Hyperammonemias alters membrane expression of GluA1 and GluA2 subunits of AMPA receptors in hippocampus by enhancing activation of the IL-1 receptor: underlying mechanisms

Lucas Taoro-Gonzalez*, Yaiza M. Arenas, Andrea Cabrera-Pastor and Vicente Felipo

Abstract

Background: Hyperammonemic rats reproduce the cognitive alterations of patients with hepatic encephalopathy, including altered spatial memory, attributed to altered membrane expression of AMPA receptor subunits in hippocampus. Neuroinflammation mediates these cognitive alterations. We hypothesized that hyperammonemia-induced increase in IL-1β in hippocampus would be responsible for the altered GluA1 and GluA2 membrane expression. The aims of this work were to (1) assess if increased IL-1β levels and activation of its receptor are responsible for the changes in GluA1 and/or GluA2 membrane expression in hyperammonemia and (2) identify the mechanisms by which activation of IL-1 receptor leads to altered membrane expression of GluA1 and GluA2.

Methods: We analyzed in hippocampal slices from control and hyperammonemic rat membrane expression of AMPA receptors using the BS3 cross-linker and phosphorylation of the GluA1 and GluA2 subunits using phosphor-specific antibodies. The IL-1 receptor was blocked with IL-1Ra, and the signal transduction pathways involved in modulation of membrane expression of GluA1 and GluA2 were analyzed using inhibitors of key steps.

Results: Hyperammonemia reduces GluA1 and increases GluA2 membrane expression and reduces phosphorylation of GluA1 at Ser831 and of GluA2 at Ser880. Hyperammonemia increases IL-1β, enhancing activation of IL-1 receptor. This leads to activation of Src. The changes in membrane expression of GluA1 and GluA2 are reversed by blocking the IL-1 receptor with IL-1Ra or by inhibiting Src with PP2.

After Src activation, the pathways for GluA2 and GluA1 diverge. Src increases phosphorylation of GluN2B at Tyr14721 and membrane expression of GluN2B in hyperammonemic rats, leading to activation of MAP kinase p38, which binds to and reduces phosphorylation at Thr560 and activity of PKCζ, resulting in reduced phosphorylation at Ser880 and enhanced membrane expression of GluA2.

Increased Src activity in hyperammonemic rats also activates PKCδ which enhances phosphorylation of GluN2B at Ser1303, reducing membrane expression of CaMKII and phosphorylation at Ser831 and membrane expression of GluA1.

Conclusions: This work identifies two pathways by which neuroinflammation alters glutamatergic neurotransmission in hippocampus. The steps of the pathways identified could be targets to normalize neurotransmission in hyperammonemia and other pathologies associated with increased IL-1β by acting, for example, on p38 or PKCδ.

Keywords: Neuroinflammation, IL-1β, IL-1 receptor, Hyperammonemia, Hepatic encephalopathy, AMPA receptors, GluA1, GluA2, Membrane expression, Neurotransmission

* Correspondence: ltaoro@cipf.es
Laboratory of Neurobiology, Centro de Investigacion Principe Felipe, Eduardo Primo Yufera 3, 46012 Valencia, Spain

© The Author(s). 2018 Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Highlights

- Hyperammonemia reduces GluA1 and increases GluA2 membrane expression in hippocampus
- Hyperammonemia reduces phosphorylation of GluA1 in Ser831 and of GluA2 in Ser880
- These effects are mediated by IL-1β and activation of IL-1 receptor and Src kinase
- Changes in GluA2 are mediated by changes in Src, GluN2B, MAP kinase p38, and PKCζ
- Changes in GluA1 are mediated by changes in Src, GluN2B, CaMKII, and PKCζ

Background

Patients with chronic liver diseases (cirrhosis, hepatitis...) may present hepatic encephalopathy with cognitive and motor alterations including attention deficits, mild cognitive impairment, and reduced spatial memory [1–4]. Hyperammonemia and inflammation are the main contributors to the neurological alterations in hepatic encephalopathy [5, 6]. Rats with chronic hyperammonemia similar to that present in patients with liver cirrhosis also show cognitive alterations, including impaired spatial learning and memory [7, 8]. Chronic hyperammonemia per se induces neuroinflammation, which mediates the cognitive alterations in rats [8, 9]. Neuroinflammation is also a main contributor to cognitive deficits in many chronic (e.g., cirrhosis, diabetes), mental (e.g., schizophrenia), and neurodegenerative (e.g., Alzheimer’s) diseases and in situations such as post-operative cognitive dysfunction or aging [10–15]. Neuroinflammation-induced cognitive impairment is therefore a highly and increasingly prevalent situation with serious health, social, and economic consequences. Neuroinflammation alters cognitive function by altering neurotransmission [15, 16]. The mechanisms by which neuroinflammation alters neurotransmission may share common aspects in different pathologies. Unveiling these mechanisms may provide therefore the bases to design new therapeutic approaches which could be applied in different highly prevalent pathologies.

Neuroinflammation impairs spatial learning by mechanisms which are beginning to be unveiled.

Sustained expression of IL-1β in hippocampus impairs spatial learning and memory [17, 18].

Spatial learning is mainly modulated by NMDA and AMPA receptors for glutamate in hippocampus [19, 20]. A main mechanism modulating glutamatergic neurotransmission and synaptic plasticity in hippocampus is the modulation of membrane expression of AMPA receptors, which is mainly mediated by changes in phosphorylation of the GluA1 subunit in Ser831 and Ser845 and of the GluA2 in Ser880 [21–26].

Neuroinflammation alters membrane expression of glutamate (AMPA, NMDA) and GABA receptors in hippocampus and impairs spatial learning [17, 27, 28]. Lai et al. [29] showed that exposure to IL-1β reduces phosphorylation in Ser831 and membrane expression of GluA1 in hippocampal neurons and this was prevented by IL-1Ra, an antagonist of IL-1 receptors. Machado et al. [30] also found that IL-1β reduced phosphorylation of GluA1 subunit at Ser831 and Ser845 60 min after contextual fear memory reactivation and that intra-hippocampal administration of IL-1β after memory reactivation also induced a decrease in surface expression and total expression of GluA1.

We have recently proposed that impaired spatial learning and memory in rats with hepatic encephalopathy due to portacaval shunts would be due to the increased levels of IL-1β in hippocampus [28]. We have also shown that both rats with hepatic encephalopathy and rats with hyperammonemia without liver failure show neuroinflammation, with increased levels of IL-1β and other pro-inflammatory markers, and altered membrane expression of AMPA receptor subunits GluA1 and GluA2 in hippocampus [7, 28, 31].

We hypothesize that hyperammonemia-induced increase in IL-1β in hippocampus would be responsible for the altered membrane expression of GluA1 and GluA2 subunits of AMPA receptors. The aims of this work were to (1) assess if increased IL-1β levels and activation of its receptor (IL-1R) are responsible for the changes in GluA1 and/or GluA2 membrane expression in hyperammonemia and (2) identify the mechanisms by which activation of IL-1R leads to altered membrane expression of GluA1 and GluA2.

The model of chronic hyperammonemia in rats used consisted in administering them an ammonium-containing diet as described in [32]. Membrane expression and phosphorylation of the GluA1 and GluA2 were analyzed in freshly isolated hippocampal slices from control and hyperammonemic rats. To assess the role of IL-1β in the changes in GluA1 and GluA2, we tested whether blocking the IL-1β receptor with the endogenous antagonist IL-1Ra reverses these changes. We also analyzed the intracellular pathways mediating the effects of IL-1β by assessing the effects of modulating different steps on GluA1 and GluA2 phosphorylation and membrane expression.

Methods

Model of chronic hyperammonemia

Male Wistar rats (120–140 g at the beginning of the diet, Charles River Laboratories, Barcelona, Spain) were made hyperammonemic by feeding them a diet containing standard diet supplemented with ammonium acetate as in [32]. In the present work, the diet contained 25% of ammonium acetate instead of 20% as in [32]. This change was made because now we house the rats in...
ventilated racks which were not used in [32]. These racks extract some ammonia, and the increase in ammonium acetate in the diet was necessary to reproduce the ammonia levels in blood obtained previously in un-ventilated cages using 20% ammonium acetate. The ammonium-containing diet was prepared as described in [33] (but using 25% ammonium acetate) and increased blood ammonia levels around threefold, an increase similar to that found in patients with liver cirrhosis. Rats remain hyperammonemic for long periods of time and reproduce many cognitive and motor alterations present in cirrhotic patients with hepatic encephalopathy and has allowed identifying some underlying mechanisms [7–9, 34–36]. Experiments were performed after 4–5 weeks in the ammonium diet, when the rats are 10–11 weeks old. The experiments were approved by the Comité de Ética y Bienestar en Experimentsion Animal, Prince Felipe Research Center-Consellería de Agricultura, Generalitat Valenciana, and carried out in accordance with the European Communities Council Directive (86/609/EEC).

**Analysis of protein content and phosphorylation in hippocampal slices by western blot**
In each experiment, four rats (two control and two hyperammonemic rats) were decapitated at 4–5 weeks of hyperammonemia and their brains quickly transferred to a plate where they were carefully dissected, separating the two hemispheres by cutting through the midline and extracting both hippocampi using thin spatulas. Once dissected, hippocampi were immersed immediately into ice-cold Krebs buffer (in mmol/L): NaCl 119, KCl 2.5, KH₂PO₄ 1, NaHCO₃ 26.2, CaCl₂ 2.5, and glucose 11, aerated with 95% O₂ and 5% CO₂ at pH 7.4. After that, hippocampi were placed longitudinally on a manual chopper and cut to obtain transverse slices (400 μm). Slices were transferred to incubation wells (15 slices in each well, from the two rats per group) without any differentiation between dorsal or ventral hippocampus and incubated for 20 min at 35.5 °C in Krebs buffer for stabilization. One hundred nanogram per milliliter of IL1Ra (R&D Systems cat# 1545-RA-025, Minneapolis, USA), 100 μM ifenprodil (Abcam cat# ab120111, Cambridge, MA), 20 μM SB239063 (Tocris cat# 1962, Bristol, UK), 10 μM rottlerin (Sigma cat# R5648, Darmstadt, Germany), or 10 μM PP2 (Sigma cat# P0042, Darmstadt, Germany) was added to the incubation buffer during 30 min to specifically inhibit IL-1 receptor, GluN2B, p38, PKCδ, and Src activities, respectively. After the treatments, slices were collected and homogenized by sonication for 20 s in a buffer (Tris-HCl 66 mM pH 7.4, SDS 1%, EGTA 1 mM, glycerol 10%, leupeptin 0.2 mg/mL, NaF 1 mM, Na orto-vanadate 1 mM). Samples were subjected to immunoblotting as in Felipo et al. [37], using antibodies against IL-1β (1:500, cat# AF-501-NA) from R&D Systems; Src (1:1000, cat# ab47405), Src phosphorylated at Tyr416 (1:1000, cat# ab40660), GluN2B phosphorylated at Ser1303 (1:1000, cat# ab81271), GluA2 phosphorylated at Ser880 (1:2000, cat# ab52180), and PKCζ phosphorylated at Thr560 (1:1000, cat# ab59412) from Abcam; GluN2B (1:1000, cat# 06-600), GluN2B phosphorylated at Tyr1472 (1:1000, cat# AB5403), GluA1 (1:1000, cat# 04-855), GluA1 phosphorylated at Ser831 (1:1000, cat# 04-823), and GluA2 (1:2000, cat# AB1768) from Millipore (Darmstadt, Germany); p38 (1:1000, cat# 9212) and p38 phosphorylated at Thr180/Tyr182 (1:500, cat# 9211) from Cell Signaling (Leiden, Netherlands); and PKCζ (1:2000, cat# sc-17,781) from Santa Cruz (Dallas, TX). As a control for protein loading, the same membranes used to quantify the amount of proteins were incubated with an antibody against Actin (1:5000, cat# ab6276) from Abcam. Secondary antibodies were anti-rabbit (cat# A8025), anti-goat (cat# A7650), or anti-mouse (cat# A3562) IgG, 1:4000 dilution conjugated with alkaline phosphatase from Sigma (St. Louis, MO). The images were captured using the ScanJet 5300C (Hewlett-Packard, Amsterdam, Netherlands), and band intensities quantified using the Alpha Imager 2200, version 3.1.2 (Alphalnnotech Corporation, San Francisco). Phosphorylation levels were normalized to the total amount of the respective proteins.

**Analysis of surface expression of receptors or CaMKII by cross-linking with BS3**
Membrane surface expression of the GluA1 and GluA2 subunits of AMPA receptors, GluN2B subunit of NMDA receptors, and CaMKII (1:1000 primary antibody from Thermo Fisher cat# MA1-048, Waltham, MA) in whole hippocampal slices was analyzed at 4–5 weeks of hyperammonemia as described by Boudreau and Wolf [38], by cross-linking with BS3 (bis(sulfosuccinimidyl) suberate, Pierce cat# 21580, Rockford, IL). After the treatments (see above), slices were added to tubes containing ice-cold Krebs buffer with or without 2 mM BS3 and incubated for 30 min at 4 °C with gentle shaking. Cross-linking was terminated by quenching the reaction with 100 mM glycine (10 min, 4 °C). The slices were homogenized by sonication for 20 s. Samples treated or not with BS3 were analyzed by western blot as describe above. The surface expression of each subunit was calculated as the difference between the intensity of the bands without BS3 (total protein) and with BS3 (non-membrane protein) as described by Cabrera-Pastor et al. [39].

**Statistical analysis**
Results are expressed as mean ± standard error. All statistical analyses were performed using the software program GraphPad Prism. Normality was assessed using the D’Agostino and Pearson Omnibus test and the Shapiro-Wilk normality tests. Differences in variances of normally
distributed data were assessed using Bartlett’s test. Data were analyzed by a parametric two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test to determine the individual and interaction effects between hyperammonemia and/or treatments on membrane expression and phosphorylation levels of proteins [40]. A confidence level of 95% was accepted as significant.

Results
Hyperammonemia alters membrane expression and phosphorylation of the GluA1 and GluA2 subunits of AMPA receptors in hippocampus
Membrane expression of the GluA1 and GluA2 subunits of AMPA receptors was altered in opposite ways in hippocampus of hyperammonemic rats. Membrane expression of GluA1 was reduced ($p < 0.05$) to 86 ± 6% in control rats (Fig. 1a), while membrane expression of GluA2 was increased ($p < 0.001$) to 136 ± 7% in control rats (Fig. 1b).

Changes in membrane expression were associated with changes in phosphorylation. Phosphorylation of GluA1 in Ser831 was reduced ($p < 0.001$) in hyperammonemic rats to 73 ± 7% in control rats (Fig. 1c), and phosphorylation of GluA2 in Ser880 was reduced ($p < 0.05$) to 84 ± 4% in control rats (Fig. 1d).

Enhanced activation of IL-1β receptor mediates the changes in membrane expression and phosphorylation of GluA1 and GluA2 in hyperammonemic rats
As it has been shown that IL-1β modulates membrane expression of AMPA receptors, we assessed its possible contribution to the effects of hyperammonemia. In hyperammonemic rats, the content of IL-1β in hippocampus was increased to 129 ± 8% in control rats ($p < 0.05$).

**Fig. 1** Blocking IL-1 receptor with IL-1Ra normalizes both phosphorylation and membrane expression of the GluA1 and GluA2 subunits in hyperammonemic rats. IL-1Ra, an antagonist of IL-1 receptor, was added to hippocampal slices. Membrane expression of GluA1 (a) and GluA2 (b) subunits and phosphorylation of GluA1 at Ser831 (c) and of GluA2 at Ser880 (d) were analyzed as described in the "Methods" section. Values are expressed as percentage of basal levels in control rats and are the mean ± SEM of 24, 34, 31, and 18 rats per group in a, b, c, and d respectively. Data were analyzed by two-way ANOVA. In a, F (1, 92) = 0.004596 for effect of HA, $p = 0.9461$; F (1, 92) = 11.77 for effect of IL1Ra, $p = 0.0009$; and F (1, 92) = 0.0368 for interaction, $p = 0.0368$. In b, F (1, 132) = 4.893 for effect of HA, $p = 0.0287$; F (1, 132) = 41.17 for effect of IL1Ra, $p < 0.0001$; and F (1, 132) = 9.953 for interaction, $p = 0.0020$. In c, F (1, 118) = 1.122 for effect of HA, $p = 0.2915$; F (1, 118) = 11.77 for effect of IL1Ra, $p = 0.0009$; and F (1, 118) = 15.73 for interaction, $p = 0.0001$. In d, F (1, 68) = 1.028 for effect of HA, $p = 0.3142$; F (1, 68) = 4.783 for effect of IL1Ra, $p = 0.0322$; and F (1, 68) = 22.11 for interaction, $p < 0.0001$. Values significantly different from control rats are indicated by asterisk and from hyperammonemic rats are indicated by "a". Bonferroni post-test: *$p < 0.05$, **$p < 0.001$, *a*$p < 0.001$
To assess if changes in membrane expression of GluA1 and GluA2 are due to enhanced activation of IL-1 receptor, we tested if blocking this receptor with the endogenous antagonist IL-1Ra reverses these changes. Perfusion of IL-1Ra increased \((p < 0.001)\) membrane expression of GluA1 in hippocampal slices of hyperammonemic rats, reaching \(124 \pm 7\%\) in control rats (Fig. 1a), in parallel with an increase \((p < 0.001)\) of phosphorylation of GluA1 in Ser831 to \(140 \pm 16\%\) in control rats (Fig. 1c).

IL-1Ra reduced \((p < 0.001)\) membrane expression of GluA2 to the same levels in control rats (Fig. 1b) in parallel with a normalization of phosphorylation of GluA2 at Ser880 (Fig. 1d).

These data show that blocking IL-1 receptor with IL-1Ra reverses the changes induced by hyperammonemia on GluA1 and GluA2 phosphorylation and membrane expression, suggesting that enhanced activation of IL-1 receptor is responsible for these alterations in hippocampus of hyperammonemic rats.

**Activation of Src kinase mediates the effects of IL-1β on GluA1 and GluA2**

We then looked for the intracellular pathways mediating the changes in membrane expression induced by enhanced activation of IL-1Ra. As activation of IL-1 receptor leads often to increased phosphorylation and activity of Src kinase [41], we tested whether it is increased in hyperammonemic rats. Phosphorylation of Src at Tyr416 is increased \((p < 0.05)\) in hyperammonemic rats to \(118 \pm 8\%\) in control rats, and blocking IL-1 receptor with IL-1Ra normalizes it, returning to \(77 \pm 13\%\) in control rats (Fig. 2a).

To assess if enhanced activation of Src mediates the changes in membrane expression of GluA1 and GluA2 in hyperammonemic rats, we tested if inhibiting Src with PP2 reverses these changes.

Perfusion of PP2 increased \((p < 0.001)\) membrane expression of GluA1 in hippocampal slices of hyperammonemic rats, reaching \(134 \pm 20\%\) in control rats (Fig. 2b), in parallel with an increase \((p < 0.05)\) of phosphorylation of GluA1 in Ser831 to \(103 \pm 13\%\) in control rats (Fig. 2c).

PP2 reduced \((p < 0.001)\) membrane expression of GluA2 to the same levels in control rats (Fig. 2d) in parallel with an increase of phosphorylation of GluA2 at Ser880 (Fig. 2e).

These data suggest that enhanced activation of Src contributes to altered phosphorylation and membrane expression of GluA1 and GluA2 in hippocampus of hyperammonemic rats.

**GluN2B, p38, and PKCζ mediate the effects of IL-1β and Src on GluA2 but not on GluA1**

On the bases of the knowledge in the literature (see the “Discussion” section), we hypothesized that Src would be altering GluA1 and GluA2 membrane expression by modulating the GluN2B subunit of NMDA receptors. Src phosphorylates GluN2B at Tyr1472 and this increases its membrane expression [42].

As shown in Fig. 3, hyperammonemia increases phosphorylation \((p < 0.05)\) of GluN2B at Tyr1472 to \(122 \pm 10\%\) in control rats (Fig. 3a) and membrane expression \((p < 0.001)\) of GluN2B to \(151 \pm 10\%\) in control rats (Fig. 3b).

Blocking IL-1 receptor with IL-1Ra normalized both phosphorylation (Fig. 3a) and membrane expression (Fig. 3b) of GluN2B in hyperammonemic rats.

Similar effects were obtained in experiments using the Src inhibitor PP2, which normalized both phosphorylation (Fig. 3c) and membrane expression (Fig. 3d) of GluN2B in hyperammonemc rats.

It has been reported that enhanced membrane expression of GluN2B leads to enhanced phosphorylation and activity of the MAP kinase p38 [43]. We therefore assessed whether phosphorylation of p38 was increased in hippocampus of hyperammonemic rats. Phosphorylation of p38 was increased \((p < 0.05)\) to \(124 \pm 7\%\) in control rats (Fig. 4a) and was normalized by treatment with IL-1Ra (Fig. 4a), the Src inhibitor PP2 (Fig. 4b), or with ifenprodil (Fig. 4c), a selective antagonist of NMDA receptors containing the GluN2B subunit [44].

To assess if enhanced activation of p38 mediates the changes in membrane expression of GluA1 and GluA2 in hyperammonemic rats, we tested if inhibiting p38 with SB239063 reverses these changes.

Perfusion of SB239063 reduced \((p < 0.01)\) membrane expression of GluA2 to the same levels in control rats (Fig. 4d) in parallel with an increase of phosphorylation of GluA2 at Ser880 (Fig. 4e). However, SB239063 did not normalize phosphorylation (Fig. 4g) or membrane expression (Fig. 4f) of the GluA1 subunit.

These data suggest that enhanced activation of p38 contributes to altered phosphorylation and membrane expression of GluA2 but not of GluA1 in hippocampus of hyperammonemc rats.

The GluA2 subunit is phosphorylated at Ser880 by protein kinase C (PKC) [45]. The above data suggest that enhanced p38 activity would reduce the activity of some isoform of PKC, resulting in reduced phosphorylation of Ser880 and increased membrane expression of GluA2. It has been shown that activated p38 binds to PKCζ, and this prevents auto-phosphorylation of PKCζ at Thr560, thus reducing its activity [46].

We therefore tested whether phosphorylation of PKCζ at Thr560 is altered in hyperammonemic rats. As shown in Fig. 5, phosphorylation of PKCζ at Thr560 was reduced \((p < 0.05)\) to \(83 \pm 6\%\) in control rats and was normalized...
by treatment with IL-1Ra (Fig. 5a), the Src inhibitor PP2 (Fig. 5b), or by blocking GluN2B with ifenprodil (Fig. 5c).

Altogether, the above data allow proposing the pathway shown in Fig. 6a for the mechanism by which hyperammonemia increases membrane expression of the GluA2 subunit of AMPA receptors in hippocampus. Hyperammonemia increases IL-1β, enhancing activation of IL-1 receptor. This leads to activation of Src, reflected in increased phosphorylation of Tyr416. Src in turn enhances phosphorylation of GluN2B at Tyr14721 and membrane expression of GluN2B, which leads to activation of p38. Activated p38 binds to and reduces phosphorylation at Thr560 and activity of PKCζ, thus resulting in reduced phosphorylation at Ser880 and enhanced membrane expression of GluA2.

CaMKII and PKCδ mediate the effects of IL-1β and Src on GluA1

The above results show that changes in phosphorylation at Ser831 and in membrane expression of GluA1 are
also mediated by enhanced activation of IL-1 receptor and of Src, but not by the p38-PKC pathway. As Ser831 may be phosphorylated by PKC or by CaMKII [47, 48], we hypothesized that changes in GluA1 would be mediated by CaMKII.

We then assessed if hyperammonemic alters the amount of CaMKII in the membrane. The amount of CaMKII in the membrane is strongly reduced \( p < 0.01 \) in membrane of hippocampal slices from hyperammonemic rats, to 54 ± 10% in control rats (Fig. 7). Moreover, this decrease is reversed by treatment with IL-1Ra (Fig. 7a) or by inhibiting Src with PP2 (Fig. 7b).

One of the mechanisms of modulation of membrane expression of CaMKII is phosphorylation of GluN2B at Ser1303. Enhancing this phosphorylation reduces membrane expression of CaMKII [49]. Phosphorylation of GluN2B at Ser1303 is increased in hyperammonemic rats (Fig. 7c, d). Moreover, blocking IL-1 receptor with IL-1Ra (Fig. 7c) or inhibiting Src with PP2 (Fig. 7d) normalizes phosphorylation of GluN2B at Ser1303. This suggests that increased phosphorylation of GluN2B at Ser1303 in hyperammonemic rats leads to reduced membrane expression of CaMKII which, in turn, reduces phosphorylation at Ser831 and membrane expression of GluA1.

Increased phosphorylation of GluN2B at Ser1303 is mediated by IL-1 receptor and by Src. However, Src is a tyrosine kinase, and may not phosphorylate Ser residues. This suggests that a serine-threonine kinase would mediate the effects of Src on phosphorylation of GluN2B at Ser1303 [50]. It has been reported that Src phosphorylates PKC\( \delta \) at Tyr311, enhancing its activity [51].

To assess if increased activity of PKC\( \delta \) mediates the increase in phosphorylation of GluN2B at Ser1303 in hyperammonemic rats, we analyzed whether a specific inhibitor of PKC\( \delta \) (rottlerin) reverses this increase. Treatment with rottlerin completely reversed the increase in

---

**Fig. 3** IL-1 receptor and Src activation lead to increased phosphorylation at Tyr1472 and membrane expression of the GluN2B subunit in hyperammonemic rats. IL-1Ra or PP2, an inhibitor of Src kinase, were added to hippocampal slices. Phosphorylation of GluN2B at Tyr1472 (a, c) and membrane expression of GluN2B (b, d) were analyzed as described in the “Methods” section. Values are expressed as percentage of basal levels in control rats and are the mean ± SEM of 27, 37, 28, and 37 rats per group in a, b, c, and d respectively. Data were analyzed by two-way ANOVA. In a, F (1, 104) = 0.3170 for effect of HA, p = 0.5746; F (1, 104) = 5.592 for effect of IL-1Ra, p = 0.0199; and F (1, 104) = 9.208 for interaction, p = 0.0030. In b, F (1, 143) = 3.642 for effect of HA, p = 0.0583; F (1, 143) = 10.33 for effect of IL-1Ra, p = 0.0016; and F (1, 143) = 9.704 for interaction, p = 0.0022. In c, F (1, 106) = 1.727 for effect of HA, p = 0.1917; F (1, 106) = 6.991 for effect of PP2, p = 0.0094; and F (1, 106) = 1.457 for interaction, p = 0.2302. In d, F (1, 143) = 8.814 for effect of HA, p = 0.0035; F (1, 143) = 5.432 for effect of PP2, p = 0.0212; and F (1, 143) = 4.963 for interaction, p = 0.0275. Values significantly different from control rats are indicated by asterisk and from hyperammonemic rats are indicated by "a". Bonferroni post-test: *p < 0.05, **p < 0.001, ***p < 0.001, a*p < 0.05, a**p < 0.01, a***p < 0.001
phosphorylation of GluN2B at Ser1303 in hyperammonemic rats (Fig. 8a). Moreover, rottlerin also increased membrane expression of CaMKII (Fig. 8b) even above (178 ± 75%) in control rats. The increase of CaMKII in the membrane was associated with an increase, also above control rats, of phosphorylation of GluA1 at Ser831 (142 ± 11% in controls, Fig. 8c) and of membrane expression of GluA1 (140 ± 37% in controls, Fig. 8d).

The above data allow proposing the pathway shown in Fig. 6b for the mechanism by which hyperammonemia reduces membrane expression of the GluA1 in hippocampus. Hyperammonemia increases IL-1β, enhancing activation of IL-1 receptor. This leads to activation of Src, reflected in increased phosphorylation of Tyr416. Src in turn activates PKCδ which enhances phosphorylation of GluN2B at Ser1303, reducing membrane expression of CaMKII and phosphorylation at Ser831 and membrane expression of GluA1.

**Discussion**

There is increasing evidence that many pathological situations, including neurodegenerative and chronic...
diseases, lead to neuroinflammation which, in turn, is a main contributor to cognitive impairment in these situations. Neuroinflammation would impair cognitive function by altering neurotransmission. Unveiling the mechanisms by which neuroinflammation alters neurotransmission would allow identifying pathways and therapeutic targets to try to reverse the alterations in neurotransmission and therefore in cognitive function in different pathological situations.

In this study, we have identified two pathways by which neuroinflammation alters membrane expression of the GluA2 and GluA1 subunits of AMPA receptors, respectively, in hippocampus of hyperammonemic rats. It is shown that hyperammonemia increases IL-1β and activation of its receptor, leading to activation of Src which increases phosphorylation at Tyr1472 and membrane expression of GluN2B, which leads to activation of p38. Activated p38 binds to and reduces phosphorylation at Thr560 and activity of PKCζ. This is associated with reduced phosphorylation of GluA2 at Ser880 and enhanced membrane expression of GluA2. On the other hand, activated Src also increases phosphorylation of PKCδ which enhances phosphorylation of GluN2B at Ser1303, reducing membrane expression of CaMKII and phosphorylation at Ser831 and membrane expression of GluA1.

Modulation of the membrane expression of AMPA receptors plays a main role in synaptic plasticity in hippocampus [52]. Long-term potentiation (LTP) is mediated by enhanced and long-term depression (LTD) by reduced membrane expression of AMPA receptors [53, 54]. LTP in hippocampus is considered the bases for spatial learning and memory [55]. Both LTP in hippocampus [56] and spatial learning and memory [7] are impaired in hyperammonemic rats. Altered modulation of GluA1 and GluA2 membrane expression in hyperammonemic rats would be a main contributor to the impairment of LTP and spatial learning.

We show here that the alterations in membrane expression of GluA1 and GluA2 in hyperammonemic rats are a consequence of neuroinflammation and of enhanced activation of IL-1 receptor by increased levels of IL-1β. It has been already shown that high levels of IL-1β impair LTP [57]. Altogether, these data support that in hyperammonemic rats, (and likely in other pathological situations) increased levels of IL-1β in hippocampus alter membrane expression of GluA1 and GluA2 subunits of AMPA receptors, which would lead to impairment of LTP and of spatial learning and memory.

We also identify the intracellular signal transduction pathways by which activation of IL-1 receptor leads to changes in phosphorylation and to opposite effects on membrane expression of GluA1 and GluA2. These pathways are presented in Fig. 6 and in the graphical abstract.

Hyperammonemia increases IL-1β, enhancing activation of IL-1 receptor. This leads to activation of Src, reflected in increased phosphorylation of Tyr416. These steps are common to the pathways leading to altered membrane expression of the GluA1 and GluA2 subunits. The changes in membrane expression of both GluA1 and GluA2 are reversed by blocking the IL-1 receptor with IL-1Ra or by inhibiting Src with PP2, thus...
confirming the contribution of these steps to the changes in membrane expression.

However, after Src activation, the pathways diverge. The enhanced activity of Src in hyperammonemic rats results in increased phosphorylation of GluN2B at Tyr1472 and membrane expression of GluN2B, which leads to activation of p38. Activated p38 binds to and reduces phosphorylation at Thr560 and activity of PKCζ, resulting in reduced phosphorylation at Ser880 and enhanced membrane expression of GluA2. Src also activates PKCδ which enhances phosphorylation of GluN2B at Ser1303, reducing membrane expression of CaMKII and phosphorylation at Ser831 and membrane expression of GluA1.

Altered membrane expression of GluA1 is also mediated by activation of IL-1 receptor and Src, but after this step, the pathway is different than for GluA2. Increased activity of Src in hyperammonemic rats also activates PKCδ which enhances phosphorylation of GluN2B at Ser1303, reducing membrane expression of CaMKII and phosphorylation at Ser831 and membrane expression of GluA1. These changes are reversed by blocking the IL-1 receptor with IL-1Ra and by inhibiting Src with PP2 or PKCδ with rottlerin, thus supporting the contribution of the pathway depicted in Fig. 6b in the changes in membrane expression of GluA1. Some reports in the literature support the existence of the steps proposed in Fig. 6b. It has already been reported that Src phosphorylates PKCδ at Tyr311, enhancing its activity [59], and that enhancing phosphorylation GluN2B at Ser1303 reduces membrane expression of CaMKII [49]. We show here that all these steps are induced sequentially in hyperammonemic rats by activation of IL-1 receptor, leading to increased membrane expression of GluA2.

Moreover, these steps would occur in neurons and not in astrocytes. This is supported by the report of Srinivasan et al. [58], who showed that IL-1β activates the p38 signaling pathway in hippocampal neurons, in contrast to the activation of NF-κB in hippocampal astrocytes, demonstrating cell type-specific signaling responses to IL-1 in the brain and yielding distinct functional responses. However, Srinivasan et al. [58] did not test if activation of p38 by IL-1β is a direct effect or if it is mediated by some previous steps. We show here that increased phosphorylation of p38 is reduced by blocking the IL-1 receptor, by inhibiting Src, or by blocking the NR2B subunit of NMDA receptors, supporting that activation of p38 by IL-1β is mediated by Src and NR2B.
membrane expression of NR2B, in phosphorylation of p38 and of PKCζ and phosphorylation and membrane expression of GluA2, indicating that activation of Src precedes all these steps. Similarly, blocking NR2B with ifenprodil prevents changes in phosphorylation of p38 and of PKCζ and phosphorylation and membrane expression of GluA2, indicating that changes in NR2B precede these steps. Finally, inhibiting p38 with SB239063 prevents changes in phosphorylation and membrane expression of GluA2. Altogether, these results show that the hyperammonemia induces sequential activation of the pathway depicted in Fig. 6a to modulate membrane expression of GluA2. Moreover, this is mediated by increased levels of IL-1β and of activation of IL-1 receptor and we identify the intracellular pathways involved.

It is noteworthy that neuroinflammation may also alter AMPA receptors by other mechanisms. TNF-α also may alter membrane expression of AMPA receptor subunits. Moreover, the effects of IL-1β and of TNF-α on membrane expression of AMPA receptors are the opposite. IL-1β reduces membrane expression of the GluA1 subunit while TNF-α increases GluA1 but reduces GluA2 membrane expression [29, 60–62]. This indicates that different types or
grades of neuroinflammation may lead to different alterations in AMPA receptor membrane expression depending on the prevalence of IL-1β or TNF-α effect [29, 60–62].

TNF-α selectively enhances membrane expression of GluA1 in hippocampal neurons and the proportion of GluA2-lacking receptors, resulting in AMPA receptors with different properties becoming calcium-permeable, inwardly rectifying and inhibited by polyamines [61]. In vivo nanoinjection of TNF-α in rats also increases synaptic expression of the GluA1 subunits with a concurrent decrease in the GluA2 subunit [62]. In contrast, IL-1β at high concentrations reduces membrane expression of GluA1 [29]. The results reported here show that, in hyperammonemic rats, increased levels of IL-1β reduces GluA1 and enhances GluA2 membrane expression through activation of IL-1 receptor.

It is also noteworthy that, although the effects of IL-1β and TNF-α on membrane expression of AMPA receptors are the opposite, both increased levels of IL-1β and of TNF-α result in altered glutamatergic neurotransmission by altering AMPA receptor function. Sustained overexpression of either IL-1β or TNF-α impairs hippocampal LTP [63]. Increased levels of IL-1β also impair spatial learning and memory [17, 64]. Some reports suggest that TNF-α also impairs spatial learning [65, 66].

A potential limitation of the present work is that the effects reported could be limited to a certain period of time in the progression of chronic hyperammonemia. The present work has been performed at 4–5 weeks of hyperammonemia. However, the type or intensity of neuroinflammation could be different at longer times of hyperammonemia, triggering other mechanisms. The
above reports indicate that neuroinflammation may alter hippocampal neurotransmission and spatial learning by different mechanisms depending on the type and grade of neuroinflammation, for example on the relative contribution of the increases in IL-1β and TNF-α [17, 29, 60–66]. This may explain the discrepancy between the effects induced by 4–5 weeks of hyperammonemia reported here and those reported in [7] showing increased GluA1 and reduced GluA2 membrane expression at 8 weeks of hyperammonemia. We have preliminary results showing that neuroinflammation in hyperammonemic rats is dynamic and changes with time, resulting in a different pattern of inflammatory markers at different types. Dynamic changes in neuroinflammation have been also reported in situations such as Parkinson’s disease [67], stroke [68, 69], ischemia [70], amyotrophic lateral sclerosis, AIDS, and multiple sclerosis [71]. These dynamic changes in neuroinflammation and its consequences on neurotransmission suggest that the treatments to reverse neuroinflammation-induced cognitive impairment should be different depending on the type and grade of neuroinflammation reached, which would be different in different pathological situations.

We show that in rats with chronic moderate hyperammonemia, similar to that present in patients with liver cirrhosis, neuroinflammation in hippocampus alters membrane expression of AMPA receptors mainly through activation of IL-1 receptor by increased levels of IL-1β. Blocking IL-1 receptor with the endogenous antagonist IL-1Ra reverses completely the alterations in AMPA receptor membrane expression. It has been shown that IL-1Ra also prevents the impairment of LTP by IL-1β [63]. This suggests that blocking this receptor would also restore spatial learning impaired by overexpression of IL-1β in different pathological situations associated with neuroinflammation, including hyperammonemic rats, and possibly, patients with minimal hepatic encephalopathy.

Conclusions
In summary, this work identifies two pathways by which neuroinflammation alters glutamatergic neurotransmission in hippocampus. Increased IL-1β and activation of its receptor leads to activation of Src, increased membrane expression of GluN2B, and activation of p38 which reduces activity of PKCa and phosphorylation of GluA2 at Ser880, increasing membrane expression of GluA2. Activated Src also increases phosphorylation of PKCδ which enhances phosphorylation of GluN2B at Ser1303, reducing membrane expression of CaMKII and phosphorylation at Ser831 and membrane expression of GluA1. These steps could be targets to normalize neurotransmission in hyperammonemia and other pathologies associated with increased IL-1β by acting, for example, on p38 or PKCδ.

Abbreviations
BS3: Bis (sulfo succinimidyl) suberate; CAMKKδ: Calcium-calmodulin dependent protein kinase II; GluA1: Glutamate ionotropic receptor AMPA type subunit 1; GluA2: Glutamate ionotropic receptor AMPA type subunit 2; GluN2B: Glutamate ionotropic receptor NMDA type subunit 2B; IL-1Ra: Interleukin 1 receptor antagonist; LTP: Long-term potentiation; PKCa: Delta isoform of protein kinase C; PKCC: Zeta isoform of protein kinase C; PP2: 4-Amino-3-(4-chlorophenyl)-1-(t-buty)-1H-pyrazolo[3,4-d]pyrimidine; SB239063: Trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2- methoxypyridimidin-4-yl)imidazole
factors to cognitive impairment in minimal hepatic encephalopathy. Metab Brain Dis. 2012;27:51–8.

7. Hernandez-Razba V, Cabrera-Pastor A, Taoro-Gonzalez L, Malaguena M, Agusti A, Llansola M, Felipo V. Hyperammonemia induces glial activation, neuroinflammation and alters neurotransmitter receptors in hippocampus, impairs spatial learning reversal by sulforaphane. J Neuroinflamm. 2016;13:41.

8. Hernandez-Razba V, Cabrera-Pastor A, Taoro-Gonzalez L, Gonzalez-Usoa A, Agusti A, Balzano T, Llansola M, Felipo V. Neuroinflammation increases GaβAεGic tone and impairs cognitive and motor function in hyperammonemia by increasing GaAT-3 membrane expression. Reversal by sulforaphane by promoting M2 polarization of microglia. J Neuroinflamm. 2016;13:83.

9. Rodrigo R, Cauili O, Gomez-Pinedo U, Agusti A, Hernandez-Razba V, Garcia-Verdugo JM, Felipo V. Hyperammonemia induces neuroinflammation that contributes to cognitive impairment in rats with hepatic encephalopathy. Gastroenterology. 2010;139:675–84.

10. Bilbo SD, Smith SH, Schwarz JM. A lifespan approach to neuroinflammatory and cognitive disorders: a critical role for glia. J Neuroimmune Pharm. 2012;7:24–41.

11. Cherniack EP. A berry thought-provoking idea: the potential role of plant polyphenols in the treatment of age-related cognitive disorders. Br J Nutr. 2012;108:794–800.

12. Boyer L, Richeri R, Dassa D, Boucekhine M, Fernandez J, Vaillant F, Padovani R, Auquier P, Lancon C. Association of metabolic syndrome and inflammation with neurocognition in patients with schizophrenia. Psychiatry Res. 2013;210:381–6.

13. Illovs B, Schwanke RG, van der Zea EA, Absalom AR, Heinemann E, van Leeuwen BL. Postoperative cognitive dysfunction: involvement of neuroinflammation and neuronal functioning. Brain Behav Immun. 2014;38:202–10.

14. Takeda S, Sato N, Morishita R. Systemic inflammation, blood-brain barrier vulnerability and cognitive/non-cognitive symptoms in Alzheimer disease: relevance to pathogenesis and therapy. Front Aging Neurosci. 2014;6:171.

15. Montoliu C, Llanos M, Felipo V. Neuroinflammation and neurological alterations in chronic liver diseases. Neuroimmunol Neuroinflamm. 2015;2:138–41.

16. Agusti A, Hernandez-Razba V, Llaneza-Acuña A, Cabrera-Pastor A, Fustero S, Llanos M, Montolivlo T, Felipo V. Sildenafil reduces neuroinflammation in cerebellum, restores GaβAεGic tone and improves motor in-coordination in rats with hepatic encephalopathy. CNS Neurosci Ther. 2017;23:386–94.

17. Moore AH, Wu M, Shafer S, Graham KA, O'Banion MK. Sustained expression of interleukin-1β in mouse hippocampus impairs spatial memory. Neuroscience. 2009;164:1484–95.

18. Hein AM, Stasko MR, Matousek SB, Scott-McKean JJ, Maier SF, Olschowka JA, Kameyama K, Roche KW, Huganir RL. Phosphorylation of the α-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluA1 subunit by activation of Ca2+-permeable AMPA receptors. Nature. 1996;381:793–8.

19. Moss SJ, Lu WY, Orser BA. Memory deficits induced by inflammation are mediated by protein kinase C alpha to AMPA receptor clusters in spines of activated microglia. J Neuroinflamm. 2012;2012:825364.

20. Esteban JA, Shi SH, Wilson C, Nurina M, Huganir RL, Malinow R. PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. Nat Neurosci. 2003;6:136–43.

21. Wang DS, Zurek AA, Lecker K, Abraham AM, Avramescu S, Davies PA, Moss SJ, Lu WW, Orser BA. Memory deficits induced by inflammation are regulated by α5-subunit-containing GABAα receptors. Cell Rep. 2012;2:488–96.

22. Wang G, Gilbert J, Man HY. AMPA receptor trafficking in homeostatic synaptic plasticity: functional molecules and signaling cascades. Neural Plast. 2012;2012:825364.
50. Liao GY, Wagner DA, Hsu MH, Leonard JP. Evidence for direct protein kinase-C mediated modulation of N-methyl-D-aspartate receptor current. Mol Pharmacol. 2001;59:960–4.

51. Murugappan S, Chari R, Palli VM, Jin J, Kunapuli SP. Differential regulation of theonine and tyrosine phosphorylations on protein kinase CB by G-protein-mediated pathways in platelets. Biochem J. 2009;417:113–20.

52. Anggono V, Huganir RL. Regulation of AMPA receptor trafficking and synaptic plasticity. Curr Opin Neurobiol. 2012;22:461–9.

53. Malinow R. AMPA receptor trafficking and long-term potentiation. Philos Trans R Soc Lond Ser B Biol Sci. 2003;358:707–14.

54. Kullmann DM. AMPA receptor attrition in long-term depression. Neuron. 1999;24:288–90.

55. Richter-Levin G, Canevari L, Bliss TV. Long-term potentiation and glutamate release in the dentate gyrus: links to spatial learning. Behav Brain Res. 1995;66:37–40.

56. Muñoz MD, Monfort P, Gaztelu JM, Felipo V. Hyperammonemia impairs NMDA receptor-dependent long-term potentiation in the CA1 of rat hippocampus in vitro. Neurochem Res. 2000;25:437–41.

57. Murray CA, Lynch MA. Evidence that increased hippocampal expression of the cytokine interleukin-1beta is a common trigger for age- and stress-induced impairments in long-term potentiation. J Neurosci. 1998;18:2974–81.

58. Srinivasan D, Yen JH, Joseph DJ, Friedman W. Cell type-specific interleukin-1beta signaling in the CNS. J Neurosci. 2004;24:6482–8.

59. Chen CL, Wang SH, Chan PC, Shen MR, Chen HC. Phosphorylation of E-cadherin at threonine 790 by protein kinase CB reduces B-catenin binding and suppresses the function of E-cadherin. Oncotarget. 2016;7:37260–76.

60. Beattie EC, Stellwagen D, Morishana W, Bresnahan JC, Ha BK, Von Zastrow M, Beattie MS, Malenka RC. Control of synaptic strength by glial TNF-α. Science. 2002;295:2282–5.

61. Stellwagen D, Beattie EC, Seo JY, Malenka RC. Differential regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha. J Neurosci. 2005;25:3219–28.

62. Ferguson AB, Christensen RN, Gensel JC, Miller BA, Sun F, Beattie EC, Bresnahan JC, Beattie MS. Cell death after spinal cord injury is exacerbated by rapid TNF alpha-induced trafficking of GluA2-lacking AMPARs to the plasma membrane. J Neurosci. 2008;28:11391–400.

63. Cunningham AJ, Murray CA, O’Neill LA, Lynch MA, O’Connor JJ. Interleukin-1 beta (IL-1 beta) and tumor necrosis factor (TNF) inhibit long-term potentiation in the rat dentate gyrus in vitro. Neurosci Lett. 1996;203:17–20.

64. Girbenti M, Newton C, Friedman H, Klein TW. Spatial learning impairment in mice infected with Legionella pneumophila or administered exogenous interleukin-1-beta. Brain Behav Immun. 1995;9:113–28.

65. Golan H, Levav T, Mendelsohn A, Huleihel M. Involvement of tumor necrosis factor-alpha in hippocampal development and function. Cereb Cortex. 2004;14:97–105.

66. Gong QH, Wang Q, Pan LL, Liu XH, Xin H, Zhu YZ. S-propargyl-cysteine, a novel hydrogen sulfide-modulated agent, attenuates lipopolysaccharide-induced spatial learning and memory impairment: involvement of TNF signaling and NF-κB pathway in rats. Brain Behav Immun. 2011;25:110–9.

67. Maia S, Arlicot N, Vieron E, Bodard S, Vergote J, Guilloteau D, Chalon S. Longitudinal and parallel monitoring of neuroinflammation and neurodegeneration in a 6-hydroxydopamine rat model of Parkinson’s disease. Synapse. 2012;66:573–83.

68. Thiel A, Radlinska BA, Paquette C, Sidel M, Soucy JP, Schirmacher R, Minuk J. The temporal dynamics of poststroke neuroinflammation: a longitudinal diffusion tensor imaging-guided PET study with 11C-PK11195 in acute subcortical stroke. J Nucl Med. 2010;51:1404–12.

69. Waltherer M, Rueger MA, Simard ML, Emig B, Jander S, Fink GR, Schoeter M. Dynamics of neuroinflammation in the macrosphere model of arterio-arterial embolic focal ischemia: an approximation to human stroke patterns. Exp Trans Stroke Med. 2010;2:22.

70. Van Ham TJ, Brady CA, Kalicharan RD, Dosterhof N, Kuipers J, Veenvlinder A, Algra A, Spilkerma KA, Peterson RT, Kampinga HH, Giepmans BN. Intravital correlated microscopy reveals differential macrophage and microglial dynamics during resolution of neuroinflammation. Dis Model Mech. 2014;7:857–69.

71. Agius LM. Neuroinflammation as the proximate cause of signature pathogenetic pattern progression in amyotrophic lateral sclerosis, AIDS, and multiple sclerosis. Patholog Res Int. 2012;2012:169270.