Presynaptic L-Type Ca^{2+} Channels Increase Glutamate Release Probability and Excitatory Strength in the Hippocampus during Chronic Neuroinflammation

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Neuroinflammation is involved in the pathogenesis of several neurologic disorders, including epilepsy. Both changes in the input/output functions of synaptic circuits and cell Ca^{2+} dysregulation participate in neuroinflammation, but their impact on neuron function in epilepsy is still poorly understood. Lipopolysaccharide (LPS), a toxic byproduct of bacterial lysis, has been extensively used to stimulate inflammatory responses both *in vivo* and *in vitro*. LPS stimulates Toll-like receptor 4, an important mediator of the brain innate immune response that contributes to neuroinflammation processes. Although we report that Toll-like receptor 4 is expressed in both excitatory and inhibitory mouse hippocampal neurons (both sexes), its chronic stimulation by LPS induces a selective increase in the excitatory synaptic strength, characterized by enhanced synchronous and asynchronous glutamate release mechanisms. This effect is accompanied by a change in short-term plasticity with decreased facilitation, decreased post-tetanic potentiation, and increased depression. Quantal analysis demonstrated that the effects of LPS on excitatory transmission are attributable to an increase in the probability of release associated with an overall increased expression of L-type voltage-gated Ca^{2+} channels that, at presynaptic terminals, abnormally contributes to evoked glutamate release. Overall, these changes contribute to the excitatory/inhibitory imbalance that scales up neuronal network activity under inflammatory conditions. These results provide new molecular clues for treating hyperexcitability of hippocampal circuits associated with neuroinflammation in epilepsy and other neurologic disorders.

Key words: glutamatergic transmission; L-type voltage-gated calcium channels; LPS; neuroinflammation; release probability; Toll-like receptor 4

Significance Statement

Neuroinflammation is thought to have a pathogenetic role in epilepsy, a disorder characterized by an imbalance between excitation/inhibition. Fine adjustment of network excitability and regulation of synaptic strength are both implicated in the homeostatic maintenance of physiological levels of neuronal activity. Here, we focused on the effects of chronic neuroinflammation induced by lipopolysaccharides on hippocampal glutamatergic and GABAergic synaptic transmission. Our results show that, on chronic stimulation with lipopolysaccharides, glutamatergic, but not GABAergic, neurons exhibit an enhanced synaptic strength and changes in short-term plasticity because of an increased glutamate release that results from an anomalous contribution of L-type Ca^{2+} channels to neurotransmitter release.

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Introduction

Inflammation influences pathogenesis and progression of most neurologic disorders, including those diseases for which the primary etiology is not inflammatory, such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, or epilepsy (Ransohoff, 2016; Vezzani et al., 2019). An imbalance of excitatory and inhibitory synaptic transmission is often associated with the pathogenesis of epilepsy, and numerous studies on the mechanisms of epileptogenesis have focused on synaptopathies (Scharfman, 2007). Neuroinflammation is generated by the synthesis and release of inflammatory molecules by resident cells of the brain, including activated microglia and astrocytes, neurons, endothelial cells of the blood–brain barrier, and blood-born macrophages (Xanthos and Sandkühler, 2014). The activation of resident cells also involves the stimulation of pattern- and danger-recognition receptors, named Toll-like receptors (TLRs), which represent an amplification pathway for proinflammatory responses (Okun et al., 2011). Among TLRs, the widely expressed subtype 4 (TLR4) (Chakravarty and Herkenham, 2005) can sense endogenous agonists, such as heat-shock proteins, high-mobility group box-1, and factors released from injured cells (Piccinini and Midwood, 2010). Several aspects of the neuroinflammatory response can be mimicked by lipopolysaccharides (LPSs), one of the best-studied immunostimulatory components of the Gram-negative bacterial wall, which acts as a high-affinity TLR4 ligand (Beutler and Rietschel, 2003; Okun et al., 2011).

Traditionally, neurons are seen as victims of neuroinflammation, as they are functionally affected by the release of glial inflammatory factors (Block et al., 2007). Notably, it has been shown that TLR4 is expressed by both cortical and hippocampal neurons (Tang et al., 2007; Hu et al., 2013), where it acts as an active cofactor of the inflammatory state via myeloid differentiation factor 88 (MyD88)-dependent and -independent pathways (Mao et al., 2012; Hu et al., 2013; G. X. Zhang et al., 2013). Hippocampal TLR4 signaling has been shown to participate in the neuroinflammatory response associated with brain trauma (Li et al., 2015) and ictogenesis (Maroso et al., 2010) by impairing various memory tasks dependent on LTP defects (Hausse-Wegrzyniak et al., 2002). However, the role of TLR4 on synaptic strength and short-term plasticity (STP) in LPS-responsive neurons has not been fully clarified. The excitatory and inhibitory synaptic strength sets the balance of the electrical activity in neural circuits by affecting STP that fine-tunes the firing patterns inside the network (Zucker and Regehr, 2002; Abbott and Regehr, 2004; Valente et al., 2016c, 2019). Presynaptic voltage-gated Ca^{2+} channels (VGCCs) play a fundamental role in STP (for a recent review, see Nanou and Catterall, 2018). Moreover, recent reports have shown that neuroinflammation leads to Ca^{2+} dysregulation (Vezzani and Viviani, 2015; Calvo-Rodríguez et al., 2017) that is contributed by an increased activity of somatodendritic L-type VGCCs (Furukawa and Mattson, 1998; for a recent review, see Navakode et al., 2018).

Our results indicate that LPS-mimicked chronic neuroinflammation induces a selective enhancement of excitatory strength, leaving the inhibitory GABAergic transmission unaffected. LPS increases both synchronous and asynchronous glutamate release and changes STP properties of excitatory synapses. Multielectrode array (MEA) recordings reveal a condition of network hyperactivity that is consistent with a selective action of LPS on excitatory synapses. We also demonstrate that the LPS-induced potentiation of excitatory transmission depends on an increased release probability (P_r) resulted by an abnormally increased expression of L-type Ca^{2+} channels at presynaptic terminals. Collectively, these

findings identify presynaptic L-type Ca^{2+} channels as a potential therapeutic target for counteracting the effects of neuroinflammation associated with neurologic disorders.

Materials and Methods

Cell cultures. All experiments were conducted in accordance with the guidelines established by the European Community Council (Directive 2010/63/EU, March 4, 2014) and were approved by the Italian Ministry of Health (authorization nos. 73/2014-PR and 1276/2015-PR). Primary cultures of hippocampal neurons were prepared from WT C57BL/6J embryos (Charles River) as previously described (Valente et al., 2012).

Mice were killed by CO_2 inhalation, and 17/18-day embryos (E17/E18) were removed immediately by caesarean section. In brief, hippocampi were dissociated by enzymatic digestion in 0.125% trypsin for 20 min at 37°C and then triturated with a fire-polished Pasteur pipette. Autaptic neurons were prepared as described previously (Valente et al., 2016a) with slight modifications. Dissociated neurons were plated at very low density (20 cells/ mm^2) on microdots (diameter of ~40–300 μm) obtained by spraying a mixture of poly-D-lysine (0.1 mg/ml) and collagen (0.25 mg/ml) on Petri dishes or glass coverslips, pretreated the day before with 0.15% agarose. Under this culture condition, each Petri dish showed ~15–20 isolated single autaptic neurons grown on poly-D-lysine microdots, with few astrocytes. Electrophysiological recordings of synaptic transmission were conducted on single and isolated autaptic neurons between 10 and 15 DIV. Low-density hippocampal neurons were prepared as previously described (Valente et al., 2016b). No antimetabolic drugs were added to prevent glia proliferation. Dissociated neurons were plated at low density (160 cells/ mm^2) on Petri dishes, previously pretreated with poly-D-lysine (0.1 mg/ml).

Only for immunocytochemistry assays, primary cultures of hippocampal neurons were prepared from postnatal GAD67-GFP knock-in mice (P0–P1). GAD67-GFP knock-in mice were generated by inserting the cDNA encoding enhanced GFP into the GAD67 locus in TT2 embryonic stem cells, as described by Tamamaki et al. (2003). Heterozygous GAD67-GFP males were mated with WT C57BL/6J females, and GFP-positive pups were identified at birth through a Dual Fluorescent Protein Flashlight (DFP-1, NIGHTSEA) and confirmed by genotyping (Prestigio et al., 2019).

Immunocytochemistry. Primary hippocampal neurons from (GAD67)-GFP knock-in mice were fixed at 13 DIV in 4% PFA/4% sucrose in PBS, pH 7.4. After several washes in PBS, cells were blocked with 3% BSA in PBS for 30 min. Samples were sequentially incubated with a primary antibody against TLR4 (1:200, Santa Cruz Biotechnology) (Chen et al., 2017), in blocking solution (3 h at room temperature or overnight at 4°C), followed by Alexa-594-conjugated secondary antibodies (Invitrogen; 1:500 for 1 h at room temperature). After several washes in PBS, coverslips were mounted using Prolong Gold antifade reagent (Invitrogen) containing DAPI for nuclear staining. Images were collected on an Olympus IX-81 microscope with an MT20 Arc/Xe lamp, 40 $\times$ objective, using the Excellence RT software (Olympus) and analyzed with ImageJ (National Institutes of Health).

To quantify the total number of synapses, neurons treated (48 h) with LPS or vehicle were fixed at 13 DIV and labeled with antibodies to the presynaptic markers Bassoon (1:1000, Synaptic Systems) (Thalhammer et al., 2017) or the vesicular glutamate transporter vGLUT1 (1:1000, Synaptic Systems) (Valente et al., 2019). Confocal images were acquired with a 60-oil immersion objective, keeping the confocal microscope settings constant (SP8, Leica Microsystems). Each image consisted of on average 30 images taken through the z plane of the cell. Bassoon-positive puncta with areas of 0.1–2 μm^2 were considered bona fide synaptic boutons. Synaptic boutons along neurites (10 μm starting from the cell body) were automatically counted using ImageJ (Esposito et al., 2019). The presynaptic localization of the L-type Ca^{2+} channel was analyzed in treated neurons by evaluating the immunoreactivity of L-type Ca^{2+} channel (1:200, Alomone Labs) (Hermosilla et al., 2017) in vGLUT1-positive excitatory boutons. The area of colocalization

puncta and the percentage of colocalization were automatically evaluated using ImageJ.

Cell viability and Sholl analysis. The Hoechst 33342 (2.5 mg/ml) and propidium iodide (0.02 mg/ml) staining solutions were added to primary hippocampal cultures, which underwent the treatment with either vehicle or LPS for 5 min at room temperature. Cell viability was observed by fluorescence after washing with free-standard external solutions. The extent of neurites arborization was evaluated at 13 DIV by Sholl analysis, as previously described (Corradi et al., 2014). To specifically identify the morphology of single cells, neurons were transfected at 11 DIV with pMCherry-C1 using Lipofectamine 2000 (Invitrogen) and fixed at 13 DIV after 48 h of treatment. All images were collected with an Olympus BX41 microscope equipped with a 40 $\times$ objective. Concentric rings each increased in radius by 10 μm were layered around the cell body until dendrites were completely encompassed and the number of intersections was automatically evaluated with the ImageJ/Sholl analysis plug-in. Neurites' total length was analyzed in the same neurons with the ImageJ/NeuronJ plug-in.

Western blotting. Total cell lysates were obtained from hippocampal neurons at 13 DIV. Cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Cell Signaling Technology). After 20 min of incubation, lysates were collected and clarified by centrifugation (10 min at 10,000 $\times$ g). Protein concentrations were determined using the Bradford assays (Bio-Rad). SDS-PAGE was performed according to Laemmli (1970), and equivalent amounts of protein were subjected to SDS-PAGE on 8% polyacrylamide gels and blotted onto nitrocellulose membranes (Whatman, Sigma Millipore). Membranes were blocked for 1 h in 5% nonfat dry milk/TBS (10 mM Tris, 150 mM NaCl, pH 8.0) plus 0.1% Triton X-100 and incubated overnight at 4°C or for 2 h at room temperature with the following primary antibodies: anti-TLR4 (1:2000, Santa Cruz Biotechnology) (Chen et al., 2017), anti-P/Q-type VGCCs (1:200, Santa Cruz Biotechnology) (Ishida et al., 2016), anti-N-type VGCCs (1:500, Sigma Millipore) (Medrihan et al., 2013), anti-L-type VGCCs (1:1000, Sigma Millipore) (Eden et al., 2016), and anti-GAPDH (1:2000, Santa Cruz Biotechnology). After several washes, membranes were incubated for 1 h at room temperature with peroxidase-conjugated anti-mouse (1:5000; Bio-Rad) or anti-rabbit (1:3000; Bio-Rad) antibodies. Bands were revealed with the ECL chemiluminescence detection system (Thermo Fisher Scientific). Immunoblots were quantified by densitometric analysis of the fluorograms (Quantity One Software, Bio-Rad) obtained in the linear range of the emulsion response.

Surface biotinylation. Hippocampal neurons at 13 DIV treated or not with LPS or with LPS+CLI-095 for 48 h were incubated with 1 mg/ml of EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific) in cold PBS for 35 min at 4°C, constantly moving. Free biotin was quenched twice with 100 mM Tris, pH 8, and once with cold PBS to remove biotin excess. Cells were then lysed in lysis buffer as described above. Total cell lysates were centrifuged at 10,000 $\times$ g at 4°C for 10 min. The supernatant fraction (0.5 mg protein) was incubated with 100 μl of NeutrAvidin-conjugated agarose beads for 3 h at 4°C, and the remaining supernatant was kept as input. After extensive washes of the beads, samples were eluted and resolved by SDS-PAGE followed by immunoblotting with antibodies against L-type Ca^{2+} channel (1:1000, Sigma Millipore) (Eden et al., 2016), actin (1:1000 Sigma Millipore), and Na^+/K^+ ATPase (1:2000, Thermo Fisher Scientific).

Patch-clamp recordings. Whole-cell patch-clamp recordings were made from autaptic or low-density hippocampal neurons as previously described (Valente et al., 2012). The membrane potentials in whole-cell recordings were uncorrected for Donnan liquid junction potentials of 9 mV (Neher, 1992). All experiments were performed at room temperature (22°C–24°C). Patch pipettes, prepared from thin-borosilicate glass (Hilgenberg), were pulled and fire-polished to a final resistance of 2–4 M Ω when filled with standard internal solution. Evoked excitatory and inhibitory postsynaptic currents (eEPSCs/eIPSCs) were recorded using an EPC-10 amplifier (HEKA Elektronik). For whole-cell recordings of synaptic transmission in autaptic neurons, cells were maintained in a standard external solution containing the following (in mM): 140 NaCl, 2 CaCl_2 , 1 MgCl_2 , 4 KCl, 10 glucose, 10 HEPES, pH 7.3 (with NaOH).

For eEPSC recordings, the external solution was supplemented with the following: D-AP5 (50 μM , Tocris Bioscience) and bicuculline (30 μM , Tocris Bioscience) to block NMDA and GABA_A receptors, respectively. To record eIPSCs, CNQX (10 μM) and CGP 58845 (10 μM) were added to the external solution to block non-NMDA and GABA_B receptors, respectively. The standard internal solution was as follows (in mM): 126 K gluconate, 4 NaCl, 1 MgSO_4 , 0.02 CaCl_2 , 0.1 BAPTA, 15 glucose, 5 HEPES, 3 ATP, 0.1 GTP, pH 7.2 (with KOH). LPS (from *Escherichia coli* O111:B) was purchased from Sigma Millipore, reconstituted in water to a final concentration of 1 mg/ml, and kept at –20°C. The TLR4 inhibitor CLI-095 was purchased from InVivoGen and was solubilized in DMSO in a stock solution of 100 $\mu\text{g}/\text{ml}$ and kept at –20°C. Neurons (10 DIV) were chronically treated with LPS or vehicle for 24, 48, 72, and 96 h. An incubation time of 48 h was adopted for the TLR4 inhibition experiments with CLI-095. The autaptic neurons under study were voltage-clamped at a holding potential (V_h) of –70 mV. Unclamped action potentials (APs) evoking PSCs were activated by a brief depolarization of the cell body to 40 mV for 0.5 ms at 0.1 Hz. ePSCs were acquired at 10–20 kHz sampling rate and filtered at 1/5 of the acquisition rate with an 8-pole low-pass Bessel filter. Recordings with leak currents >100 pA or series resistance >15 M Ω were discarded. Data acquisition was performed using PatchMaster programs (HEKA Elektronik). ePSCs were inspected visually, and only events that were not contaminated by spontaneous activity were considered. To calculate the peak current during an isolated stimulus or a train of stimuli, we first subtracted an averaged trace containing the stimulus artifact and the AP current, lacking any discernable synaptic current (i.e., synaptic failures). Such traces were easily identified toward the end of a train of stimuli, when synaptic depression was maximal. These traces were averaged and scaled to the peak Na^+ current contaminating the PSCs. To analyze the paired-pulse ratio (PPR), 2 brief depolarizing pulses were applied to autaptic neurons with an interpulse interval of 50 ms. For each couple of PSCs, PPR was calculated as the I2/I1 ratio, where I1 and I2 are the amplitudes of the PSCs evoked by the conditioning (1) and test (2) stimuli, respectively. To correctly estimate the amplitude of I2, the baseline of I2 was defined as the final value of the decay phase of I1 and the amplitude of I2 was calculated by subtracting the residual amplitude of I1 from the peak value of I2. In the analysis of synaptic responses during high-frequency stimulation (HFS), the interpulse interval was shorter than the time needed for an ePSC to return to baseline, so ePSCs overlapped partially. Thus, to correctly estimate the ePSC amplitude, the baseline of each event was defined as the final value of the decay phase of the preceding ePSC and the amplitude of ePSC(n) was calculated by subtracting the residual amplitude of ePSC(n–1) from its peak value. The size of the readily releasable pool of synchronous release (RRPsyn) and the probability that any given synaptic vesicle (SV) in the RRP will be released (P_r) were calculated using the cumulative amplitude analysis (Schneggenburger et al., 2002). The cumulative amplitude plot was determined by summing up peak EPSC amplitudes during 80 repetitive stimuli applied at 40 Hz. This analysis assumes that depression during the steady-state phase is limited by a constant recycling of SVs with an equilibrium occurring between released and recycled SVs and that P_r during the train approaches the 1 value (Schneggenburger et al., 1999). The cumulative amplitude profiles of the last 30–40 data points were fitted by linear regression and back-extrapolated to time 0. The intercept with the y axis gave the RRPsyn and the ratio between the amplitude of the first PSC (I1) and the RRPsyn yielded the P_r . Miniature EPSCs (mEPSCs) were recorded from low-density hippocampal neurons in standard external solution containing the following: TTX (1 μM ; Tocris Bioscience), D-AP5 (50 μM), CGP 58845 (10 μM), and bicuculline (30 μM). The amplitude and frequency of mEPSCs were calculated using a peak detector function with appropriate threshold amplitude and threshold area using the Minianalysis program (Synaptosoft). The delayed asynchronous release of excitatory autapses was evoked by a tetanic stimulation lasting 2 s at 40 Hz. Asynchronous release was estimated by measuring the charge (pA $\cdot$ ms) of spontaneous PSCs that follow the stimulation train, in 9 consecutive time windows of 1 s. The first 5 ms of eEPSCs evoked before train was used to evaluate the synchronous charge. As the extent of spontaneous network activity preceding the train may influence

asynchronous release induced by the train, we measured the charge of spontaneous release 1 s before the train and subtracted it from values of asynchronous release after HFS.

Patch-clamp recordings of VGCC currents in low-density hippocampal cells were performed on pyramidal neurons morphologically identified by their teardrop-shaped somata and characteristic apical dendrite after 12–16 DIV (Pozzi et al., 2013). Series resistance was compensated 80% (2 μs response time), and the compensation was readjusted before each stimulus. Recordings with either leak currents >100 pA or series resistance >20 M Ω were discarded. Voltage-clamp recordings of VGCCs were acquired at 20 kHz and low-pass filtered at 4 kHz using an external solution containing the following (in mM): 140 NaCl, 5 BaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.4 (with NaOH 1 M). TTX (1 μM), D-AP5 (50 μM), CNQX (10 μM), CGP 58845 (10 μM), and bicuculline (30 μM) were added to the external solution to block spontaneous APs, NMDA, non-NMDA, GABA_A, and GABA_B receptors, respectively. The intracellular solution was composed of the following (in mM): 90 CsCl, 20 TEA-Cl, 10 EGTA, 10 glucose, 1 MgCl₂, 4 ATP, and 15 phosphocreatine, pH 7.4 (with 1 M CsOH). The external solution containing the Ca^{2+} channel blockers, nifedipine (Sigma Millipore, 5 μM), ω -conotoxin GVIA (PeptaNova, 1 μM), and ω -agatoxin IVA (PeptaNova, 1 μM), was applied with a gravity-driven, local perfusion system at a flow rate of 200 $\mu\text{l}/\text{min}$ positioned within 100 μm of the neuron under study. In all voltage-clamp experiments, V_h was set at -70 mV. Voltage-gated inward currents were evoked by stepping V_h from -70 to 60 mV for 10 ms with 5 mV increments with 2 s interpulse intervals. The current density ($J = \text{nA}/\text{pF}$) was obtained by dividing the peak inward current by the cell capacitance.

MEA recordings. Neuronal activity was recorded using a multiwell MEA system (Maestro, Axion BioSystems). The MEA plates used (M768-tMEA-48W, Axion BioSystems) contain 48 wells, each with a square grid of 16 electrodes (50 μm electrode diameter; 350 μm center-to-center spacing) that create a 1.1×1.1 mm recording area. MEAs, coated by depositing a 20 μl drop of poly-L-lysine (0.1 mg/ml, Sigma Millipore) over each recording area, were incubated overnight. Dissociated hippocampal neurons were plated at a final density of 50,000 neurons per well and incubated with Neurobasal medium supplemented with 1% Glutamax, 2% B27, and 1% penicillin-streptomycin. One-third of the medium was replaced with fresh medium every week. Spiking activity from hippocampal networks grown onto MEAs was monitored and recorded using the Axion BioSystems hardware (Maestro amplifier and Middle-man data acquisition interface) and the Axion's Integrated Studio software (AxIS 2.4). Neuronal cultures on MEAs were treated with LPS (10 ng/ml) or vehicle at DIV 12. After 48 h, MEA plates were set on the Maestro apparatus and their activity recorded for 10 min at 37°C. Networks were then acutely exposed to the VGCC L-type blocker nifedipine (5 μM), and recordings were carried on for a further 10 min. After 1200 $\times$ amplification, raw data were digitized at 12.5 kHz/channel and stored for subsequent offline analysis. Spike detection and spike train data analysis were computed using the Axion BioSystems software NeuralMetricTool. To study the effect of LPS on firing and bursting properties, only wells that contained ≥ 4 active electrodes (≥ 5 spikes/min) were considered for further analysis. Extracellular APs were detected by adaptive threshold crossing ($7 \times$ the SD of the rms noise on each channel) on 200 Hz high-pass-filtered traces. Bursts within single channels were identified by applying an interspike interval (ISI) threshold algorithm, which defines bursts as collections of a minimum number of spikes ($N_{\text{min}} = 5$) separated by a maximum ISI (ISI_{max}) of 100 ms (Chiappalone et al., 2005).

Statistical analysis. Data are expressed as mean $\pm$ SEM for number of cells (n) or mouse preparations as detailed in the figure legends. Normal distribution of data was assessed using the D'Agostino-Pearson's normality test. The F test was used to compare variance between two sample groups. To compare two normally distributed sample groups, the Student's unpaired or paired two-tailed t test was used. To compare two sample groups that were not normally distributed, the nonparametric Mann-Whitney U test was used. To compare more than two normally distributed sample groups, we used one- or two-way ANOVA, followed by the Bonferroni's test. In cases in which data were

not normally distributed, nonparametric one- and two-way ANOVAs (Kruskal-Wallis's and Friedman's two-way ANOVA tests, respectively) were used, followed by the Dunn's multiple comparison test. Alpha levels for all tests were 0.05% (95% CIs). Statistical analysis was conducted using OriginPro-8 (OriginLab) and Prism (GraphPad Software) software.

Results

The strength of glutamatergic, but not GABAergic, synapses is enhanced by chronic LPS treatment

LPS extracted from *E. coli* has been extensively used to investigate the mechanisms of brain inflammation both *in vivo* and *in vitro* (Tanaka et al., 2006; Deng et al., 2012). Here, we studied synaptic transmission and STP parameters in autaptic hippocampal neurons on LPS treatment. The use of primary autaptic cultures offers the advantage of recording synaptic currents from isolated neurons, activating a defined and homogeneous population of synapses (Valente et al., 2016a). Moreover, the extremely low-cell density allows a precise control of the extracellular environment over time.

To examine the functional effects of neuroinflammation on glutamatergic and GABAergic transmission, autaptic hippocampal neurons were exposed to LPS (10 ng/ml) and studied by whole-cell patch-clamp recordings (Fig. 1A). Postsynaptic currents (ePSCs) evoked by single APs were recorded in excitatory and inhibitory neurons (10–15 DIV) and identified as excitatory (eEPSCs) or inhibitory (eIPSCs) based on their kinetics and sensitivity to either CNQX (10 μM) or bicuculline (30 μM), respectively (Fig. 1B). When autaptic neurons were exposed for increasing times (24, 48, 72, and 96 h) to LPS, we observed a twofold increase in the eEPSC amplitude that was virtually absent after acute (5 min) treatment (Fig. 1C, top). Interestingly, neither acute nor chronic LPS treatments affected the eIPSC amplitude (Fig. 1C, bottom).

To explore the mechanism underlying the specific increase of eEPSC amplitude after long-lasting LPS treatment, we studied the distribution of TLR4 expression in primary excitatory and inhibitory neurons (12–15 DIV) from glutamate decarboxylase 67 (GAD67)-GFP knock-in mice (Fig. 1D) (Prestigio et al., 2019). We found that TLR4 is expressed in virtually all inhibitory (GFP-positive) and excitatory (GFP-negative) hippocampal neurons (Fig. 1E) and that its expression was not affected by chronic treatment with LPS (Fig. 1F). However, TLR4 were directly implicated in the LPS-induced increase of eEPSC amplitude since the presence of CLI-095 (1 μM), a cyclohexene derivative that specifically suppresses TLR4 signaling, completely abolished the effects of LPS on the postsynaptic current (Fig. 1G) (Matsunaga et al., 2011). Moreover, the prolonged (48 h) treatment of hippocampal neurons with LPS did not affect neuronal viability (Fig. 2A,B) or the number of Bassoon-positive synaptic puncta (Fig. 2C,D). At the same time, the morphology of individual Cherry-labeled neurons exposed to LPS and analyzed by Sholl analysis showed the absence of LPS-induced changes in dendritic nodes and in total dendritic length (Fig. 2E,F).

TLR4 activation by LPS affects STP of excitatory autapses

We next investigated the effects of LPS on STP of excitatory and inhibitory synapses. We first analyzed the response to paired stimuli (PPR at various ISIs), which also provides an indirect estimation of the P_r of SVs (Saviane and Silver, 2006; Fioravante and Regehr, 2011). Excitatory autapses treated with LPS for 48 h displayed a smaller facilitation at the shortest intervals with respect to vehicle-treated neurons ($\text{ISI} < 100$ ms; Fig. 3A,B).

Figure 1. Chronic treatment with LPS induces a TLR4-dependent enhancement of evoked glutamatergic, but not GABAergic, postsynaptic currents in autaptic hippocampal neurons. **A**, Phase-contrast micrograph of a typical glutamatergic (top) and GABAergic (bottom) autaptic hippocampal neurons. The nature of both types of neurons was confirmed by retrospective kinetics and pharmacological analysis of postsynaptic currents. Scale bar, 100 μm . **B**, Representative eEPSCs (top) and eIPSCs (bottom) of autaptic hippocampal neurons after 48 h treatment with vehicle (veh; black and blue represent excitatory and inhibitory neurons, respectively) evoked by a single 0.5 ms step from a V_h of -70 mV to 40 mV, applied at a frequency of 0.1 Hz (inset). Currents were fully blocked by the application of specific AMPA and GABA A receptor antagonists CNQX (10 μM , gray, top) and bicuculline (30 μM , gray, bottom), respectively. In all traces, stimulation artifacts were blanked for clarity. **C**, Time course (5 min and 24, 48, 72, 96 h) of the mean changes in eEPSC (top) and eIPSC (bottom) amplitude after vehicle and LPS treatments (eEPSCs, 5 min: veh, $n = 13$, LPS, $n = 13$; 24 h: veh, $n = 38$, LPS, $n = 40$; 48 h: veh, $n = 47$, LPS, $n = 57$; 72 h: veh, $n = 30$, LPS, $n = 27$; 96 h: veh, $n = 14$, LPS, $n = 12$; eIPSCs, 5 min: veh, $n = 6$, LPS, $n = 6$; 24 h: veh, $n = 7$, LPS, $n = 7$; 48 h: veh, $n = 26$, LPS, $n = 29$; 72 h: veh, $n = 6$, LPS, $n = 7$; 96 h: veh, $n = 6$, LPS, $n = 6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; paired/unpaired Student's t test/Mann–Whitney U test. **D**, Representative images of TLR4 immunostaining (red) in hippocampal neurons (16 DIV) from the GAD67-GFP knock-in mouse. DAPI staining was used to label cell nuclei (blue). Merge panel represents one inhibitory neuron (GFP-positive; green) and one excitatory neuron (GFP-negative) that are both labeled with TLR4 (red) and DAPI (blue). Scale bar, 100 μm . **E**, Bar graph showing the percentage of TLR4-positive cells in GAD67- (black) and GAD67+ (blue) neurons. Data are shown as mean $\pm$ SEM. **F**, Western blot analysis of TLR4 and GADPH protein levels in hippocampal neurons treated with LPS for 0, 2, 6, 24, and 48 h. Molecular weight markers (kDa) are indicated on the right. TLR4 levels are shown as a percentage change relative to control (0 h). **G**, Bar graph showing the percentage change of EPSC amplitude after LPS and CLI treatments. Data are shown as mean $\pm$ SEM. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2. Long-lasting LPS treatment does not affect neuronal viability, synaptic density, and dendritic arborization. **A**, Representative merged phase-contrast and fluorescence images represent uptake of Hoechst 33258 and propidium iodide in low-density cultures of hippocampal neurons treated with either vehicle (veh) or LPS (10 ng/ml) for 48 h. Scale bar, 200 μm . **B**, Results are presented as the percentage of cell viability in both treatments (veh: 10.94 ± 0.969 of 2667 total neurons; LPS: 11.08 ± 0.49 of 2637 total neurons from two independent preparations). **C**, Representative images of dendrites of hippocampal neurons chronically exposed to either vehicle or LPS stained with presynaptic marker Bassoon. Scale bar, 10 μm . **D**, Quantitative analysis of synaptic puncta counted on 30 μm dendrite tracts starting 10 μm from the cell body. Data are mean $\pm$ SEM from three independent preparations. **E**, Representative Cherry-transfected neurons exposed (48 h) to either vehicle or LPS. Sholl rings were drawn at 20- μm -diameter intervals starting from the cell body center. **F**, Quantification of the number of intersections (left) and total process length. Data are mean $\pm$ SEM from three independent preparations (veh, $n = 37$; LPS, $n = 32$).

←
DAPI (blue). Scale bar, 50 μm . **E**, Bar plot of the TLR4 expression level in GAD67[−] and GAD67⁺ cells, respectively ($n = 3$ independent culture preparations). **F**, Representative immunoblot (left) and quantification of the expression levels (right) of TLR4 in total cell lysates of LPS-treated primary hippocampal neurons at various times of treatment. GAPDH was used as a control of equal loading. Data (mean $\pm$ SEM, $n = 3$) were normalized to GAPDH and expressed as percentages of untreated neurons. **G**, Mean ($\pm$ SEM) percentage changes in eEPSC amplitude with respect to vehicle in neurons treated with LPS for various times, neurons treated for 48 h with LPS in the presence of the TLR4 inhibitor CLI-095 (1 μM), and neurons treated with CLI-095 alone. H (* $p < 0.05$, ** $p < 0.01$ versus 5 min LPS; # $p < 0.01$, ### $p < 0.001$ versus LPS+CLI 48h; one-way ANOVA/ Bonferroni's multiple comparison tests.)

Similar PPR decrements (at ISI = 50 ms) were also observed on LPS treatment lasting from 24 to 96 h, but not after acute LPS treatment (5 min). When cells were exposed to LPS in the presence of the TLR4 inhibitor CLI-095 (48 h), the effect of LPS on PPR was virtually abolished, while the treatment with CLI-095 alone was ineffective (Fig. 3C). No changes in PPR (ISI = 50 ms) were observed in inhibitory autapses after either acute or chronic exposure to LPS (10 ng/ml) (Fig. 3D). Such a decrease in short-term facilitation of excitatory synapses suggests that an increase in the P_r is likely responsible for the LPS/TLR4-induced increase of EPSC amplitude.

Figure 3. Chronic LPS decreases the PPR of glutamatergic, but not GABAergic, hippocampal autapses. **A**, Representative eEPSCs evoked in hippocampal autaptic neurons after 48 h treatment with either vehicle (black) or LPS (10 ng/ml; red). Currents were evoked by stimulating the cell under study with two 0.5 ms steps to 40 mV separated by 20 ms ISI applied at 0.1 Hz with a V_h of -70 mV (inset). **B**, eEPSC PPR (I_2/I_1) as a function of ISI ranging from 20 to 10,000 ms for cells treated 48 h with either vehicle (black; $n = 17$) or LPS (red; $n = 18$). **C**, Time course (5 min and 24, 48, 72, 96 h) of mean changes in eEPSC PPR after either vehicle or LPS treatment (5 min: veh, $n = 14$, LPS, $n = 14$; 24 h: veh, $n = 38$, LPS, $n = 40$; 72 h: veh, $n = 30$, LPS, $n = 27$; 96 h: veh, $n = 14$, LPS, $n = 12$). The last two column couples represent the PPR changes in cells treated for 48 h with LPS in the presence of CLI-095 ($1 \mu\text{M}$; green column) or with CLI-095 alone ($1 \mu\text{M}$; gray column) compared with that treated with vehicle (veh, $n = 18$, LPS + CLI-095, $n = 22$; veh, $n = 14$, CLI-095, $n = 17$). **D**, Left, Representative eIPSCs evoked by 0.5 ms steps to 40 mV separated by 50 ms (inset) in GABAergic hippocampal autaptic neurons after 48 h treatment with either vehicle (blue) or LPS (10 ng/ml; red). Right, Time course (5 min, 24, 48, 72, 96 h) of the mean changes in eIPSC PPR after treatment with either vehicle or LPS (5 min: veh, $n = 6$, LPS, $n = 6$; 24 h: veh, $n = 6$, LPS, $n = 6$; 48 h: veh, $n = 26$, LPS, $n = 29$; 72 h: veh, $n = 6$, LPS, $n = 7$; 96 h: veh, $n = 6$, LPS, $n = 6$). In all traces, the stimulation artifacts were blanked for clarity. Data are mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; paired Student's t test/Mann–Whitney U test.

We next analyzed excitatory synaptic transmission in response to HFS (2 s at 40 Hz) in autaptic neurons treated with vehicle, LPS, or LPS+CLI-095 for 48 h (Fig. 4A). The time course of eEPSC amplitude, normalized to the amplitude of the first eEPSC, revealed a fast and significant decrease of the facilitation during the first stimuli of the train, followed by a progressive depression to a steady-state current (Fig. 4B). This effect was particularly clear-cut when the normalized EPSC amplitude was calculated for the 11th, 12th, and 13th eEPSCs in the train and was totally lost in the presence of CLI-095 (Fig. 4C,D).

The effect of chronic (48 h) LPS treatment on activity-dependent synaptic depression was investigated using 2 s stimulation trains at 20, 10, and 5 Hz (Fig. 4E). LPS-treated excitatory autapses stimulated at 20 and 10 Hz confirmed the impaired early facilitation and the increased depression (calculated for the last three eEPSCs in the train) observed with the 40 Hz train. The enhancement of synaptic depression was dramatic at 20 Hz, attenuated at 10 Hz, and disappeared at 5 Hz (Fig. 4F,G).

Post-tetanic potentiation (PTP), another form of STP, depends on the concurrent increase of P_r and the size of the readily releasable SV pool (RRP_{syn}) originating from enhanced intraterminal Ca^{2+} levels and SV mobilization, respectively (Zucker and Regehr, 2002; Valente et al., 2012). Autaptic neurons were stimulated at 0.1 Hz for 120 s; then a short HFS (2 s at 40 Hz) was applied before the stimulation frequency was returned to 0.1 Hz for further 200 s to allow a complete recovery of the eEPSC amplitude (Fig. 4H). Chronic LPS treatment (48 h) significantly

reduced PTP, an effect that was fully suppressed by the TLR4 blocker CLI-095 (Fig. 4I).

Collectively, these results clearly demonstrate that LPS, through TLR4 activation, potently affects STP of hippocampal glutamatergic synapses.

LPS increases the probability of synchronous glutamate release

The increased eEPSC amplitude, the decrease in facilitation and PTP, and the increase in synaptic depression all strongly suggest a predominant presynaptic action of LPS. To investigate in depth the mechanisms of the LPS-induced effects, mEPSCs were recorded in the presence of TTX ($1 \mu\text{M}$) at the soma of hippocampal neurons cultured at low density and clamped at -70 mV (Fig. 5A). While the mEPSC amplitude reflects the quantal size of neurotransmitter (SV content and postsynaptic effect), the mEPSC frequency depends on the number of active synapses and the probability of spontaneous release of single SVs (Stevens, 1993). No effects of LPS were observed on mEPSC amplitude, frequency, decay and rise times, or charge (Fig. 5B–F), largely excluding LPS effects at the postsynaptic level or on the density of synaptic connections (see also Fig. 2).

We next studied how LPS affects quantal parameters of evoked glutamate release by cumulative EPSC amplitude analysis (Schneeggenburger et al., 1999; Valente et al., 2012). To do this, a 2 s 40 Hz stimulation train (Fig. 4A) was used to induce a complete depletion of the RRP_{syn} (see Materials and Methods). The

Figure 4. Chronic LPS decreases synaptic facilitation and PTP at excitatory autapses. **A**, Representative recordings of eEPSCs evoked by a short tetanic stimulation (2 s at 40 Hz; inset) in cells treated for 48 h with vehicle (black), LPS (red), or LPS+CLI-095 (green). **B**, Plot of the normalized mean eEPSC amplitude versus time during tetanic stimulation (2 s at 40 Hz) under the conditions shown in **A**. Inset, An expanded time scale of 800 ms allows to better appreciate the differences in synaptic depression. **C**, Representative current traces showing the first 13 eEPSCs evoked during tetanic stimulation in cells treated with vehicle (black), LPS (red), or LPS+CLI-095 (green). **D**, Graph bars of the mean ($\pm$ SEM) ratio of the averaged amplitude of the 11th, 12th, and 13th eEPSCs, normalized to the first eEPSC of the short tetanic stimulation shown in **C**. **E**, Representative recordings of the first and last three eEPSCs evoked by 2 s tetanic stimulation at 20, 10, and 5 Hz administered to vehicle- and LPS-treated autaptic hippocampal neurons. **F**, Graph bars of the mean ($\pm$ SEM) averaged amplitude of the last three eEPSCs in the 2 s train, normalized to the first eEPSC of the respective train (20 Hz: veh, $n = 8$; LPS, $n = 10$; 10 Hz: veh, $n = 8$; LPS, $n = 12$; 5 Hz: veh, $n = 8$; LPS, $n = 12$). **G**, Plot of normalized mean eEPSC amplitude versus time during 2 s trains of APs at various frequencies in vehicle- and LPS-treated autaptic cells (left, 20 Hz; middle, 10 Hz; and right, 5 Hz). **H**, Representative recordings of the experimental protocol used to investigate PTP. Single stimuli applied at basal stimulation frequency (0.1 Hz) were followed by a tetanic stimulation (2 s at 40 Hz), and repeated until the eEPSC amplitude returned to the pretetanus level. The maximal PTP level was observed 10 s after the tetanus, and the eEPSC amplitude recovered the baseline value within 200 s. **I**, Bar plot of the maximum PTP value mean ($\pm$ SEM) expressed, for each condition, as the percentage increase with respect to the mean baseline EPSC. The following replications were used for the statistical analysis of **B**, **D**, and **H**. Black symbols/column represent veh ($n = 59$). Red symbols/column represent LPS ($n = 59$). Green symbols/column represent LPS+CLI-095 ($n = 21$). In all traces, the stimulation artifacts were removed for clarity. Data are mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; one-way ANOVA/Bonferroni's multiple comparison tests or unpaired Student's t test/Mann–Whitney U test.

Figure 5. TLR4 activation by chronic LPS increases the probability of synchronous glutamate release. **A**, Representative traces of mEPSCs from low-density hippocampal neurons treated for 48 h with either vehicle or LPS (10 ng/ml) (black and red, respectively). **B–F**, Analysis of mEPSCs. Left to right, Bar graphs represent the mean $\pm$ SEM of amplitude, frequency, 80% decay time, 10%–90% rise time, and area of mEPSCs calculated for vehicle-treated ($n = 29$, black bars) and LPS-treated ($n = 26$, red bars) neurons. **G**, Representative cumulative amplitude profiles recorded in autaptic neurons treated for 48 h with vehicle, LPS, or LPS+CLI-095 (black represents veh, $n = 59$; red represents LPS, $n = 59$; green represents LPS+CLI-095, $n = 21$) and stimulated for 2 s at 40 Hz. The last 40–30 data points in the range 0.5–2 s were fitted by linear regression and back-extrapolated to time 0 (solid lines) to estimate the RRP_{syn} size. **H**, Left to right, Mean $\pm$ SEM of amplitude of the first eEPSC in the train (eEPSC₁), RRP_{syn} size, and P_r estimated for cells treated with vehicle (black bars, $n = 59$), LPS (red bars, $n = 59$), or LPS+CLI-095 (green bars, $n = 21$). **I**, Average number of SVs in the RRP_{syn} (N_{syn}) in vehicle- and LPS-treated neurons obtained from the ratio between the RRP_{syn} and the mean mEPSC amplitude. **J**, Representative traces showing asynchronous release evoked by a tetanic stimulation (2 s at 40 Hz) in autaptic hippocampal neurons treated for 48 h with vehicle (black), LPS (red), or LPS+CLI-095 (green). Insets, Traces are shown in an expanded time scale. **K**, Mean synchronous charge from neurons treated as shown in **J** (veh, $n = 59$; LPS, $n = 65$; LPS+CLI-095, $n = 18$), stimulated with one AP 10 s before the tetanus. Synchronous charge was estimated by measuring the area of the eEPSC in a time window of 5 ms following its activation. **L**, Time course of asynchronous charge calculated by measuring the area of spontaneous EPSCs evoked by HFS. The area was calculated in 9 time windows, each lasting 1 s (veh, $n = 59$; LPS, $n = 65$; LPS+CLI-095, $n = 18$). Inset, The charge of the third, fourth, and fifth bins after HFS is plotted. In all traces, the stimulation artifacts were blanked for clarity. Data are mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; one-way ANOVA/Bonferroni's multiple comparison tests and unpaired Student's t test/Mann–Whitney U test.

cumulative profile of eEPSC amplitude showed a rapid rise followed by LPS followed by a slower linear increase (Fig. 5G). While the RRP_{syn} size was not affected, chronic LPS treatment induced a significant increase in the P_r (calculated as the ratio between the first eEPSC in the train and RRP_{syn}) that paralleled the increase in eEPSC amplitude and was totally abolished by CLI-095 treatment (Fig. 5H). The unchanged number of SVs in the RRP_{syn} (N_{syn}), calculated by normalizing RRP_{syn} to the mean mEPSC amplitude, demonstrating that LPS does not affect the SV priming process (Fig. 5I).

Finally, we investigated the LPS effects on the HFS (2 s at 40 Hz)-induced asynchronous glutamate release generated by the intraterminal Ca^{2+} buildup and persisting for tens/hundreds of milliseconds to seconds (Fig. 5J) (Kaeser and Regehr, 2014). As previously reported (Medrihan et al., 2013; Valente et al., 2016a), HFS-induced asynchronous release was measured as the charge transferred in a 9 s time window following the stimulation (1 s bins). Both the synchronous charge (evoked by a single stimulus applied 10 s before the train) and the asynchronous charge were increased by chronic LPS treatment compared with vehicle-treated neurons, an effect that was totally abolished by CLI-095 (Fig. 5K,L).

The effects of LPS/TLR4 on excitatory transmission involve presynaptic L-type Ca^{2+} channels

The increased P_r suggests that an increase in either Ca^{2+} sensitivity or Ca^{2+} influx occurs at the presynaptic terminals after chronic LPS treatment. Presynaptic active zones contain distinct VGCC subtypes as primary sources of Ca^{2+} influx that triggers SV exocytosis. In particular, N and P/Q types of VGCCs are the main actors mediating Ca^{2+} -triggered neurotransmitter release in the CNS (Bean, 1989; Seabrook and Adams, 1989; Mintz et al., 1992; Luebke et al., 1993; Wheeler et al., 1994; Dunlap et al., 1995; Wright and Angus, 1996). On the contrary, L-type VGCCs are not activated when APs invade presynaptic terminals (Miller, 1987; Kullmann et al., 1992; Dunlap et al., 1995; Reuter, 1996); and, accordingly, the blockade of L-type of VGCCs has little effect on synaptic transmission in various brain areas (Kamiya et al., 1988; Linás et al., 1989; Horne and Kemp, 1991; Kullmann et al., 1992; Mintz et al., 1992; Turner et al., 1993; J. F. Zhang et al., 1993; Wheeler et al., 1994). Here, we studied the contribution of P/Q-, N-, and L-type of VGCCs to the LPS-induced increase of eEPSC amplitude in hippocampal autaptic neurons. Autapses were stimulated at 0.1 Hz for over 2 min with a single AP to obtain a stable eEPSC baseline before applying ω -agatoxin (1 μM), ω -conotoxin (1 μM), or nifedipine (5 μM) to block P/Q-, N-, and L-type VGCCs, respectively (Fig. 6A,C) and recording the eEPSC amplitude over the following 5 min. As previously reported (Takahashi and Momiyama, 1993; Reid et al., 1997), the presynaptic Ca^{2+} influx in vehicle-treated neurons was mainly mediated by P/Q- and N-type channels ($\sim 85\%$), with no effects of blockade of L-type VGCCs with nifedipine (Fig. 6B,E) and a minimal contribution ($\sim 15\%$) of ω -agatoxin- and ω -conotoxin-resistant R-type VGCCs (Fig. 6F, left). Interestingly, in neurons chronically (48 h) treated with LPS, nifedipine brought about a $\approx 30\%$ decrease in eEPSC amplitude, while P/Q- and N-type VGCCs blockers exerted the same extent of inhibition previously observed in vehicle-treated neurons (Fig. 6D,E). These results demonstrate that LPS treatment affects the composition of presynaptic Ca^{2+} channels subtypes responsible for glutamate release by replacing R-type with a “new” L-type VGCCs at the active zone (Fig. 6F, right). Thus, the increase of P_r that underlies the increased excitatory strength and STP changes induced by

chronic LPS appears to be mediated by an increased Ca^{2+} influx because of the “ectopic” presynaptic expression of L-type VGCCs at presynaptic terminals.

Activation of TLR4 by LPS increases Ca^{2+} current because of overexpression of the L-type Ca^{2+} channels

To investigate the mechanism underlying the contribution of L-type VGCCs to the increased P_r in LPS-treated neurons, we recorded VGCC currents in visually identified excitatory neurons (see Materials and Methods). Ca^{2+} currents, elicited by voltage steps ranging from -60 to 60 ($V_h = -70$ mV, step duration = 200 ms), were recorded in vehicle- and LPS-treated neurons (48 h) in the presence or absence of nifedipine (5 μM ; Fig. 7A).

The analysis of the current density (J ; nA/pF)/voltage relationship (J/V) revealed that nifedipine significantly reduced VGCC- J in both vehicle- and LPS-treated neurons (Fig. 7B). Interestingly, the mean VGCC- J recorded at -10 mV was significantly higher in LPS-treated neurons than in vehicle-treated cells, while such difference was completely lost in the presence of nifedipine (Fig. 7B). Accordingly, the percentage of blockade of Ca^{2+} conductance by nifedipine was significantly larger in LPS-treated neurons than in vehicle-treated ones (Fig. 7C). The analysis of J/V curves of pure L-type currents, obtained by subtracting nifedipine-insensitive currents from the total Ca^{2+} currents, clearly showed that LPS treatment dramatically enhanced L-type currents (Fig. 7D).

To dissect the molecular basis of these results, we analyzed the total expression level of Cav1.2 ($\alpha 1c$; L-type), Cav2.1 ($\alpha 1a$; P/Q-type), and Cav2.2 ($\alpha 1b$; N-type) in cultured hippocampal neurons treated for 48 h with either vehicle or LPS by quantitative immunoblotting. LPS significantly increased ($\sim 50\%$) the expression of the L-type Cav1.2 subunit, without altering the levels of the other VGCC subtypes (Fig. 7E,F).

This result confirmed previous data reporting a similar increase of the L-type VGCC expression in rat infused with LPS (Hopp et al., 2015a). However, it is still unclear whether such an increase can be ascribed to new protein synthesis or to a slowdown of channel turnover. qPCR analysis revealed that the mRNA coding for the Cav1.2 subunit of neurons treated for 24 h with LPS was unchanged with respect to vehicle-treated neurons (Fig. 7G). This result demonstrates that the LPS-induced increase in Cav1.2 expression is attributable to a slowdown of the protein turnover possibly because of a more efficient channel trafficking to the membrane, as also underlined by the increased L-type Ca^{2+} current density.

To confirm this hypothesis, we investigated whether chronic LPS treatment affected the membrane exposure of L-type VGCCs by surface biotinylation of neurons treated with vehicle, LPS, or LPS+CLI-095, respectively. LPS treatment significantly increased both the total and extracellular levels of L-type VGCCs compared with vehicle-treated neurons. This effect was abolished by TLR4 blockade with CLI-095 (Fig. 7H,I), demonstrating the role of TLR4 activation by LPS in the increased membrane exposure of L-type VGCCs.

LPS induces mislocalization of L-type Ca^{2+} channels at glutamatergic presynaptic terminals

To further demonstrate the abnormal localization of L-type Ca^{2+} channels at excitatory presynaptic terminals, vehicle- and LPS-treated neurons were double-immunostained for Cav1.2 subunits and the presynaptic marker vGLUT1. As previously shown with Bassoon staining, no LPS-induced changes were observed in the density of glutamatergic synapses (Fig. 8A,B).

Figure 6. Presynaptic L-type VGCCs participate in the LPS-induced increase of eEPSC amplitude. **A, C**, Representative EPSCs evoked in hippocampal autaptic neurons after 48 h treatment with either vehicle (black) or LPS (10 ng/ml; red) after the acute application of ω -agatoxin ($1 \mu\text{M}$, Agtx), ω -conotoxin ($1 \mu\text{M}$, Cntx), or nifedipine ($5 \mu\text{M}$, Nfd). Currents were evoked by stimulating the cell with a 0.5 ms step to 40 mV applied at 0.1 Hz ($V_h = -70$ mV). **B, D**, Mean ($\pm$ SEM) amplitude of eEPSCs evoked in autaptic neurons treated with either vehicle (black) or LPS (red) before and after perfusion with Agtx, Cntx, and Nfd (Agtx: veh, $n = 10$; LPS, $n = 9$; Cntx: veh, $n = 11$; LPS, $n = 14$; Nfd: veh, $n = 10$; LPS, $n = 12$). **E**, Percent eEPSC inhibition by Agtx, Cntx, and Nfd calculated from the recordings of **B, D**. In all traces, the stimulation artifacts were blanked for clarity. **F**, Pie chart of the contribution of the various VGCC subtypes to the eEPSC amplitude in vehicle-treated (black) and LPS-treated (red) neurons based on the experiments shown in **E**. Data are mean $\pm$ SEM. $**p < 0.01$; $***p < 0.001$; paired/unpaired Student's t test/Mann–Whitney U test.

When vGLUT1-positive puncta were immunostained for Cav1.2 subunits, we observed an increased area and percentage of colocalization of Cav1.2 with an increased overlap of the vGLUT1/Cav1.2 immunoreactivity intensity profiles in LPS-treated neurons (Fig. 8C).

All these data demonstrate that LPS changes the subcellular localization of L-type channels, spreading them to presynaptic terminals of excitatory neurons where they can participate in glutamate release.

Chronic LPS induces hyperactivity in hippocampal networks dependent on L-type Ca^{2+} channels

To study, at the network level, the impact of the LPS-induced mislocalization of L-type VGCCs at excitatory synapses, we used MEA recordings of the reverberating spontaneous electrical activity of primary hippocampal networks (14–17 DIV) treated with either vehicle or LPS for 48 h (Fig. 9A). Spontaneous network activity was investigated in terms of both sparse isolated

spikes and organized high-frequency bursts (Fig. 9B) (van Pelt et al., 2004; Chiappalone et al., 2007; Vajda et al., 2008). Chronic LPS treatment significantly increased the mean firing and bursting rates (Fig. 9C,D, left columns), while burst duration ($p = 0.763$), intraburst ISI ($p = 0.462$), and burst percentage ($p = 0.942$; veh = 58 and LPS = 56 for all parameters) were unaffected. Notably, the firing and bursting rates in neurons treated with LPS strongly decreased after perfusion with nifedipine and became lower than those recorded in vehicle-treated neurons (Fig. 9C,D, right). The much larger decrease of the firing and bursting rates induced by nifedipine blockade in LPS-treated neurons (Fig. 9E) confirms the abnormally higher contribution of L-type VGCCs to the excitatory strength and network activity in neurons chronically treated with LPS.

Discussion

It is widely demonstrated that a large variety of insults causing inflammation in the brain, such as brain trauma, tumors, and

Figure 7. Chronic LPS treatment increases the expression and the membrane exposure of L-type VGCCs. **A**, Representative voltage-gated Ca^{2+} currents recorded in excitatory hippocampal neurons treated for 48 h with either vehicle (back/gray traces) or LPS (red/brown traces; 10 ng/ml) in the absence (ctrl; black/red traces) or presence (gray/brown traces) of nifedipine (Nfd). **B**, Current density versus voltage relationships under the conditions described in **A** (veh: ctrl $n = 11$ /Nfd $n = 11$; LPS: ctrl $n = 13$ /Nfd $n = 13$). **C**, Percent inhibition by Nfd on the current density at -10 mV evoked in cells treated with either vehicle (left) or LPS (right). **D**, L-type current density versus voltage relationship in neurons treated with either vehicle (black line/symbols) or LPS (red line/symbols) obtained by subtracting the Nfd-insensitive current density from the total Ca^{2+} current density. **E**, Representative immunoblots of the expression levels of Cav1.2, Cav2.1, and Cav2.2 subunits in total lysates of primary hippocampal neurons at 13 DIV treated with either vehicle or LPS (10 ng/ml) for 48 h. GAPDH immunoreactivity was included as control of equal loading. **F**, Quantification of the expression of VGCC subtypes normalized on GAPDH expression and expressed as mean $\pm$ SEM percentages of the relative control ($n = 5$ independent preparations). **G**, Mean $\pm$ SEM of L-type VGCC mRNA determined by qPCR after 24 h of treatment with either vehicle or LPS ($n = 3$ independent preparations). **H**, Representative immunoblots of cell surface biotinylation performed in primary hippocampal neurons treated for 48 h with vehicle, LPS (10 ng/ml), or LPS + CLI-095 ($1 \mu\text{M}$). Total cell lysates (input), biotinylated (cell surface, extracellular), and nonbiotinylated (intracellular, intracellular) fractions were analyzed by immunoblotting. Na/K-ATPase and actin were included as markers of the plasma membrane and cytosolic fractions, respectively. **I**, L-type VGCC expression in the membrane and intracellular pools was normalized on Na/K-ATPase and actin expression ($n = 3$ independent preparations). Data are mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; one-way ANOVA/Bonferroni's tests and unpaired Student's t test/Mann–Whitney U test.

infections (Annegers et al., 1988, 1996; Allan and Rothwell, 2001; Singhi, 2011; Ngugi et al., 2013; Vezzani et al., 2016), predispose to the occurrence of seizures (Pitkänen and Sutula, 2002). In parallel, seizures could be a trigger of neuroinflammation by inducing proinflammatory cytokine genes (Minami et al., 1991). Albeit a tight correlation exists between neuroinflammation and

epilepsy (van Vliet et al., 2018; Vezzani et al., 2019), the cellular mechanisms by which neuroinflammation leads to neuronal hyperactivity are still unclear.

Here, we investigated the role of neuroinflammation in promoting neuronal hyperactivity using acute and chronic LPS treatments, a component of Gram-negative bacteria (Tanaka et

Figure 8. Long-lasting LPS treatment increases the expression of L-type Ca^{2+} channels at glutamatergic synapses. **A**, Top, Representative images of neurites of hippocampal neurons exposed to either vehicle or LPS for 48 h and double-immunostained with Cav1.2 antibodies (green) and the excitatory presynaptic marker vGLUT1 (red). Merge panels represent synaptic puncta in which Cav1.2 and vGLUT1 colocalize (yellow; arrows). Bottom, Representative immunoreactivity intensity profiles of the colocalization experiments showing the increased occurrence of overlap between Cav1.2- and vGLUT1-positive puncta in the samples chronically treated with LPS. **B**, Quantification of the density of vGLUT1-positive puncta counted on 30 μm dendrite tracts starting from the neuronal body. Data are mean $\pm$ SEM from three independent experiments, each conducted in duplicate (veh = 30; LPS = 29). **C**, Quantification of the absolute Cav1.2/vGLUT1 colocalization area (top) and of the Cav1.2/vGLUT1 colocalization expressed in percent of the total Cav1.2-immunopositive area (bottom) in vehicle- and LPS-treated neurons (veh = 66; LPS = 97). Data are mean $\pm$ SEM. ** $p < 0.01$ (unpaired Student's t test/Mann–Whitney U test).

al., 2006; Deng et al., 2012). Previous reports showed that LPS reduces the seizure threshold and increases neuronal death after status epilepticus (Sayyah et al., 2003; Heida and Pittman, 2005; Auvin et al., 2007; Galic et al., 2008) and that anti-inflammatory drugs rescue the proepileptogenic effects of LPS (Sayyah et al., 2003). The increased seizure propensity in response to LPS treatment could be because of an excitation/inhibition imbalance. Indeed, a potentiation of excitatory transmission was shown in hippocampal slices of mice chronically treated (3 weeks) with LPS (Galic et al., 2008), and even shorter *ex vivo* LPS treatment of acute hippocampal slices enhanced eEPSCs of CA1 pyramidal neurons in response to Shaffer collateral stimulation (Gao et al., 2014).

In this study, we found that long-term (24–96 h) treatment of primary hippocampal neurons with LPS increased eEPSC, but not eIPSC, amplitude, generating an excitation/inhibition imbalance in synaptic transmission. The LPS effect was absent during acute treatments, did not affect cell viability, neuronal morphology or total synaptic connectivity, and was mediated by TLR4 (Matsunaga et al., 2011).

Although TLR4 is expressed in both excitatory and inhibitory neurons, LPS effects were specific for excitatory synapses. However, we cannot exclude that longer LPS treatments could also affect inhibitory transmission, as described in hippocampal organotypic slices (Hellstrom et al., 2005). Importantly, the selectivity of LPS action for excitatory transmission was observed in very low-density autaptic hippocampal neurons, a condition that strongly limits paracrine effects of cytokines released by neighboring cells.

Using quantal analysis, we found that the LPS-induced eEPSC potentiation was entirely attributable to an increase in the probability of glutamate release. Consistently, various forms of

STP relying on Ca^{2+} buildup and depending on the initial P_r , such as facilitation, PTP, and synaptic depression, (Zucker and Regehr, 2002) were significantly affected by LPS. Asynchronous glutamate release following HFS, relying on Ca^{2+} buildup, was also increased in LPS-treated neurons, suggesting that an increased Ca^{2+} influx in response to AP is the common mechanism for all the effects of LPS on excitatory strength. Indeed, we found that chronic LPS treatment, via TLR4 activation, increases the expression and the membrane exposure of L-type VGCCs. It is well established that L-type VGCCs do not participate in neurotransmitter release under physiological conditions and that glutamate release induced by single APs is exclusively dependent on N-, P/Q-, and, to a lesser extent, R-type VGCCs (Wheeler et al., 1994, 1996; Scholz and Miller, 1995; Reid et al., 1997). On the contrary, we observed that chronic treatment with LPS favored the ectopic localization of L-type VGCCs at excitatory presynaptic terminals and their direct contribution to the increase of the P_r of glutamate SVs and of eEPSC amplitude. Indeed, in chronically LPS-treated neurons evoked glutamate release becomes sensitive to nifedipine, testifying the spread of L-type VGCCs from somatic and dendritic sites to excitatory presynaptic terminals (Ahlijanian et al., 1990; Westenbroek et al., 1990).

Both Cav2.1 and Cav2.2 channels, sustaining P/Q- and N-type Ca^{2+} currents, respectively, densely colocalize with syntaxin-1 at nerve terminals (Cohen et al., 1991; Westenbroek et al., 1992, 1995), from which they can be isolated as a complex with SNARE proteins (Bennett et al., 1992; Yoshida et al., 1992; Lvque et al., 1994). The SNARE proteins syntaxin-1A and SNAP-25 specifically interact with Cav2.2 and Cav2.1 channels by binding to the *synprint* region in the second intracellular loop. On the contrary, Cav1.2 channels, which conduct L-type Ca^{2+} current, are typically ineffective in supporting synaptic transmission. Interestingly, it

Figure 9. LPS-induced network hyperactivity depends on L-type VGCCs. **A**, Phase-contrast micrograph of a hippocampal network (14 DIV) cultured onto an MEA chip (top). Representative single-channel signals extracted from vehicle-treated (black trace) and LPS-treated (red trace) networks after high-pass filtering at 200 Hz (bottom). **B**, Raster plots of the spiking activity of vehicle-treated (left) and LPS-treated (right) cultures in a 30 s time window (top). Each bar represents a spike. Each horizontal line indicates an electrode. Magnification of 2 s of activity for both groups studied (bottom). **C**, **D**, Firing and bursting rates in networks treated for 48 h with either vehicle or LPS (basal) and acutely exposed to nifedipine (+ Nfd; $n = 3$ preparations, 58 independent chips for vehicle, 56 for LPS). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; two-way ANOVA/Bonferroni's tests. **E**, Percent inhibition of firing and bursting rates induced by Nfd in vehicle-/LPS-treated networks normalized to the basal condition ($n = 3$ preparations, $n = 26$ and $n = 23$ independent experiments for vehicle and LPS, respectively). Data are mean $\pm$ SEM. Intragroup and intergroup differences in the Nfd effects were evaluated using one-sample ($***p < 0.001$) and two-sample Student's t tests ($**p < 0.01$), respectively.

was reported that the insertion of the Cav2.1 *synprint* region in Cav1.2 is sufficient to target Cav1.2 to the synapse and establish synaptic transmission initiated by L-type Ca^{2+} currents (Mochida et al., 2003). Moreover, *synprint* peptides, by competitively inhibiting the interactions between SNAREs and Cav2.2/Cav2.1 channels, increase late asynchronous release by uncoupling of the N- and P/Q-type VGCCs from the active zone (Mochida et al., 1996). Thus, the lack of the *synprint* region in Cav1.2 channels may be responsible of the LPS-induced increased asynchronous release because of a “loose” coupling of the channels to the active zone.

Previous reports have highlighted that neuroinflammation may lead to neuronal Ca^{2+} dysregulation and trigger excitotoxicity (Vezzani and Viviani, 2015; Calvo-Rodríguez et al., 2017). It has been reported that elevated levels of proinflammatory cytokines and oxidative stress can increase the L-type VGCCs activity

and ryanodine receptor expression (Furukawa and Mattson, 1998; Hopp et al., 2015a). Ca^{2+} dysregulation resulting from increased L-type VGCCs activity was also observed in pacemaker neurons of the substantia nigra pars compacta and locus ceruleus in experimental models of Parkinson's disease (Surmeier et al., 2011; Surmeier and Schumacker, 2013). Indeed, inhibition of L-type VGCCs improves motor scores of Parkinsonian patients (Ilijic et al., 2011) and has neuroprotective effects in the substantia nigra and locus ceruleus of LPS-induced rat model of chronic neuroinflammation (Hopp et al., 2015b). Similar increases in Ca^{2+} influx via L-type VGCCs were previously reported in aging associated with brain inflammation (Tamaru et al., 1991; Thibault and Landfield, 1996; Veng et al., 2003; Norris et al., 2010; Núñez-Santana et al., 2014). The “ Ca^{2+} hypothesis” of neurodegeneration holds that an excessive Ca^{2+} influx through somatic and postsynaptic L-type VGCCs eventually alters

activity-dependent gene expression impacting on long-term plasticity and higher brain functions (Landfield and Pitler, 1984; Núñez-Santana et al., 2014; Oh et al., 2016; for review see Navakkode et al., 2018). While we confirm that neuroinflammation enhances L-type VGCCs expression and somatic Ca^{2+} currents, we uncover an abnormal contribution of L-type VGCCs to excitatory synaptic strength and plasticity as a novel mechanism of LPS-induced inflammation acting at the presynaptic level.

Epileptic seizures in neuroinflammation are generated by an initially localized hyperexcitability that spreads into neuronal networks and is not restricted by inhibitory mechanisms (Gullo et al., 2014; Vezzani et al., 2019). Indeed, chronic LPS treatment increased the “seizure-like” propensity in primary networks, an effect that was markedly sensitive to blockade of L-type channels. These results confirm, at the network level, that the excitation/inhibition imbalance induced by LPS plays a crucial role in the enhancement of network excitability under proinflammatory conditions.

In conclusion, our results show that LPS modulation of glutamatergic transmission in the hippocampus increases glutamate P_r . This effect dramatically modifies the STP properties and thereby the filtering activity of excitatory synapses. While excitatory synapses under control conditions display a very low initial P_r and operate as high-pass filters (Abbott and Regehr, 2004), LPS switches them to a low-pass filter mode. These properties are of particular interest in light of the reported reduction of β/γ oscillations (20–80 Hz) observed with multielectrode EEG recordings in freely moving rats treated with LPS (Albrecht et al., 2018). Thus, our data offer a mechanistic explanation to the increased seizure susceptibility and disruption of synchronized oscillatory activity that are associated with neuroinflammation.

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