We, thus, tested whether the LPS-mediated EPSC increase was caused by TLR4 signaling. To this aim, we applied LPS on slices from TLR$4^{-/-}$ KO mice (32). LPS application to TLR$4^{-/-}$ slices failed to increase the EPSC frequency ($n = 6$) (Fig. 2*C*), although neuronal activity recorded from mutant was not significantly different from activity from WT mice (1.8 ± 0.4 Hz vs. 1.7 ± 0.4 Hz, respectively). This finding shows that LPS acts through TLR4 to modulate the frequency of EPSCs.

Together, these experiments show that microglia are required for LPS to modulate the frequency of AMPAergic EPSCs through the TLR4 pathway and support a model in which microglial activation is the primary step of this modulation.

LPS-Mediated Frequency Increase Involves Purines. Microglia cells release numerous signaling factors, among which TNF-α, NO, and ATP have been described to increase the frequency of EPSC (33–35). We tested the involvement of these molecules in the LPS-mediated modulation of EPSC. First, TNF-α is one of the earliest chemokines released by microglia after activation (31). Glial TNF-α has been shown to rapidly increase EPSC frequency at AMPAergic synapses with no associated change in amplitude (33) and be necessary for astrocytic modulation of neuronal activity (36). We tested LPS application on slices from TNF-α KO mice (37). In these KO mice, basal spontaneous EPSC frequency was 1.8 ± 0.3 Hz. In six of eight neurons [Kolmogoroph–Simirnov (KS) test < 0.05], LPS induced a significant increase in EPSC frequency (*t* test; $P < 0.01$; $n = 8$) that was not significantly different from the one recorded in WT mice (Fig. 3*A*). Second, an increase of NOS2 has been reported to increase the frequency of EPSC in the neocortex (38). Because NOS2 is up-regulated during inflammation, we tested LPS application on slices from NOS2$^{-/-}$ mice. We found in NOS2$^{-/-}$ slices ($n = 11$) no significant difference in the LPS response compared with WT (Fig. S2*A*), ruling out an involvement of NO from NOS2. Finally, ATP has been shown to modulate the frequency of EPSC without affecting their amplitudes (34). We test the involvement of ATP in LPS-mediated modulation of EPSC by applying LPS in the presence of the broad spectrum purinergic antagonists Reactive Blue-2 (RB-2; 2 μM) or pyridoxalphosphate-6-azophenyl-20,40-disulphonic acid (PPADS; 50 μM). Both antagonists reduced basal frequency (Fig. S1*C*) and blocked the LPS-mediated increase in EPSC frequency (Fig. 3*B*). These data suggest that purinergic receptors are necessary for the modulation of synaptic activity by microglial activation.

RB-2 and PPADS are broad spectrum antagonists of purinergic receptors with partially overlapping targets. P2Y1 receptor (P2Y1R) is one of the few receptors blocked by both antagonists. We, therefore, investigated the involvement of P2Y1R in the

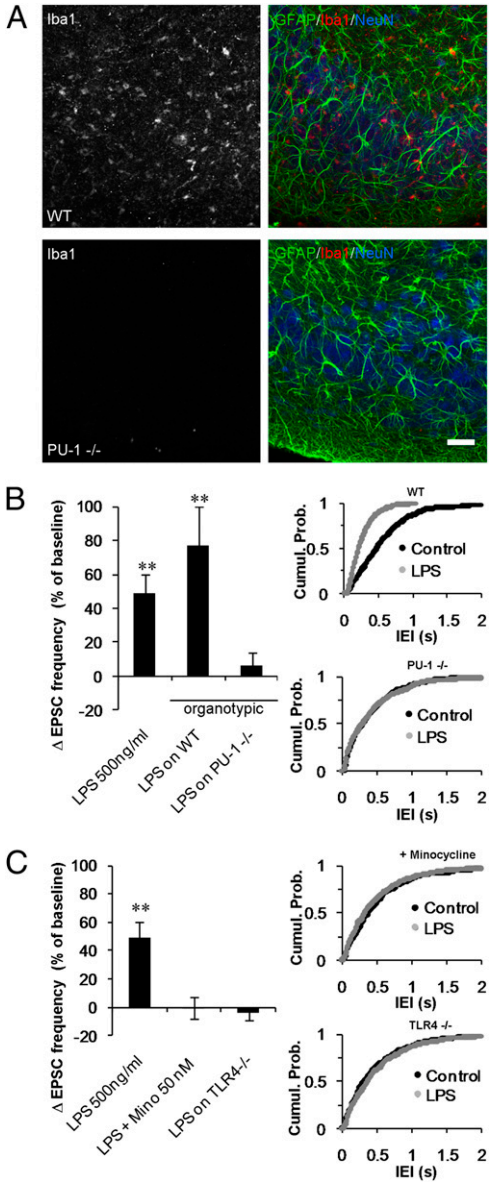

Fig. 2. LPS-induced EPSC frequency increase is mediated by the TLR4 pathway and requires microglia. (*A*) Triple immunostainings showing the expression of Iba1 (red in *Right*), a microglial marker, GFAP (green in *Right*), an astrocyte marker, and NeuN (blue in *Right*), a neuronal marker in organotypic slices from WT (*Upper*) or Pu-1$^{-/-}$ mice (*Lower*). (Scale bar: 50 μm.) (*B, Left*) Histogram representing the change in EPSC frequency on LPS application on WT acute slices ($n = 12$) and WT organotypic slices ($n = 8$) and the absence of response in Pu-1 ($n = 9$) organotypic slice culture (mean ± SEM, *t* test, $^{**}P < 0.01$). (*B, Right*) Corresponding representative cumulative probability plots for IEI before (black circles) and after (gray circles) application of LPS in WT (*Upper Right*) and Pu1$^{-/-}$ (*Lower Right*). In WT slices, seven of nine cells responded to LPS (KS test; $P < 0.01$). Histogram in *C Left* shows that the response to LPS is blocked by minocycline and absent in slices from mice lacking TLR4 (TLR4$^{-/-}$; mean ± SEM, *t* test, $^{**}P < 0.01$). (*C Right*) Corresponding representative probability plots for IEI before (black circles) and after (gray circles) application of LPS in the presence of minocyclin (*Upper Right*) and TLR4$^{-/-}$ (*Lower Right*).

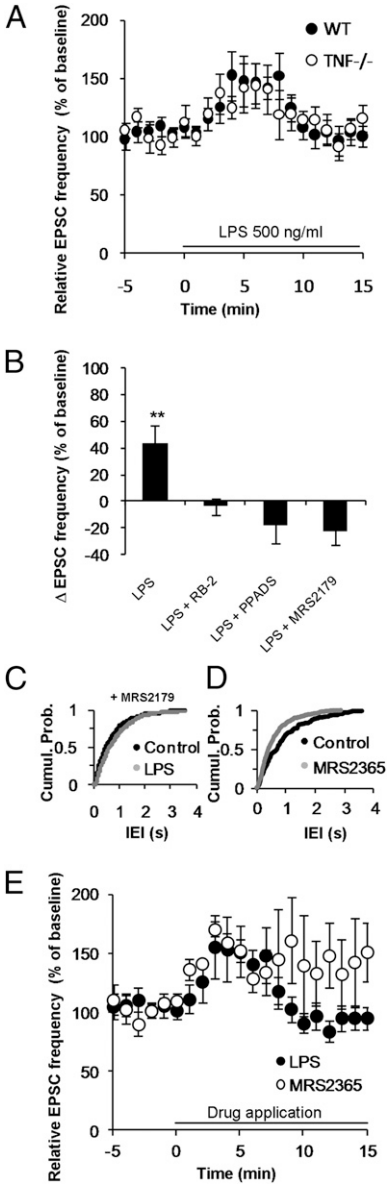

Fig. 3. LPS-induced EPSC frequency increase involves purines acting on P2Y1. (*A*) TNF-α signaling is not involved in the microglial modulation of EPSC frequency, because the LPS response was not significantly different in slices from TNF-α (open circles) -deficient mice compared with WT mice (black circles). (*B*) The LPS-mediated EPSC frequency increase was blocked by broad spectrum purinergic antagonists RB-2 ($n = 7$) and PPADS ($n = 7$) and the P2Y1-specific antagonist MRS2179 ($n = 5$, mean ± SEM, *t* test, $^{**}P < 0.01$). (*C*) Representative cumulative probability plots for IEI in the presence of MRS2179 before (black circles) and after (gray circles) LPS application. MRS2179 blocked the response to LPS in five of five recorded cells. (*D*) Representative cumulative probability plots for IEI before (black circles) and after (gray circles) MRS2365 application; seven of nine cells responded by an increased frequency in the presence of MRS2365 (KS test < 0.01). (*E*) The P2Y1 agonist MRS2365 (open circles) mimics the effect of LPS (black circles) on EPSC frequency ($n = 9$, mean ± SEM).

LPS-mediated modulation of EPSC using the specific antagonist MRS2179 (39). EPSC frequency increase in response to LPS application was completely abolished in the presence of the P2Y1R antagonist MRS2179 (30 μM; $n = 5$) (Fig. 3 *B* and *C*). Conversely, the specific P2Y1R agonist MRS2365 (1 μM) (40) mimicked the LPS response and induced a rapid increase in the frequency of EPSCs by 49.8 ± 17.5% ($n = 9$) (Fig. 3 *D* and *E*) without affecting their amplitudes (Fig. S2*B*). Noteworthy, the effect of the P2Y1R agonist occluded the effect of LPS, because the increase in EPSC frequency observed on application of MRS2365 was not synergized by application of LPS ($n = 10$) (Fig. S2*C*). This finding indicates that the LPS-mediated modulation of EPSC occurs through the P2Y1 regulatory pathway.

Together, these experiments show that the rapid communication from microglia to neurons after stimulation by LPS is mediated by purines through P2Y1R.

P2Y1R Located on Astrocytes Acts Downstream of Microglial ATP to Modulate EPSC Frequency. In the hippocampus, P2Y1Rs are only expressed by astrocytes (Fig. S2*D*) and interneurons (41–43). The effect of microglia on excitatory neurotransmission should, therefore, be mediated by astrocytes and/or interneurons. We previously showed that blocking $GABA_AR$ with picrotoxine did not prevent the LPS-mediated modulation of EPSC, suggesting that interneurons are not necessary. We also tested the involvement of interneurons by recording the inhibitory PSCs (IPSCs) using a chloride-based internal solution to visualize inward $GABA_A$ currents when neurons were held at −70 mV. Recordings were made in the presence of NBQX and D-APV to isolate GABAergic currents. Under these conditions, basal frequency of IPSC was 9.9 ± 0.5 Hz, and LPS application had no effect on the frequency of $GABA_A$ currents (Fig. 4*A*) in five of five cells (not significant; KS test > 0.24). This result also confirmed that interneurons are not involved in the microglial-induced increase in PSC frequency and allowed us to hypothesize that astrocytes are cellular intermediates in this regulation.

To functionally show that astrocytes are involved in the regulation of AMPAergic EPSC frequency by microglial activation, we first tested the effect of LPS application when astrocytic function was impaired. We used the glial metabolic blocker fluoroacetate (FAC) at 1 mM for 30 min. These conditions are moderate compared with other studies (44, 45) and are most probably specific within the time range of our experiments. FAC treatment did not change the basal neuronal activity (1.3 ± 0.3 Hz; $n = 7$) (Fig. S2 *E* and *F*) but blocked the LPS-induced increase in EPSC frequency (Fig. 4*B*). The lack of LPS effect in the presence of FAC was not because of a direct alteration of neuronal functions or metabolism, because application of KCl (10 mM) in the presence of FAC still induced an increase in EPSC frequency (Fig. S2*F*). This finding suggests that astrocytes are necessary for LPS-mediated modulation of EPSC.

The above-described experiments show that both microglia and astrocytes are necessary for the LPS-mediated modulation of EPSC and favor a model in which microglia act upstream of astrocytes. However, an action of microglia downstream of astrocytes cannot be ruled out. To further investigate the role of astrocytes, we reasoned that, if P2Y1R from astrocytes is signaling downstream of microglia, then we should be able to mimic the effect of LPS application by stimulating P2Y1R even in the absence of microglia. To test this hypothesis, we applied the purinergic agonist MRS2365 onto organotypic slices cultured from Pu-$1^{-/-}$ or WT neonates and monitored the EPSCs. We found that, in Pu-$1^{-/-}$ slices, MRS2365 induced a significant increase in EPSC frequency comparable with the increase observed on application of LPS (Fig. 4*C*). This finding shows that purinergic signaling onto astrocytes acts downstream of microglial activation to regulate EPSCs.

LPS Binds to TLR4 Expressed by Microglia and Induces the Release of ATP. Our data suggest that astrocytes activity is triggered by activation of microglia. This finding implies that microglia stimulated by LPS can rapidly produce ATP. To unambiguously identify the source of ATP and because there is no accurate method to identify ATP releasing cells in living tissue, we used primary cultures stimulated with LPS. In healthy brain, TLR4 mRNA has only been detected in microglia (46, 47) (Fig. S3), but a few studies have described that cultured astrocytes express TLR4 and/or respond to LPS (48, 49). In most cases, however, astrocytes cultures are contaminated with microglia (50), and the presence of microglia was rarely tested. Because contamination of astrocytes by microglia could bias the results, we first designed a protocol to obtain microglia-free astrocytes cultures. Pure astrocytes were obtained by pretreating the cultures with L-leucine-methyl esther (LME). This drug is selectively toxic to microglia (51, 52) and has already been used to obtain microglia-free cultures of astrocytes (53). The purity of the microglial and astrocyte cultures was assessed by quantitative RT-PCR (qRT-PCR) (Fig. 5*A*), immunohistochemistry, and Western blot (Fig. S4 *A* and *B*). This assessment allowed us to confirm that nontreated astrocytes cultures consistently contained microglia (Fig. S4*C*). Next, using these purified cultures, we assessed the expression of TLR4 and showed (using qRT-PCR) that mRNAs encoding for TLR4 and its associated protein CD14 were only detected in microglial cells and not in LME-treated microglia-free astrocyte cultures (Fig. 5*B*). We also showed that, in line with the expression profile, the binding of Alexa-568–tagged LPS was restricted to microglia (Fig. 5*C*).

Finally, we measured the production of ATP by microglia or astrocytes on application of LPS using a luciferin/luciferase luminescent assay. As shown in Fig. 5*D*, microglia released significant amounts of ATP 5 min after application of LPS when they were plated at high concentrations (10^6 microglia/mL) (Fig. S4*D*). On the contrary, pure LME-treated astrocytes failed to elicit an increase of ATP concentration (Fig. 5*E*), although they were confluent. This finding shows that, on application of LPS, the primary source of ATP is microglia. Remarkably, we found that low concentrations of microglia, which did not allow ATP detection on application of LPS, responded to LPS by a rapid and strong increase in ATP concentration when mixed with pure LME-treated confluent astrocytes (Fig. 5*E*). This latest result indicates that the default of ATP release by pure astrocyte is not caused by the treatment with LME and that LPS-mediated response is caused by

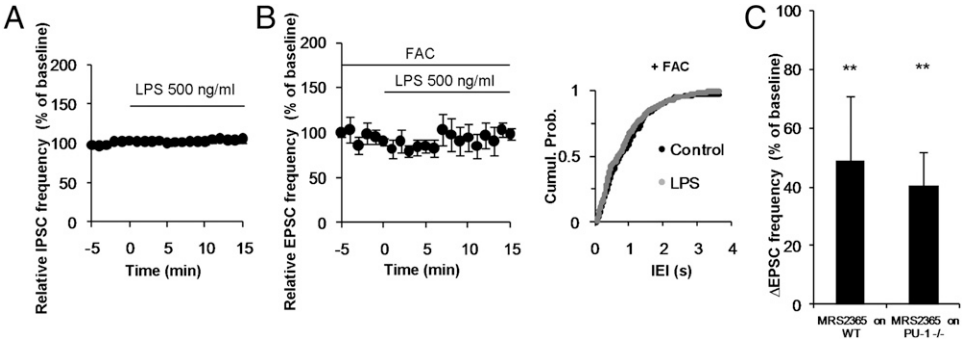

Fig. 4. Astrocytes mediate the microglial modulation of EPSC frequency. (*A*) LPS application did not increase the frequency of spontaneous IPSC ($n = 5$, mean ± SEM). (*B Left*) The LPS-mediated EPSC frequency increase was prevented by treatment with FAC (1 mM, $n = 7$, mean ± SEM). (*B Right*) Representative cumulative probability plots for IEI before (black circles) and after (gray circles) application of LPS in the presence of FAC (1 mM). (*C*) In organotypic slices cultured from Pu-1 mice, P2Y1 agonist (MRS2365) application induced an increase of EPSC frequency ($n = 9$) similar to the increase in WT ($n = 8$, mean ± SEM, *t* test, $^{**}P < 0.01$).

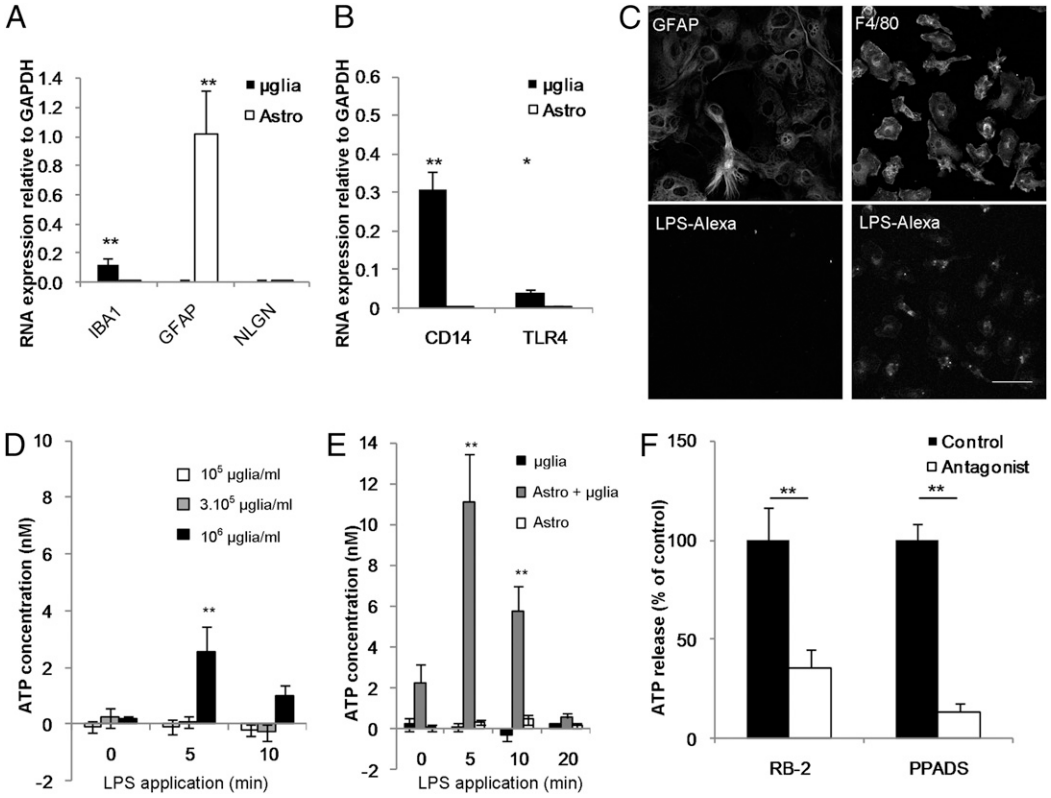

Fig. 5. Microglia activation by LPS induces the release of ATP that recruits astrocytes. (*A*) RNA expression levels relative to GAPDH were used to test the purity of the cultures of astrocytes (white bars) and microglia (black bars). GFAP, Iba1, and Neuroligin 1 transcripts were used as markers of astrocytes, microglia, and neuron, respectively (84). (*B*) TLR4 and CD14 mRNA are only detected in microglia by qRT-PCR using pure cultures of microglia (black bars) and microglia-free culture of astrocytes (white bars, $n = 3$ cultures, mean ± SEM, ANOVA, $^*P < 0.05$, $^{**}P < 0.01$ compared with Nlgn1). (*C*) Cultured astrocytes identified by GFAP immunostaining (*Upper Left*) do not bind Alexa-568–tagged LPS (*Lower Left*). In contrast, Alexa-568 LPS (*Lower Right*) colocalized with cultured microglia identified by F4/80 immunostaining (*Upper Right*). (*D*) LPS application on pure microglial cultures induces the production of ATP when microglia were plated at high concentration (mean ± SEM, ANOVA, $^{**}P < 0.01$, $n = 6$ cultures). (*E*) LPS application to pure confluent astrocytes (Astro; white bars) or pure microglia at low concentration (μglia; 3×10^5 cells/mL; black bars) does not induce the production of ATP. LPS application to mixed cultures containing microglia at a low concentration added to confluent LME-treated astrocyte cultures (Astro + μglia; gray bars) induced the production of ATP ($n = 6$, average ± SEM). (*F*) LPS-mediated ATP release in mixed cultures (black bars) was prevented by RB-2 ($n = 12$ wells, six cultures) or PPADS ($n = 10$ wells, five cultures, mean ± SEM, $^{**}P < 0.01$; white bars).

a synergistic effect of microglia and astrocytes. Similar to the neuronal response to LPS application, the increased ATP production by mixed cultures on LPS application was transient, and ATP levels in the extracellular solution returned to baseline after 20 min. The release of ATP induced by LPS in this mixed cultures was significantly reduced by the presence of PPADS or RB-2 (Fig. 5*F*), confirming that the communication between microglia and astrocytes was mediated by purinergic receptors. These results suggest that, in response to LPS, microglia rapidly release small amounts of ATP that recruit astrocytes through purinergic receptors. Astrocytes could, in turn, amplify the signal through an ATP-induced ATP release (54), recruiting neighboring astrocytes.

Astrocytes Recruited by Microglia Increase CA1 EPSC Frequency Through mGluR Activation. We next characterized the mechanism linking astrocytes to neurons on microglial activation. Activation of astrocytic P2Y1R has been shown to trigger the release of glutamate (41, 55). In the hippocampal stratum radiatum, the astrocytic glutamate is known to bind to neuronal receptors such as postsynaptic NMDA receptors to generate slow inward currents (SICs) (4, 5) or mGluR type 1 receptors to modulate AMPAergic synapses (6, 7). We have shown earlier that NMDA receptors are not involved in the response to LPS (Fig. 1*C*), and in agreement with previous work showing that P2Y1R activation does not trigger SICs (56), we did not find a significant increase in SIC frequency. We, therefore, focused our attention on metabotropic glutamate receptors. Application of the mGluR5 antagonist MPEP (100 μM) abolished the effect of LPS application (Fig. 6*A*). This finding implicates mGluR5 in the astrocyte-mediated regulation of neuronal activity by microglial activation. In this model, purinergic stimulation of astrocytes activates downstream neuronal mGluR to enhance the EPSC frequency. To validate this hypothesis, we applied MPEP before the application of P2Y1 agonist MRS2365. We found that the mGluR5 antagonist MPEP totally prevented the P2Y1-induced EPSC frequency increase (Fig. 6*A*), confirming that mGluR signaling acts downstream of P2Y1 receptors, probably on neurons as previously shown (6, 7).

Finally, we used EM to assess the presence of mGluR5 in synapses enwrapped by astrocyte processes. Astrocytes were identified by a preembedding staining for the astrocytes-specific Glutamine Synthase-6 (57), and mGluR5 immunoreactivity was then revealed using immunogold particles. When we analyzed mGluR5 immunoreactivity on synapses next to astrocyte processes (100 synapses), we found that mGluR5 had no favored localization and could be found in both pre- (36%) and postsynaptic (56%) neuronal membranes processes (Fig. 6*B*). Hence, this EM study further supports a glutamate release by astrocyte that would act on neuronal mGluR5 receptors.

Microglial-Induced EPSC Frequency Increase Is Sufficient to Promote Bursting Activity in Epilepsy Model. The above-described results show that microglial activation transiently enhances the frequency

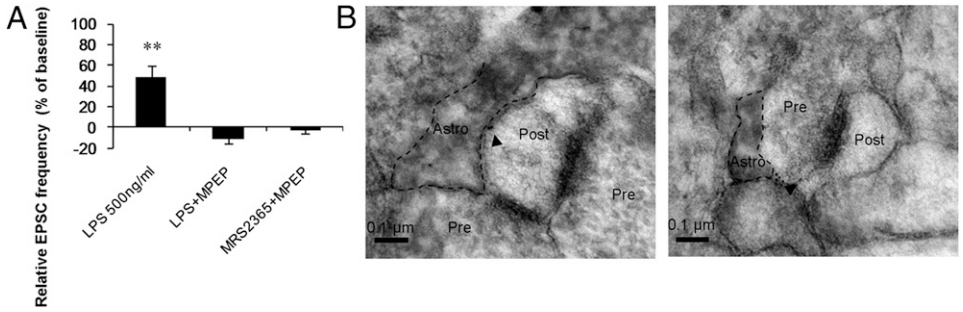

Fig. 6. Microglial activation triggers glutamate release from astrocytes that acts on neuronal mGluRs. (*A*) Application of LPS in the presence of the mGluR5 antagonist MPEP prevented the EPSC frequency increase (n = 9, average ± SEM). Application of MPEP prevented the EPSC frequency increase by the P2Y1 agonist MRS2365 (n = 8, mean ± SEM). (*B*) Electron micrograph showing mGluR5 immunoreactivity (arrowheads; 10-nm gold particles) located on the postsynaptic (post) membrane (*Left*) and the presynaptic (pre) membrane (*Right*) juxtaposed to astrocytic processes (astro) identified by Glutamine Synthetase immunoreactivity (DAB accumulation) and outlined by dashed lines.

of excitatory activity. This finding raises the question of the biological relevance of such regulation. Therefore, we determined if this additional excitatory input resulting from microglial activation could impact the whole synaptic network. In particular, microglial activation is consistently observed during epilepsy (58), and we explored if such activation could promote seizures. Under normal conditions, the microglial-induced increase in EPSC frequency was never associated with the occurrence of bursting activity in slices, indicating that the inhibitory drive was sufficient to cope with microglia-mediated excitation. However, when the generation of seizures was facilitated by the presence of extracellular medium containing low Mg^{2+} and picrotoxin (100 µM) (Fig. 7*A*), we found that application of LPS frequently induced bursting activity corresponding to seizure-like activity (Fig. 7*B*). This finding shows that, when the balance between excitation and inhibition is impaired as in genetic models of epilepsy (59, 60), microglial modulation of synaptic activity could be sufficient to increase the probability of epileptiform waves.

Discussion

We now report that activation of microglia, which is the earliest step of inflammation, can rapidly modulate the excitatory neurotransmission. The data that we have described support a model in which activation of microglia induces a rapid production of ATP. Microglial ATP then recruits astrocytes that amplify the ATP production and release glutamate. The astrocytic glutamate then increases the EPSC frequency through neuronal mGluR5.

LPS Specifically Activates Microglia. We used LPS as a specific tool to activate microglia. It has been proposed that LPS can directly bind and activate nonmicroglial cell types. However, the specificity of LPS binding to microglia in the adult brain is supported by our data and the literature. First, we confirmed that TLR4, the LPS receptor, is only expressed by microglia (46, 61) using pure microglia cultures and binding experiments. A transient expression of TLR4 has been reported in cultured embryonic cortical neurons (62), but this expression is debated (63–65). The expression of TLR4 has occasionally been detected in astrocytes and neurons under pathological conditions (62, 66). However, TLR4 has never been detected in healthy adult hippocampal neurons or astrocytes (46, 66–69). In addition, a direct effect of LPS on neurons was functionally excluded by the fact that LPS had no effect on brain slices from Pu-$1^{-/-}$ animals lacking microglia. The expression of TLR4 has also been detected in cultured astrocytes (48, 49). However, we found that astrocyte cultures are consistently contaminated by microglia (Fig. S4*C*) (50), and in pure cultures, TLR4 and its accessory protein CD14 were not expressed by astrocytes (61). Furthermore, data mining of Gene Expression Omnibus DNA array experiments performed on purified cells confirmed that TLR4 and CD14 are not expressed by adult astrocytes, oli-

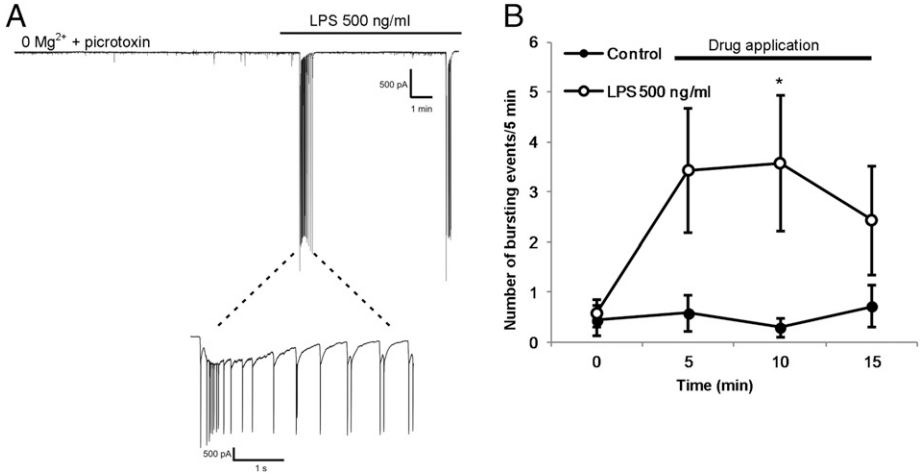

Fig. 7. Microglial activation exacerbates seizure activity. (*A*) Representative trace showing that microglial activation enhances the epileptiform bursting activity when neurons were recorded in medium with picrotoxin and low Mg2+. (*B*) Plots of the number of bursting events per 5 min for each cell (n = 7) before and after application of saline (black circles) or LPS (open circles; average bursting activity in 5 min ± SEM, ANOVA, *$P < 0.05$).

godendrocytes, or endothelial cells. Finally, the specific action of LPS on microglia is functionally supported by our in vitro assay showing that pure astrocytes cannot produce ATP on LPS application and our experiments showing that LPS application on brain slices lacking microglia has no effect on EPSCs. Noteworthy, acute brain slices were used to monitor the effect of microglial activation on neuronal activity. The activation state of microglia in such slices and on LPS application may somehow differ from in vivo conditions. Therefore, we cannot exclude that some aspects of the mechanisms that we have now described might be different in vivo. However, in these slices, microglia cells express low levels of the inflammatory marker CD11b (Fig. S4*E*), which are similar to the levels expressed in vivo. This finding suggests that microglia cells are not basally activated. Acute brain slices are, therefore, relevant tools to study the impact of microglial activation.

Microglial Activation Recruits Astrocytes by Purinergic Signaling. We have revealed that microglial ATP is the primary messenger of the LPS-mediated modulation of EPSC. Using mutant mice, we ruled out the involvement of TNF-α and NO, which are major intermediates of inflammation. This finding shows that the regulatory pathway involved in the LPS-mediated regulation of EPSC is independent of those pathways recently described by TNFα-dependent modulation of astrocytic glutamate release (36). LPS induced a transient increase in the frequency of EPSC. We found that, on LPS application, the time course of EPSC increase paralleled the time course of ATP release by microglia. This finding suggests that the transient EPSC response could be because of the depletion of a releasable pool of ATP. However, TLR4 endocytosis after binding to LPS cannot be excluded. In the extracellular space, ATP is rapidly degraded by ectonucleotidase (70). This finding implies that it must be released close to its receptors. However, the affinity of P2Y1R is higher for ADP than for ATP (71), allowing for a wider range of efficiency of ATP released from microglia. Microglia display highly ramified processes that are tightly interwoven with astrocytic processes (16). Astrocytes are, therefore, good candidates as intermediates between microglial ATP and synaptic regulation. Our functional observations also support such a role of intermediates. In the hippocampus, P2Y1Rs are only detected on astrocytes and interneurons (42, 72), but we functionally ruled out any contribution of interneurons. In addition, activation of P2Y1R in microglia-deficient brain slices from a Pu-$1^{-/-}$ mouse mimicked the effect of microglial activation on EPSC. Therefore, astrocytes are likely to act downstream of microglial activation to regulate EPSC. Such downstream action of astrocytes is also supported by the fact that the synaptic response to microglial activation was abolished by FAC application. FAC is incorporated into astrocytes (73) in which it blocks the production of glutamate (74). It has been shown that incubation of astrocytes with up to 25 mM FAC for 3 h induced no reduction of astrocyte ATP levels (75). Finally, FAC has been successfully used to specifically abolish astrocytes to neuron communication (44, 56, 76). We also revealed that microglial production of ATP on activation is amplified by astrocytes. Altogether, our results support a model in which microglia produces small amounts of ATP that bind to P2Y1R on astrocytes. This microglial ATP induces the production of ATP by astrocytes that recruit other astrocytes to produce more ATP. A degradation of ATP into adenosine probably also occurs, thereby decreasing the excitatory effect of LPS by acting on presynaptic A1 receptors (77). The negative effect of adenosine might explain the decreased frequency of sEPSC recorded on application of the P2Y1 antagonists (PPADS, MRS2179) and MPEP.

In addition to promoting ATP release, activation of astrocytes by P2Y1R is known to trigger glutamate exocytosis (41). In the CA1 region of the hippocampus, glutamate released by astrocytes facilitates the release of neurotransmitter by activation of group I mGluR presumably located on presynaptic neurons (6, 7). We have now shown that the mGluR5 antagonist MPEP abolishes the LPS-induced increase in EPSC frequency. This finding supports the notion that astrocytes release glutamate and facilitate synaptic transmission through metabotropic glutamate receptors on stimulation by microglial ATP.

Microglial Activation Enhances Excitatory Network. Microglial activation is the primary stage of brain inflammation. Activation of microglia and astrocytes is a prominent feature of temporal lobe epilepsy and most animal models of recurrent seizures (78). Inflammation is even thought to be causal of epileptic seizure. For instance, injection of LPS into cortex produces focal epileptiform discharges within 5 min after stimulation (79). Fever, a symptom of inflammation, is known to promote febrile seizures in infants or mice with appropriate genetic backgrounds (80). Our work now provides the basis to understand the molecular and cellular cascades by which inflammation triggers seizures. More generally, inflammation and synaptic dysfunctions are two early symptoms of most, if not all, neurological diseases, and for that reason, this signaling might be relevant in many other pathologies. Finally, the role of microglial activation could not be restricted to pathologies. Actually, an increasing number of endogenous TLR ligands have now been described (81), and we speculate that the mechanisms that we have now deciphered will be relevant during physiological situations.

Materials and Methods

The experimental procedures have been approved by the Charles Darwin Committee for Animal Experimentation (Ce5/2010/066 and Ce5/2010/029).

Electrophysiology. Hippocampal slices from 15- to 21-d-old C57Bl6/J mice were cut to 400-μm thickness using a vibratome (Leica). The patch pipette used for recordings had a 4–5 MΩ resistance when filled with the following internal solution: 115 mM $CsMeSO_3$, 20 mM CsCl, 10 mM Hepes, 0.1 mM EGTA, 4 mM Mg-ATP, 0.4 mM Na_3-GTP, and 10 mM Na-phosphocreatine. To record inhibitory postsynaptic currents, we used an intrapipette solution containing 135 mM CsCl, 10 mM Hepes, 0.1 mM EGTA, 4 mM MgATP, 0.4 mM Na_3GTP, and 10 mM Na-phosphocreatine. The extracellular perfusion medium contained 124 mM NaCl, 3.1 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose and was saturated with 95% O_2 and 5% CO_2. Throughout the experiment, cells were held at −70 mV; the access resistance (16.8 ± 0.2 Mohm) was periodically tested, and if the access resistance changed by more than 10% or was greater than 20 MΩ at the beginning of the recording, the cells were discarded. Whole-cell patch-clamp recordings were performed with a Multiclamp 700B amplifier. The signal was filtered at 2–5 kHz and was acquired at 5–10 kHz. Drugs were bath-applied.

Organotypic Slice Cultures. PU-1–$GFP^{-/-}$ mice (82) were backcrossed 12–13 times, and C57Bl6 and C57Bl6 were used as controls. Homozygous mice die at birth. For this reason, organotypic hippocampal slice cultures were prepared from postnatal day 0 neonates. The hippocampi were dissected, and transversal slices (400 μm) were obtained using a McIllwain tissue chopper (Mickle Laboratory) and cultured according to the protocol developed by Stoppini et al. (83). Slices were placed on Millicell-CM inserts (Millipore) and maintained in slice culture medium for 10–14 d. The culture medium was made of 10.63 g/L MEM (50-019-PC; Cellgro) reconstituted in milliQ water, 20% heat-inactivated horse serum, 1 mM L-glutamine, 1 mM CaCl2, 2 mM $MgSO_4$, 1 mg/L insulin (I5500; Sigma-Aldrich), 61 μM ascorbic acid (A4034; Sigma-Aldrich), 11 mM D-glucose, 5 mM NaHCO3, and 30 mM Hepes (223–778; Boehringer). The pH was adjusted to 7.27. In all experiments, slice cultures were maintained in a CO_2 incubator at 36 °C. Electrophysiological recordings were performed using extracellular and intrapipette solutions identical to experiments performed in acute slices.

Analysis of Synaptic Currents. Recording analysis was performed using clampfit 10.0 (Molecular Device). The template chosen for each current event consisted of at least 20 chosen events. Only single-peak events were accepted during subsequent visual control. Number of events, amplitude, rise time, decay time, and interevent interval were extracted using clampfit 10.0 (Molecular Device). Frequency analysis was done by calculating the number of events during a 60-s period. Spontaneous frequency of PSC was found to be highly variable between cells. For this reason, we calculated the relative

frequency for each cell. The baseline frequency was calculated by averaging the frequencies within the 5 min before LPS application. Relative frequency was then expressed as a percentage of this baseline. In histograms, each bar represents the average relative difference 3 min before LPS application vs. 3 min during LPS application. Interevent intervals and amplitudes were compared 3 min before application of LPS and 3 min during LPS application on individual cells using normalized cumulative probability with a binning of 10 and the nonparametric KS two-sample test. Each group contained more than 60 events.

Cell Cultures. Mixed microglia–astrocyte primary cultures were prepared from the cortices of newborn C57Bl6 mice (postnatal day 1) as described (14). Briefly, tissue was dissociated by titration using a Pasteur pipette, and cells were seeded in DMEM containing 10% heat-inactivated FBS (BioWest) on culture dishes or glass coverslips coated with poly-DL-ornithine hydrobromide (Sigma) and placed in a humidified incubator at 37 °C with 5% CO_2. Medium was changed at days 1 and 3, and cells were used between 14 and 21 d in culture. Astrocytes were grown for about 7 d to reach confluence, and microglia was collected after about 10 d. Pure astrocytes were obtained by treating the mixed cultures with cytosine arabinoside (5 μM) and LME (75 mM). Pure microglial cells were collected from mixed microglia–astrocytes cultures by shaking. Controlled mixed cultures were obtained by seeding freshly collected microglia onto LME-treated pure confluent astrocytes (cocultures microglia–astrocyte). Cocultures or pure cultures were maintained for 24 h in DMEM + 10% FBS before ATP measurement, immunocytochemistry, or RNA and protein isolation.

ATP Measurements. ATP release was measured by luminometry. During the assay, the cells were maintained at 36 °C and 5% CO_2, and ATP concentration was monitored before and after 0, 5, and 10 min of LPS treatment (50 ng/mL) using luciferin–luciferase assay (ATPlite kit; Perkin-Elmer) and a luminometer (Berthold). Each sample was run in triplicate. Absolute quantities were obtained from an ATP standard. The responses to LPS were compared with control wells to which culture medium was added instead of LPS.

Statistics. Data were analyzed using R and expressed as means ± SEM when appropriate. Statistical differences were determined using Student *t* test or ANOVA for multiple comparisons (Scheffé test). Differences were considered statistically significant at $P < 0.05$.

Additional information concerning Western blots, qRT-PCR, immunohistochemistry, and EM is presented in *SI Materials and Methods*.

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