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Extracellular cGMP Reverses Altered Membrane Expression of AMPA Receptors in Hippocampus of Hyperammonemic Rats: Underlying Mechanisms

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Abstract

Chronic hyperammonemia impairs spatial memory by altering membrane expression of GluA1 and GluA2 subunits of AMPA receptors in hippocampus. Intracerebral administration of extracellular cGMP to hyperammonemic rats restores spatial memory and membrane expression of AMPA receptors. The underlying molecular mechanisms remain unknown and cannot be analyzed in vivo. The aims of the present work were to (1) assess whether extracellular cGMP reverses the alterations in membrane expression of GluA1 and GluA2 in hippocampus of hyperammonemic rats ex vivo and (2) identify the underlying mechanisms. To reach these aims, we used freshly isolated hippocampal slices from control and hyperammonemic rats and treated them ex vivo with extracellular cGMP. Extracellular cGMP normalizes membrane expression of GluA2 restoring its phosphorylation in Ser880 because it restores PKCζ activation by Thr560 auto-phosphorylation, which is a consequence of normalization by extracellular cGMP of phosphorylation and activity of p38 which was increased in hyperammonemic rats. Normalization of p38 is a consequence of normalization of membrane expression of the GluN2B subunit of NMDA receptor, mediated by a reduction in its phosphorylation in Tyr1472 due to reduction of Src activation, which was over-activated in hyperammonemic rats. Extracellular cGMP also restores membrane expression of GluA1 increasing its phosphorylation at Ser831 because it restores CaMKII membrane association and phosphorylation in Thr286. All these effects of extracellular cGMP are due to a reduction of hippocampal IL-1β levels in hyperammonemic rats, which reduces IL-1 receptor-mediated Src over-activation. Reduction in IL-1β levels is due to the reduction of microglia activation in hippocampus of hyperammonemic rats.

Keywords Cyclic-GMP · AMPA receptors · Hippocampus · Hyperammonemia · Interleukin 1 beta · Microglia

Introduction

Around 40% of patients with liver cirrhosis suffer minimal hepatic encephalopathy (MHE), with psychomotor slowing, motor alterations, mild cognitive impairment, and reduced spatial memory that reduce their ability to perform daily tasks, increase the risk of accidents, falls, and hospitalization, and reduce quality of life and life span [1–4]. MHE affects more than two million people in the USA and a similar number in the European Union [5]. MHE is therefore an important clinical, social, and economic problem [1].

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Chronic moderate hyperammonemia is a main contributor to the neurological alterations in MHE and most therapeutic approaches are directed to reduce ammonia levels [6–9]. Rats with chronic hyperammonemia similar to that present in patients with liver cirrhosis also show cognitive alterations, including impaired spatial learning and memory [10–12]. Hyperammonemia alters cognitive function by altering neurotransmission [13–16]. Reduced extracellular cGMP levels in some brain areas in hyperammonemic rats contribute to cognitive and motor alterations in hyperammonemia. Increasing extracellular cGMP improves learning of a Y-maze task, spatial learning and memory and motor coordination in hyperammonemic rats [11, 17, 18].

In hippocampus, hyperammonemia alters membrane expression of the GluA1 and GluA2 subunits of AMPA receptors, which seems to be a main contributor to impairment of spatial learning and memory in hyperammonemic rats [10, 11]. In vivo administration of extracellular cGMP in brain normalizes membrane expression of AMPA receptors in

hippocampus and spatial reference memory of hyperammonemic rats [11]. The mechanisms by which extracellular cGMP modulates membrane expression of GluA1 and GluA2 and normalizes it in hippocampus of hyperammonemic rats has not been studied. Identifying these mechanisms would improve the knowledge about how to improve cognitive impairment in hyperammonemia and MHE and may identify new therapeutic targets.

These molecular mechanisms cannot be analyzed in detail in rats in vivo. We have recently shown that freshly isolated ex vivo slices from hippocampus or cerebellum of hyperammonemic rats retain the alterations in membrane expression of AMPA receptors and is a good system to analyze the mechanisms modulating trafficking of GluA1 and GluA2 and their alterations in hyperammonemia [18, 19].

The aims of the present work were (1) to assess whether extracellular cGMP reverses the alterations in membrane expression of GluA1 and GluA2 in hippocampus of hyperammonemic rats ex vivo and (2) identify the underlying mechanisms.

Trafficking of GluA1 is mainly modulated by phosphorylation of Ser831 and/or Ser845, which increase membrane expression [20, 21]. Ser831 may be phosphorylated by protein kinase C (PKC) or by calcium-calmodulin-dependent protein kinase II (CaMKII) [22, 23], while Ser845 is mainly phosphorylated by cAMP-dependent protein kinase (PKA) [24]. Trafficking of GluA2 is mainly modulated by phosphorylation of Ser880 by PKC, which reduces membrane expression of GluA2 [25, 26].

We have recently shown that hyperammonemia reduces GluA1 and increases GluA2 membrane expression in hippocampus by reducing phosphorylation of GluA1 in Ser831 and of GluA2 in Ser880 [19]. Changes in GluA2 are mediated by changes in Src, GluN2B, MAP kinase p38 and PKCζ while changes in GluA1 are mediated by changes in Src, GluN2B, CaMKII and PKCδ [19].

We therefore used freshly isolated hippocampal slices from hyperammonemic rats, incubated or not ex vivo with extracellular cGMP to assess if extracellular cGMP reverses the alterations in membrane expression of GluA1 and GluA2 and to identify the underlying mechanisms.

Results

Extracellular cGMP Reverses Hyperammonemia-Induced Alterations in Phosphorylation and Membrane Expression of GluA1 and GluA2 Subunits of AMPA Receptors in Hippocampus

Membrane expression of the GluA1 subunit of AMPA receptors is positively regulated by phosphorylation in Ser831 [27]. In hippocampal slices from hyperammonemic rats, phosphorylation of GluA1 in Ser831 is reduced to 72 ± 7% of control slices (Fig. 1a) and membrane expression is reduced in parallel to 76 ± 5% of control slices (Fig. 1b).

Membrane expression of the GluA2 subunit of AMPA receptors is negatively regulated by phosphorylation in Ser880 [28]. In hippocampal slices from hyperammonemic rats, GluA2 phosphorylation in Ser880 is reduced to 77 ± 4% of control slices (Fig. 1c) while membrane expression of GluA2 is increased to 136 ± 7% of control slices (Fig. 1d).

Ex vivo treatment of hippocampal slices from hyperammonemic rats with extracellular cGMP normalized both phosphorylation of GluA1 at Ser831 (104 ± 14% of controls) and membrane expression of GluA1 (122 ± 9% of controls) (Fig. 1a, b). Extracellular cGMP also normalized phosphorylation of GluA2 at Ser880 (113 ± 11% of controls) and membrane expression of GluA2 (113 ± 11% of controls) (Fig. 1c, d).

Extracellular cGMP-Mediated Normalization of Phosphorylation and Membrane Expression of GluA1 in Hyperammonemic Rats Is Mediated by Normalization of Phosphorylation and Membrane Expression of CaMKII

Phosphorylation in Ser831, which increases membrane expression of GluA1 subunit, is mediated by CaMKII [27], whose calcium-mediated auto-phosphorylation in Thr286 is mandatory for its activation. It has been reported that hyperammonemia reduces phosphorylation of GluA1 at Ser831 by reducing the association of CaMKII to the membrane [19]. We therefore assessed if extracellular cGMP normalizes membrane expression of CaMKII. As shown in Fig. 2a, in slices from hyperammonemic rats, the amount of CaMKII associated to the membrane is reduced to 54 ± 9% of control rats ($p < 0.001$). Treatment with extracellular cGMP increased CaMKII in the membrane of hyperammonemic rats to normal levels (102 ± 39% of control rats) and also increased it in control rats to 200 ± 35% of basal levels (Fig. 2a).

We also found that phosphorylation of CAMKII in Thr286 is reduced in hippocampal slices from hyperammonemic rats to 71 ± 9% of control slices ($p < 0.05$) and extracellular cGMP restored it to normal levels (127 ± 13% of control slices, $p < 0.001$) (Fig. 2b).

In Hippocampal Slices from Hyperammonemic Rats, Extracellular cGMP Restores Phosphorylation of GluA2 at Ser880 by Reversing p38-Mediated Inhibition of PKCζ

Phosphorylation of GluA2 in Ser880 is mediated by PKC [28]. It has been also reported that interaction of p38 kinase with PKCζ blocks auto-phosphorylation of PKCζ on Thr-

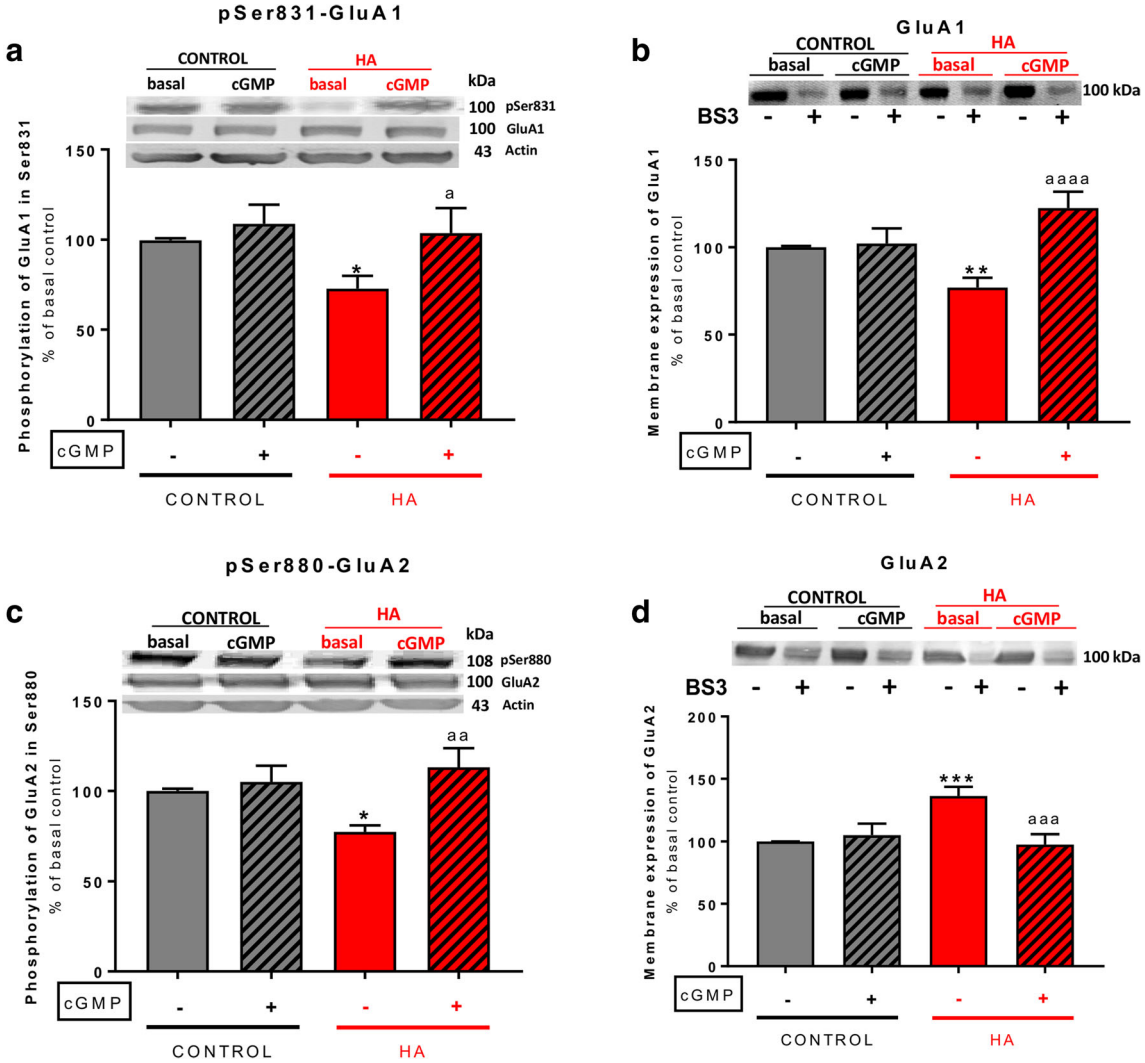

Fig. 1 Extracellular cGMP counteracts hyperammonemia-induced alterations in membrane expression and phosphorylation of GluA1 and GluA2 AMPA subunits in the hippocampus. Hippocampal slices from control and hyperammonemic rats were incubated with cGMP. After that, slices were processed, as it is said in Methods, and membrane expression of GluA1 (**b**) and GluA2 (**d**) and its phosphorylation of Ser880 (**a**) and Ser831 (**c**) respectively were assessed by immunoblot. Values are expressed as percentage of basal protein levels in control rats and are the mean ± SEM (standard error of the mean) of 52, 38, 22, and 44 rats per group in A, B, C, and D, respectively. Data were analyzed by two-way ANOVA and Tukey's post hoc test, with values significantly different from control rats are indicated by asterisk and from hyperammonemic rats are indicated by "a" (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$, $^{aaaa}p < 0.0001$)

560, which is required for PKCζ activation [29]. We have recently shown that hyperammonemia-induced reduction in phosphorylation of GluA2 at Ser880 is due to reduced PKCζ auto-phosphorylation at Thr560 which, in turn, is due to the increased p38 phosphorylation and activity [19]. We therefore assessed the effects of extracellular cGMP on PKCζ auto-phosphorylation at Thr560 and on p38 phosphorylation.

Extracellular cGMP restored PKCζ phosphorylation at Thr560 in slices from hyperammonemic rats, increasing it from 76 ± 4% to 125 ± 10% of control rats (Fig. 3a). This was associated with a reduction in p38 phosphorylation (and activity) from 136 ± 9% to 86 ± 11% of control slices (Fig. 3b). Extracellular cGMP increased PKCζ phosphorylation in Thr560 in the slices from control rats to 128 ± 13% of basal values (Fig. 3a).

Normalization of p38 Activity in Slices from Hyperammonemic Rats by Extracellular cGMP Would Be Due to Normalization of Phosphorylation by Src and Membrane Expression of the GluN2B Subunit of NMDA Receptor

In hippocampus of hyperammonemic rats, p38 is over-activated because of the enhanced entry of calcium through GluN2B-containing NMDA receptors, which membrane expression is increased due to increased Tyr1472 phosphorylation [19, 30].

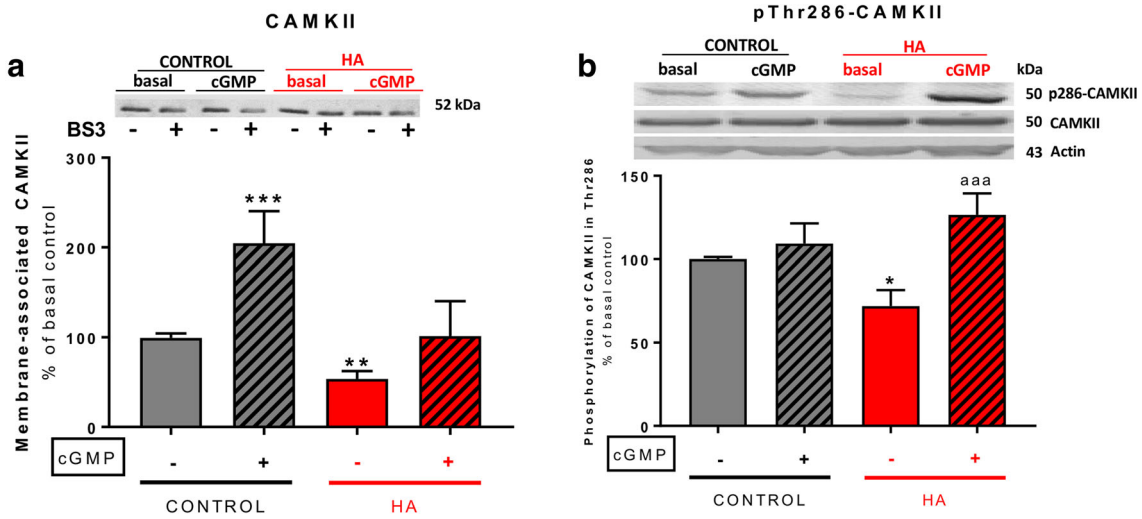

Fig. 2 Extracellular cGMP restores membrane association and phosphorylation of CaMKII in the hippocampus of hyperammonemic rats. Hippocampal slices from control and hyperammonemic rats were incubated with cGMP and processed to analyze membrane association of CaMKII (**a**) or Thr286 phosphorylation of CaMKII (**b**). Values are expressed as percentage of basal protein levels in control rats and are the mean ± SEM of 13 and 14 rats per group in **a** and **b**, respectively. Data were analyzed by two-way ANOVA and Tukey's post hoc test, with values significantly different from control rats are indicated by asterisk and from hyperammonemic rats are indicated by "a" ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $^{aaa}p < 0.001$)

We therefore assessed the effects of extracellular cGMP on these parameters. We found that extracellular cGMP normalizes membrane expression of GluN2B, reducing it from 150 ± 9% to 103 ± 7% of control slices (Fig. 3c). This is associated with a reduction of its phosphorylation at Tyr1472 from 127 ± 11% to 97 ± 7% of control slices (Fig. 3d).

Tyr1472 of GluN2B is phosphorylated by the tyrosine kinase Src, which activity is increased in hippocampus of hyperammonemic rats due to increased phosphorylation at Tyr416 [19, 30]. We therefore assessed whether extracellular cGMP reduces activation of Src in slices from hyperammonemic rats. As shown in Fig. 4a, extracellular cGMP normalizes Src phosphorylation, reducing it from 128 ± 8% to 94 ± 8% of control slices.

Reduction of Src Phosphorylation by Extracellular cGMP in Slices from Hyperammonemic Rats Would Be Due to Reduced IL-1β Levels, Likely Due to Reduced Activation of Microglia

Tyrosine kinase Src is activated by phosphorylation in Tyr416 due to IL-1β signalling through IL-1 receptor [31]. IL-1β content and activation of IL-1 receptor are increased in hippocampus of hyperammonemic rats [19]. We therefore assessed whether extracellular cGMP modulates IL-1β amount. As shown in Fig. 4b, extracellular cGMP reduces IL-1β levels from 133 ± 7% to 105 ± 8% of control slices.

Hyperammonemic rats show activated microglia in hippocampus [10, 11], which may release IL-1β [32]. We therefore analyzed by immunohistochemistry the effects of extracellular cGMP on microglia activation. Microglia cells show a ramified morphology in its resting state and turn into amoeboid-shaped cells when they are activated by extracellular stimuli. In their activated state, microglia processes retract, reducing its perimeter, and the size of the cell body size increases, so measurement of area and perimeter of microglia can be used as an indirect method to quantify its activation [33–35].

Microglia cells are strongly activated in hippocampal slices from hyperammonemic rats, as indicated by the strong reduction in its area to 79 ± 7 μm^2 compared to 200 ± 10 μm^2 in control slices (Fig. 5a) and in its perimeter to 64 ± 6 μm compared to 141 ± 9 μm in control slices (Fig. 5b) leading to an amoeboid-like shape (Fig. 5c). Extracellular cGMP treatment significantly increases both area and perimeter of microglia to 137 ± 9 μm^2 and 98 ± 6 μm, respectively (Fig. 5a, b) indicating a significant reduction of its activation in slices from hyperammonemic rats, although it remained slightly higher than in control rats (Fig. 5c).

Discussion

The results presented here show the detailed molecular mechanisms by which extracellular cGMP modulates hyperammonemia-induced alterations in hippocampal membrane expression of AMPA receptors. Interestingly, this modulation seems to be mainly due to a cGMP-induced reduction in the neuroinflammatory response observed in hippocampus of hyperammonemic rats, with increased IL-1β levels and microglia activation.

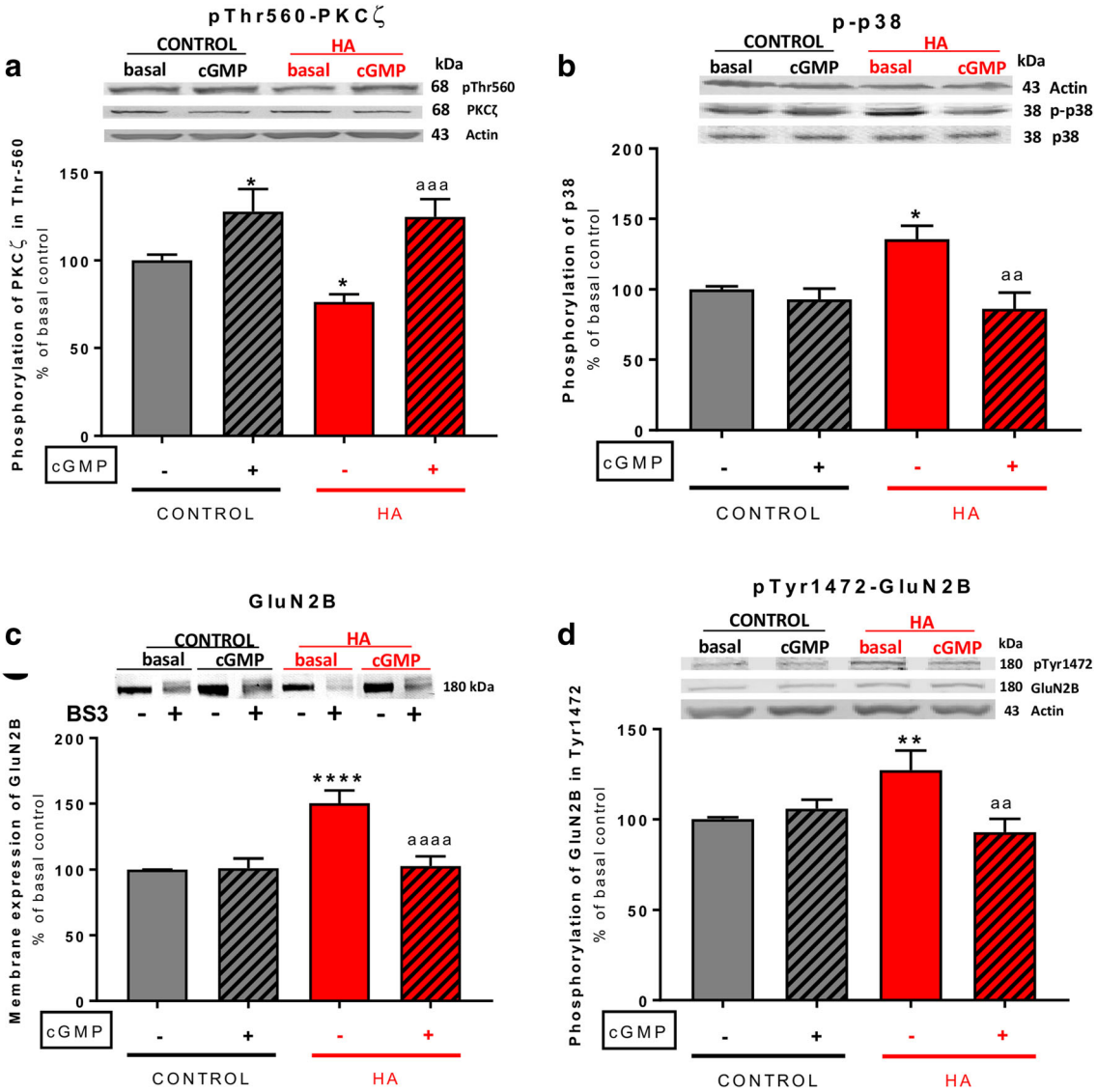

Fig. 3 In the hippocampus of hyperammonemic rats, extracellular cGMP restores phosphorylation of PKCζ and normalizes phosphorylation of p38 and GluN2B as well as its membrane expression. Hippocampal slices from control and hyperammonemic rats were incubated with cGMP and processed to analyze Thr560 phosphorylation of PKCζ (**a**), phosphorylation of p38 (**b**), membrane expression of GluN2B (**c**), and Tyr1472 phosphorylation of GluN2B (**d**). Values are expressed as percentage of basal protein levels in control rats and are the mean ± SEM of 21, 26, 53 and 35 rats per group in A, B, C and D, respectively. Data were analyzed by two-way ANOVA and Tukey's post hoc test, with values significantly different from control rats are indicated by asterisk and from hyperammonemic rats are indicated by "a" ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$, $^{aaaa}p < 0.0001$)

Previous studies have shown that extracellular cGMP is reduced in brain in vivo in hyperammonemic rats and that increasing extracellular cGMP by intracerebral administration restores learning of a Y-maze task, motor coordination and spatial reference memory in hyperammonemic rats [11, 17, 18]. While restoration of learning in the Y-maze and of motor coordination by cGMP is due to normalization of extracellular GABA levels in cerebellum [15, 18], restoration of spatial learning is due to normalization of membrane expression of AMPA receptor membrane in hippocampus [11]. To unveil the molecular mechanisms by which cGMP is normalizing membrane expression of AMPA receptors, here we have used an ex vivo approach that allows us to analyze it and the underlying signal transduction pathways in freshly isolated slices from hyperammonemic rats.

As previously reported [19], phosphorylation of GluA1 subunit at Ser831 and of GluA2 subunit at Ser880 are reduced in hippocampal slices of hyperammonemic rats, which would explain the reduced membrane expression of GluA1 and the enhanced membrane expression of GluA2 [19]. The results reported show that ex vivo treatment with extracellular cGMP normalizes membrane expression of both GluA1 and GluA2 in hippocampal slices from hyperammonemic rats by normalizing their phosphorylation in those two key residues.

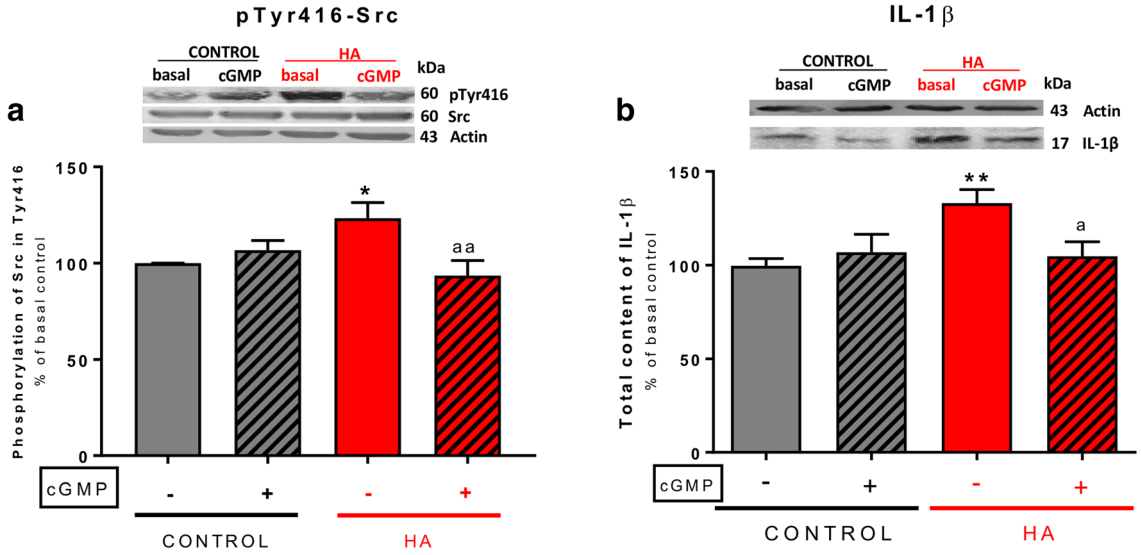

Fig. 4 Extracellular cGMP reverses hyperammonemia-induced increase in IL-1β levels and phosphorylation of Src in the hippocampus. Hippocampal slices from control and hyperammonemic rats were incubated with cGMP and processed to analyze Tyr416 phosphorylation of Src (**a**) and IL-1β levels (**b**). Values are expressed as percentage of basal protein levels in control rats and are the mean ± SEM of 35 and 24 rats per group in A and B, respectively. Data were analyzed by two-way ANOVA and Tukey's post hoc test, with values significantly different from control rats are indicated by asterisk and from hyperammonemic rats are indicated by "a" (*$p < 0.05$, **$p < 0.01$, $^{a}p < 0.05$, $^{aa}p < 0.01$)

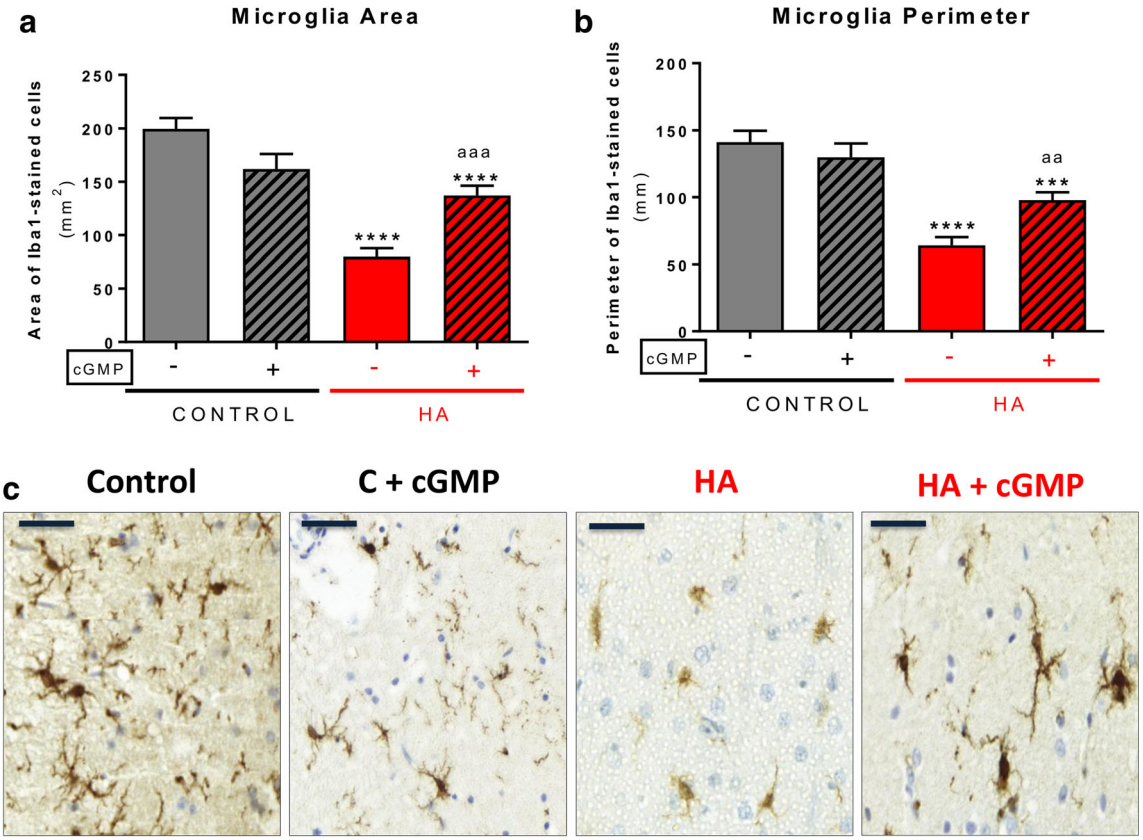

Fig. 5 Extracellular cGMP reduces microglia activation in the hippocampus of hyperammonemic rats. Immunohistochemistry was performed as indicated in methods in fixed hippocampal slices from control and hyperammonemic rats after treatment with cGMP, using an antibody against IBA-1. Representative images are shown (**c**) where scale bar is 50 μm length. To analyze activation of microglia, area (**a**) and perimeter (**b**) of IBA-1-stained microglia cells were measured using the ImageJ analysis software as described in Methods. Values are the mean ± SEM of the microglia cells area (μm^2) and perimeter (μm) in at least 10 fields (56×) per slice, while at least five slices per group were quantified. Data were analyzed by two-way ANOVA and Tukey's post hoc test. Values significantly different from control rats are indicated by asterisks and from hyperammonemic rats are indicated by "a" (***$p < 0.001$, ****$p < 0.0001$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$)

The mechanisms by which hyperammonemia alters phosphorylation and membrane expression of GluA1 and GluA2 have a few common initial steps: activation of IL-1 receptor and of the protein kinase Src. This is supported by the fact that incubation of hippocampal slices from hyperammonemic rats with the antagonist of IL-1 receptor (IL-1Ra) or with Src inhibitor (PP2) reverse such alterations [19]. However, after activation of Src, the mechanisms leading to altered membrane expression of GluA1 and GluA2 involve different signalling pathways. Reduced phosphorylation of GluA1 at Ser831 would be due to reduced amount of CaMKII in the membrane in hyperammonemic rats, which is also restored by inhibiting IL-1 receptor or Src [19]. We show here that, in addition, hyperammonemia also reduces phosphorylation of CaMKII at Thr286 in hippocampus and, therefore its activity. This reduced CaMKII activity would also contribute to the reduced phosphorylation of GluA1. We also show that extracellular cGMP normalizes both phosphorylation (and activity) and subcellular localization at the membrane of CaMKII. This would be the mechanism by which extracellular cGMP normalizes phosphorylation and membrane expression of GluA1.

As mentioned above, the initial step leading to alterations in CaMKII in hippocampus of hyperammonemic rats is activation of Src. We show here that extracellular cGMP normalizes Src activity which, in turn, would be responsible for normalization of CaMKII.

Concerning GluA2, it has been reported that Ser880 phosphorylation is reduced in hyperammonemia as a consequence of p38-mediated PKCζ inhibition. This is supported by the fact that incubation with the p38 inhibitor SB239063 restores Thr560-PKCζ phosphorylation, which is reduced in hippocampal slices from hyperammonemic rats [19], so we analyzed those two steps. Ex vivo treatment with extracellular cGMP in hippocampal slices from hyperammonemic rats reduces p38 phosphorylation, which is basally augmented, increasing activation of PKCζ by auto-phosphorylation in Thr560.

Over-activation of p38 by phosphorylation in the hippocampus of hyperammonemic rats is due to an increase in GluN2B-containing NMDA receptor membrane expression because of increased Src phosphorylation in Tyr1472 [19, 31]. This is supported by the fact that incubation with ifenprodil, a specific antagonist of GluN2B-containing NMDA receptors, reverses the increase in p38 phosphorylation and normalizes phosphorylation and membrane expression of GluA2 in hippocampal slices from hyperammonemic rats [19]. Here, we show that extracellular cGMP treatment normalizes both Src-mediated phosphorylation in Tyr1472 and membrane expression of GluN2B in hippocampal slices from hyperammonemic rats. This would be the pathway through which extracellular cGMP normalizes membrane expression of GluA2.

A main contributor to increased Src activation in hippocampus of hyperammonemic rats is an over-activation of the IL-1 receptor, because of increased IL-1β levels [31]. We show here that extracellular cGMP would reduce Src phosphorylation by reducing the increased levels of IL-1β.

Neuroinflammation in hyperammonemic rats, with the associated increase in IL-1β, seems to be triggered by microglial activation [10, 11, 36]. We therefore assessed whether extracellular cGMP reduces microglial activation in hippocampal slices from hyperammonemic rats and we found that this is actually the case. Therefore, as summarized in Fig. 6, the results reported support that extracellular cGMP reduces microglial activation in hippocampus of hyperammonemic rats, reducing IL-1β levels and activation of IL-1 receptor. This would reverse the over-activation of Src and the changes in membrane expression and activity of CaMKII, thus reversing the effects on phosphorylation and membrane expression of GluA1 (Fig. 6). On the other hand, normalization of Src activity would also normalize phosphorylation and membrane expression of the GluN2B subunit of NMDA receptors and calcium entry through it, thus leading to normalization of p38 and PKCζ activities and, subsequently of phosphorylation and membrane expression of GluA2 (Fig. 6).

This report unveils therefore the detailed steps of the signal transduction pathways through which extracellular cGMP restores membrane expression of AMPA receptors and, subsequently spatial memory.

The mechanisms and pathways altered in hyperammonemia could be altered similarly in other pathologies. For example, it has been shown that cGMP levels are reduced in cerebrospinal fluid (CSF) of patients with mild Alzheimer's disease. It can be assumed that reduced levels of cGMP are reflecting reduced levels of extracellular cGMP in the brain, including the hippocampus. Moreover, CSF cGMP levels showed a significant association with clinical dementia and amyloid pathology in Alzheimer's disease patients [37]. A similar decrease in cGMP in CSF has been reported in Creutzfeldt-Jakob disease [38]. It has been proposed that reduction of cGMP levels at early stages of Alzheimer's disease might participate in the cognitive decline [37].

This is in agreement with reports showing that increasing cGMP by treatment with the phosphodiesterase 5 inhibitor sildenafil restores cognitive function without affecting β-amyloid burden in a mouse model of Alzheimer's disease. The authors propose that these data further strengthen the potential of sildenafil as a therapeutic agent for Alzheimer's disease [39]. Similar effects have been obtained with tadalafil, another inhibitor of phosphodiesterase 5, which also increases cGMP levels [40].

Sildenafil also restores cognitive function in rats with hepatic encephalopathy [41] and in mice with Huntington's disease [42] and regulation of hippocampal

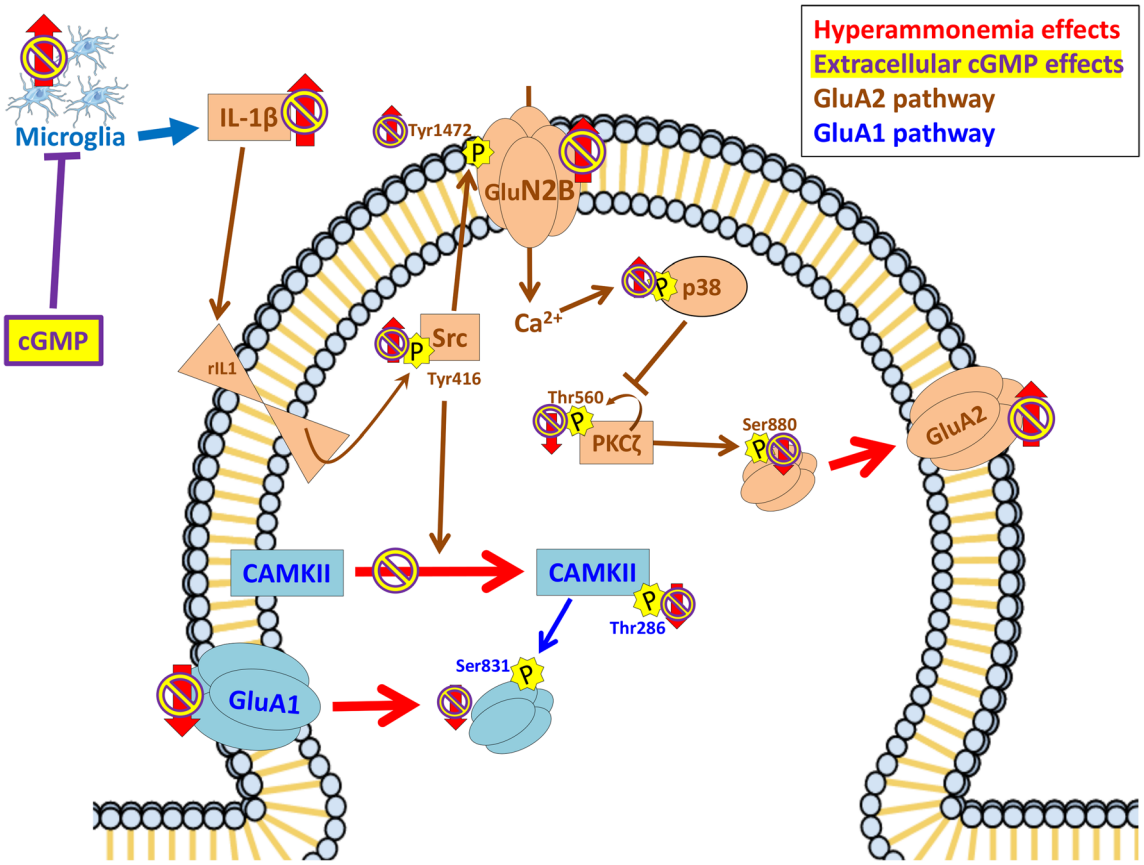

Fig. 6 Scheme summarizing the mechanisms by which extracellular cGMP reverses hyperammonemia-induced alterations in membrane expression of GluA1 and GluA2 subunits of AMPA receptor in the hippocampus. Extracellular cGMP normalizes both GluA1 and GluA2 membrane expression by two different mechanisms. Hyperammonemia induces a series of alterations (in red) that increase membrane expression of GluA2 (in brown) in the hippocampus. Extracellular cGMP normalizes membrane expression of GluA2 restoring Ser880 phosphorylation because it reduces phosphorylation of p38 and restores PKCζ activation by Thr560 phosphorylation. Normalization of p38 is a consequence of normalization of membrane expression of the GluN2B subunit of the NMDA receptor, which is due to a reduction in its phosphorylation in Tyr1472 due to reduced Src activation. On the other hand, extracellular cGMP also reverses hyperammonemia-induced alterations (in red) in GluA1 membrane expression (in blue). Extracellular cGMP restores membrane expression of GluA1 increasing its Ser831 phosphorylation because of a restoration of CaMKII membrane association and phosphorylation in Thr286. All these effects of extracellular cGMP are due to a reduction of hippocampal IL-1β levels in hyperammonemic rats, which reduces IL-1 receptor-mediated Src activation. Reduction in IL-1β levels is due to the reduction of microglia activation in hippocampus of hyperammonemic rats

cGMP levels has been also proposed as a candidate to treat cognitive deficits in hepatic encephalopathy and in Huntington's disease [41, 42].

These reports suggest that reduced extracellular cGMP levels in brain are involved in many neurological and neurodegenerative diseases. It is very likely that reduced extracellular cGMP in these pathologies is associated with altered membrane expression of AMPA receptors in hippocampus which, in turn, would lead to impaired spatial memory and learning. The increase in extracellular cGMP in these pathologies could modulate the same signal transduction pathways identified here, leading to normalization of AMPA receptors and cognitive function, as described for chronic hyperammonemia.

The beneficial effects of increasing cGMP on cognitive function would be mediated by reduced neuroinflammation. The role of cGMP in modulating neuroinflammatory response has been reported in several studies. In cultured microglia, the pro-inflammatory effects of beta-amyloid and LPS-induced activation of NO synthase are blocked by increasing cGMP levels with analogues like 8-Br-cGMP or degradation inhibitors like zaprinast [43, 44]. In a neonatal mouse stroke model treatment with sildenafil after stroke reduces microglia/macrophages density [45]. Sildenafil also reduces neuroinflammation in hippocampus and cerebellum of rats with portacaval anastomosis [41, 46]. In spite of many evidences on the anti-inflammatory effects of cGMP, other authors have shown that inhibition of cGMP degradation by zaprinast increases basal and LPS-induced pro-inflammatory cytokine production (IL-1β and TNFα) in microglia and astrocytes and microglia mixed primary cultures [47]. In contrast to these results, we have previously shown that intracerebral administration of extracellular cGMP reduces TNFα levels in the hippocampus of hyperammonemic rats [11].

The mechanisms by which extracellular cGMP induces anti-inflammatory effects and reduces neuroinflammation are not well known. In vivo intracerebral administration of extracellular cGMP normalizes TNF-a, microglia activation and membrane expression of AMPA receptors in hippocampus and spatial reference memory in hyperammonemic rats [11]. This treatment also reduces neuroinflammation in cerebellum, including microglia and astrocyte activation. This is associated with reduced extracellular GABA in cerebellum and restoration of motor coordination. It remains unclear whether cGMP has a main effect directly on microglia or has an effect upstream. Cabrera-Pastor et al. (2018) [48] showed that hyperammonemic rats show increased nuclear NF-kB in microglia, which is normalized by extracellular cGMP. This effect could be mediated by reduced membrane expression of the TNFα receptor TNFR1 [48]. However, it is not known yet if this effect occurs directly in microglia or upstream in other cell type.

Cabrera-Pastor et al. [11] showed that intracerebral administration of extracellular cGMP in vivo reduces hippocampal microglia activation and TNFα levels, but not IL-1β levels in hippocampus of hyperammonemic rats. We show now that ex vivo administration of cGMP to hippocampal slices from hyperammonemic rats reduces microglia activation and IL-1β levels. This discrepancy would be due to the different experimental conditions. In the study of Cabrera-Pastor et al. [11] hyperammonemic rats extracellular cGMP was administered intracerebrally chronically during 4 weeks to living rats, while in the present work extracellular cGMP has been administrated acutely for only 30 min to hippocampal slices from hyperammonemic rats. We show here that short-term exposure to extracellular cGMP is enough to modulate neuroinflammation and some signalling pathways, including reduction of IL-1β levels and of microglia activation. It is possible that chronic administration of extracellular cGMP could induce different effects and modulate different mechanisms and processes. Another possible reason by which extracellular cGMP could reduce IL-1β levels in the present work but not in [11] could be exposure to a different concentration of extracellular cGMP. In the present work, we have used it at 40 nM and it would be expected that it remains around this concentration during the 30 min incubation. In contrast, in the in vivo treatment in [11] extracellular cGMP was administered through osmotic pumps filled with 240 μM cGMP, which released 0.25 μl (0.06 nmols of cGMP) per hour during 28 days. The actual level of extracellular cGMP reached in vivo under these conditions is not known and would depend on the volume to which it is diffused and on its degradation rate. However, it is likely that the extracellular concentration of cGMP reached in vivo would be lower than the 40 nM concentration used here ex vivo. This could explain why IL-1β levels are reduced ex vivo but not in vivo.

In summary, as shown in Fig. 6, we unveil the molecular mechanisms by which increasing extracellular cGMP normalizes membrane expression of AMPA receptor subunits. Extracellular cGMP reduces microglial activation in hippocampus of hyperammonemic rats, reducing IL-1β levels and activation of IL-1 receptor. This would reverse the overactivation of Src and the changes in membrane expression and activity of CaMKII, thus reversing the effects on phosphorylation and membrane expression of GluA1 (Fig. 6). On the other hand, normalization of Src activity would also normalize phosphorylation and membrane expression of the GluN2B subunit of NMDA receptors and calcium entry through it, thus leading to normalization of p38 and PKCζ activities and, subsequently of phosphorylation and membrane expression of GluA2 (Fig. 6).

Materials and Methods

Rats with Chronic Hyperammonemia Male Wistar rats (220–250 g) were made hyperammonemic by feeding them a diet containing ammonium acetate for 4–6 weeks as in [49], modified to contain 25% ammonium acetate as in [19]. Animals were distributed into two groups: control and chronic hyperammonemia. A total of 53 rats per group were used in the study. The experiments were approved by the Comite de Etica y Bienestar en Experimentacion 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 Control and hyperammonemic rats were decapitated and their brains transferred into ice-cold Krebs buffer. Hippocampi were dissected and transverse slices (400 μm) were obtained as in [19].

Slices were transferred to incubation wells and, after 20 min at 35.5 °C in Krebs buffer for stabilization, 40 nM cGMP was added to the incubation buffer during 30 min. 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 [50], 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), GluA2 phosphorylated at Ser880 (1:2000, cat# ab52180), PKCζ phosphorylated at Thr560 (1:1000, cat# ab59412) and Actin (1:5000, cat# ab6276) 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 Signalling (Leiden, Netherlands); PKCζ (1:2000, cat# sc-17,781) from Santa Cruz (Dallas, TX); and CaMKII (1:1000, cat# MA1-048) and CaMKII phosphorylated at Thr286 (1:500, cat# MA1-047) from Thermo Fisher (Waltham, MA). 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 (AlphaInnotech Corporation, San Francisco). Phosphorylation levels were normalized to the total amount of the respective proteins.

Analysis of Membrane Expression of Receptors by Cross-Linking with BS3 Membrane expression of the GluA1 and GluA2 subunits of AMPA receptors and GluN2B subunit of NMDA receptors in whole hippocampal slices was analyzed as in [19], by cross-linking with BS3 (bis(sulfosuccinimidyl) suberate, Pierce, 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 shacking. 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 in [51].

Analysis of Microglial Activation by Immunohistochemistry in Hippocampal Slices After incubation with cGMP (see above) 400 μm hippocampus slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h. Then, the tissue was placed inside histology cassettes and processed for permanent paraffin embedding on a Leica ASP 300 tissue processor (Leica Microsystems, Nussloch, Germany). Five μm thick paraffin-embedded sections were cut and mounted on coated slide glass. The tissue sections were then processed with the Envision Flex+kit (DAKO, Santa Clara, CA, USA) to block endogenous peroxidase activity for 5 min and then incubating with the primary antibody: anti-Iba1 (Wako, 019-19741; 1:300 for 30 min). The reaction was visualized by Envision Flex+ horseradish peroxidase for 20 min and finally diaminobenzidine for 10 min. Sections were counterstained with Mayer's hematoxylin (DAKO S3309; Ready to use) for 5 min.

Analysis of microglial activation was performed measuring the area and perimeter of each microglia cell, stained with Iba1, using ImageJ (National Institute of Health, Bethesda, MD, USA) (1.48v). The region of interest was selected and using Auto Local Threshold and Analyze Particles functions, the intensity thresholds and size filter were applied. To measure the area and perimeter of each microglia cell, the Bernsen method was used and 2000–20,000 sized filters were applied. For each rat, at least 10 fields (56×) were quantified while at least five slices per group were quantified. The result was expressed as percentage of the mean ± SEM of area (μm^2) and perimeter (μm) of all the microglia cells quantified in slices from control rats.

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 Tukey post hoc test to determine the individual and interaction effects between hyperammonemia and/or cGMP on phosphorylation, membrane expression of receptors, total amount of proteins and immunohistochemical parameters. A confidence level of 95% was accepted as significant.

Authors' Contributions LTG: most ex vivo experiments with fresh slices, membrane expression experiments and western blot analysis; LTG and ACP: analysis and interpretation of data, drafting of the manuscript; YMA: immunohistochemical studies and analysis; ACP and VF: study concept, design and supervision, analysis and interpretation of data; VF: obtained funding, writing of the manuscript.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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