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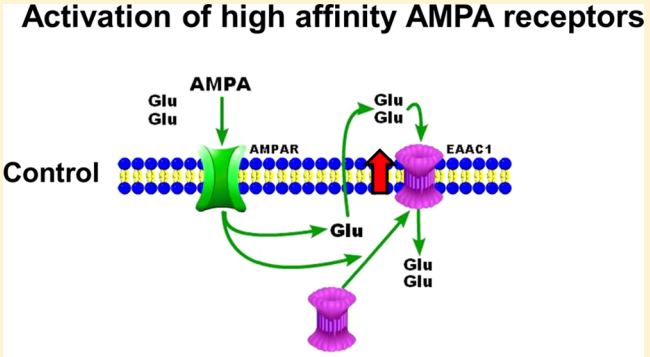
Roles of the NMDA Receptor and EAAC1 Transporter in the Modulation of Extracellular Glutamate by Low and High Affinity AMPA Receptors in the Cerebellum in Vivo: Differential Alteration in Chronic Hyperammonemia

Andrea Cabrera-Pastor, Lucas Taoro, Marta Llansola, and Vicente Felipo*

Laboratory of Neurobiology, Centro de Investigación Príncipe Felipe, Calle Eduardo Primo Yufera, 3, 46012 Valencia, Spain

ABSTRACT: The roles of high- and low-affinity AMPA receptors in modulating extracellular glutamate in the cerebellum remain unclear. Altered glutamatergic neurotransmission is involved in neurological alterations in hyperammonemia, which differently affects high- and low-affinity AMPA receptors. The aims were to assess by in vivo microdialysis (a) the effects of high- and low-affinity AMPA receptor activation on extracellular glutamate in the cerebellum; (b) whether chronic hyperammonemia alters extracellular glutamate modulation by high- and/or low-affinity AMPA receptors; and (c) the contribution of NMDA receptors and EAAC1 transporter to AMPA-induced changes in extracellular glutamate. In control rats, high affinity receptor activation does not affect extracellular glutamate but increases glutamate if NMDA receptors are blocked. Low affinity AMPA receptor activation increases transiently extracellular glutamate followed by reduction below basal levels and return to basal values. The reduction is associated with transient increased membrane expression of EAAC1 and is prevented by blocking NMDA receptors. Blocking NMDA receptors with MK-801 induces a transient increase in extracellular glutamate which is associated with reduced membrane expression of EAAC1 followed by increased membrane expression of the glutamate transporter GLT-1. Chronic hyperammonemia does not affect responses to activation of low affinity AMPA receptors. Activation of high affinity AMPA receptors increases extracellular glutamate in hyperammonemic rats by an NMDA receptor-dependent mechanism. In conclusion, these results show that there is a tightly controlled interplay between AMPA and NMDA receptors and an EAAC1 transporter in controlling extracellular glutamate. Hyperammonemia alters high- but not low-affinity AMPA receptors.



KEYWORDS: *AMPA receptors, NMDA receptors, extracellular glutamate, hyperammonemia, glutamate uptake, EAAC1 glutamate transporter*

Glutamate modulates cognitive and motor functions through the activation of ionotropic (iGluRs) and metabotropic glutamate receptors. There are three main types of iGluRs: NMDA, AMPA and kainate receptors. AMPA receptors are composed of four types of subunits (GluR1-GluR4). Activation of AMPA receptors allows the entry of Na^+. Receptors lacking GluR2 subunits are also permeable to Ca^{2+}.[1,2] There are high- and low-affinity AMPA receptors in the brain which seem to be interconvertible states of the same receptors.[3,4] Most AMPA receptors are of the low affinity type[4] and contain the GluR2 subunit,[5] which prevents Ca^{2+}transport. In agreement with this, Cabrera-Pastor et al.[6] suggested that in the cerebellum in vivo high affinity AMPA receptors transport Na^+, K^+, and Ca^{2+}, while low affinity receptors transport mainly Na^+ and K^+ but not Ca^{2+}. Moreover, Cabrera-Pastor et al.[6] also showed that in the cerebellum in vivo low AMPA concentrations (0.1 mM) are enough to nearly completely activate Ca^{2+}-permeable AMPA receptors, which have higher affinity for AMPA than Ca^{2+}-impermeable receptors.[6] AMPA receptor activation may lead to NMDA receptor activation.[6−8]

Activation of NMDA or AMPA receptors increases extracellular glutamate.[9−17] However, excessive or sustained activation of iGluRs is neurotoxic, and extracellular glutamate must be rapidly reduced. Glutamate transporters terminate the excitatory signal by rapid glutamate uptake into cells. There are three main glutamate transporters: GLAST and GLT-1 (astrocytic) and EAAC1 (neuronal). EAAC1 seems to play a main role in the rapid modulation of glutamatergic synaptic transmission.[18,19] For example, Levenson et al.[20] showed that induction of long-term potentiation in the CA1 region of the hippocampus is associated with an NMDA receptor-dependent translocation of the EAAC1 glutamate transporter to the plasma membrane and increased uptake of glutamate. Waxman et al.[19] reported a dual modulation of the glutamate transporter EAAC1

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by NMDA receptors. Under basal conditions, NMDA receptors enhance EAAC1 membrane expression, which is also enhanced by mild activation of NMDA receptors (i.e., with 10 μM NMDA). However, robust activation of NMDA receptors (i.e., with 100 μM NMDA) or AMPA receptors (i.e., with 100 μM AMPA + cyclothiazide) reduces membrane expression of EAAC1.

To make compatible rapid neurotransmission with keeping properly low glutamate levels, there is a rapid interaction between iGluRs activation and surface expression of EAAC1.[19] The sequence of events would be activation of NMDA and/or AMPA receptors, increased extracellular glutamate, increased EAAC1 surface expression, increased glutamate uptake, and rapid decrease of extracellular glutamate. This allows a transient increase of glutamate and a rapid return to low levels while maintaining proper glutamatergic neurotransmission.

Altered glutamatergic neurotransmission is involved in cognitive and motor alterations in many pathological situations including chronic hyperammonemia and hepatic encephalopathy.[21−26] Patients with chronic liver diseases have reduced capacity to eliminate ammonia in the liver and, as a consequence, show hyperammonemia, which increases ammonia levels in the brain and is a main contributor to the cognitive and motor alterations in patients with hepatic encephalopathy.[27] Altered glutamatergic neurotransmission due to hyperammonemia contributes to the neurological alterations in hepatic Encephalopathy.[21,25] Clarifying the mechanisms by which hyperammonemia alters glutamatergic neurotransmission may identify targets to improve neurological function in hepatic encephalopathy.[24]

Hyperammonemia impairs the glutamate-nitric oxide (NO)-cGMP pathway in the cerebellum in vivo, which is responsible for the reduced ability to learn a Y maze task.[28−30] The function of this pathway is modulated by different neurotransmitter receptors including $GABA_A$[30] and high and low affinity AMPA receptors.[31,6] Hyperammonemia affects differently high- and low-affinity AMPA receptors. Hyperammonemia reduces activation of Ca^{2+}-permeable AMPA receptors but increases Na^+ entering through high affinity AMPA receptors, resulting in NMDA receptor activation.[6]

The modulation of extracellular glutamate by AMPA receptors has not been studied in the cerebellum in vivo even in normal rats.

The aims of this work were to assess (a) the effects of activation of high- and low-affinity AMPA receptors on extracellular glutamate in the cerebellum in vivo; (b) whether chronic hyperammonemia alters the modulation of extracellular glutamate by high- and/or low-affinity AMPA receptors; and (c) the contribution of NMDA receptors and EAAC1 transporter to AMPA-induced changes in extracellular glutamate.

RESULTS

Time Course of the Modulation of Extracellular Glutamate and the Membrane Expression of EAAC1 by the Activation of Low Affinity AMPA Receptors. The activation of low affinity AMPA receptors was achieved by using 0.3 mM AMPA in the in vivo microdialysis studies and 2 mM AMPA in the studies on membrane expression of EAAC1 in cerebellar slices. In control rats, administration of AMPA (0.3 mM) through the microdialysis probe induced a significant increase in extracellular glutamate, reaching 158−189% of basal levels in the two fractions after AMPA administration (fractions 5−6 in Figure 1A). This increase was transient and was followed by a reduction of extracellular glutamate below the basal levels before AMPA administration. Glutamate levels were maintained at 47−50% of basal levels in fractions 8−11 (fractions 4−7 after AMPA administration) (Figure 1A). After this period, extracellular glutamate returned to basal values in fractions 12−13 (Figure 1A).

Hyperammonemia did not affect the transient increase in glutamate or the subsequent decrease and normalization induced by 0.3 mM AMPA (Figure 1A). The basal levels of extracellular glutamate were significantly ($p < 0.01$) higher in hyperammonemic rats (0.32 ± 0.04 μM) than in control rats (0.22 ± 0.02 μM). To assess whether activation of NMDA receptors contributes to the increase or the decrease in extracellular glutamate induced by 0.3 mM AMPA, we tested whether these changes are prevented by blocking NMDA receptors with MK-801 (0.5 μM).

As shown in Figure 1B, addition of MK-801 induced per se a rapid transient increase in extracellular glutamate, which reached 323 ± 108% of basal levels in control rats and was not different (275 ± 124%) in hyperammonemic rats. This transient increase was also followed by a transient decrease below basal levels in fraction 3 after MK-801 administration (fraction 7 in Figure 1B), reaching 51 ± 7% and 41 ± 7% of basal levels in control and hyperammonemic rats, respectively.

Blocking of NMDA receptors with MK-801 did not prevent the increase in glutamate induced by 0.3 mM AMPA, which reached similar levels in the presence (Figure 1B) and the absence (Figure 1A) of MK-801. This indicates that it is not mediated by a NMDA receptor activation. However, the sustained reduction of extracellular glutamate below basal levels induced in fractions 4−6 after 0.3 mM AMPA administration (fractions 8−11 in Figure 1A) was completely prevented in the presence of MK-801, both in control and hyperammonemic rats (Figure 1B). This suggests that it is mediated by NMDA receptor activation.

To assess its possible contribution to the changes in extracellular glutamate, we analyzed the time course of the effects of activation of low affinity AMPA receptors on membrane expression of EAAC1 in cerebellar slices by adding 2 mM AMPA. At early stages (8 min), AMPA induced a decrease in the membrane expression of EAAC1 to 49 ± 14% of basal levels, followed by an increase at 15 min to 186 ± 24% of basal levels, and returned to basal values (125 ± 15% of basal) at 30 min (Figure 1C).

To assess whether activation of NMDA receptors contributes to the increase in EAAC1 membrane expression induced by 0.3 mM AMPA at 15 min, we tested whether this change is prevented by blocking NMDA receptors with MK-801 (0.5 μM). As shown in Figure 1C, MK-801 prevented the increase in membrane expression of EAAC1 at 15 min, which remained at 91 ± 21% of basal levels. This suggests that it is mediated by NMDA receptor activation.

As is the case for extracellular glutamate, the effects of 2 mM AMPA or MK-801 in slices from hyperammonemic rats were similar to those from control rats. As shown in Figure 1C, under basal conditions, the membrane expression of EEAC1 is lower (62 ± 9% of controls, $p < 0.05$) in hyperammonemic rats than that in control rats. This was associated with a significant ($p < 0.05$) 39% increase in extracellular glutamate to 0.32 ± 0.04 μM compared to 0.23 ± 0.02 μM in control rats.

Activation of High Affinity AMPA Receptors Induces a Transient Increase in Extracellular Glutamate through Activation of NMDA Receptors in Hyperammonemic Rats but Not in Control Rats. Activation of high affinity AMPA

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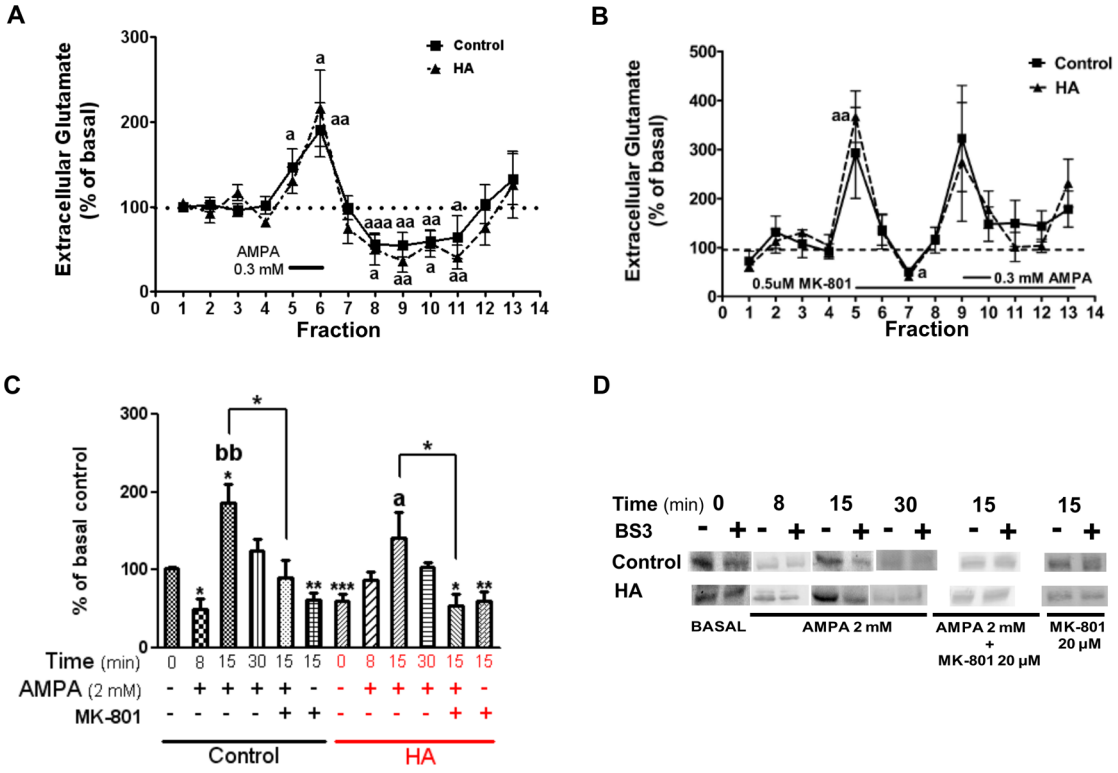

Figure 1. Effects of activation of low affinity AMPA receptors on extracellular glutamate and on membrane expression of EAAC1 in control and hyperammonemic rats. (A,B) Microdialysis was performed in the cerebellum in vivo in male rats. Perfusion was carried out in the absence (A) or the presence (B) of MK-801 (0.5 μM). The solid short lines indicate the fraction during which AMPA (0.3 mM) was applied. The longer lines indicate the application of MK-801. The dotted line indicates the basal levels. Data are presented asa percentage of basal values (mean of fractions 1−4). Values are the mean ± SEM of 8−14 rats per group. (C and D) Cerebellar slices from control or hyperammonemic (HA) rats were incubated with 2 mM AMPA during 8, 15, and 30 min, 20 μM MK-801 (MK), or its mixture, and the amount of EAAC1 present in the membrane was quantified as described in Methods and is expressed as a percentage of control slices. Values are the mean ± SEM of 7−13 rats. Values significantly different from basal values are indicated by a ($p < 0.05$), aa ($p < 0.01$), or aaa ($p < 0.001$). Values significantly different from control values are indicated with asterisks: *($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Values significantly different between incubation time 8 and 15 min are indicated by bb ($p < 0.01$).

receptors was achieved by using 0.1 mM AMPA in the in vivo microdialysis studies and 0.3 mM AMPA in the studies on membrane expression of EAAC1 in cerebellar slices. In the microdialysis studies, the effects of hyperammonemia and the responses to activation of AMPA receptors are completely different when 0.1 mM AMPA was used instead of 0.3 mM.

Perfusion of 0.1 mM AMPA induced a strong increase in extracellular glutamate in hyperammonemic rats but not in control rats. In hyperammonemic rats, glutamate reached 368−254% of basal levels ($p < 0.001$) in the two fractions after the addition of AMPA. However, in control rats 0.1 mM AMPA did not increase extracellular glutamate, which remained at 112−92% of basal levels in the two fractions after the addition of AMPA (Figure 2A). AMPA at 0.1 mM did not induce the delayed reduction of glutamate below basal values induced by 0.3 mM AMPA.

We also assessed the possible contribution of NMDA receptors to glutamate increase by blocking them with MK-801 (Figure 2B). As also occurs in Figure 1B, MK-801 induced a transient increase in extracellular glutamate, which was similar in control and hyperammonemic rats.

The response to 0.1 mM AMPA was completely different in the presence than in the absence of MK-801, both in hyperammonemic and in control rats. In hyperammonemic rats, MK-801 completely prevented the increase in glutamate induced by 0.1 mM AMPA. Glutamate remained at 90−94% of basal values before AMPA addition. In control rats, in the presence of MK-801, 0.1 mM AMPA induces a strong increase in extracellular glutamate, reaching 304−159% of basal levels in the 3 fractions after AMPA administration.

It seems that the more plausible explanation for the different effects on extracellular glutamate of 0.1 and 0.3 mM AMPA and the different mechanisms involved in these effects are that 0.1 and 0.3 mM AMPA activate different forms of AMPA receptors. As described in the first section there are high and low affinity AMPA receptors. It seems therefore likely that 0.1 mM AMPA activates high affinity (GluR2 lacking) AMPA receptors, while 0.3 mM AMPA activates low affinity (GluR2-containing) AMPA receptors.

To assess its possible contribution to the changes in extracellular glutamate, we analyzed the effects of activation of high affinity AMPA receptors on membrane expression of EAAC1 in cerebellar slices by adding 0.3 mM AMPA. At early stages (8 min), AMPA induced a slight increase in the membrane expression of EAAC1 to 123 ± 7% of basal values, followed by a further increase at 15 min to 180 ± 32% of basal values (Figure 2C). The effects were similar in hyperammonemic rats (Figure 2C).

Effects of Blocking NMDA Receptors with MK-801 on Membrane Expression of EAAC1 and GLT-1 Glutamate Transporters. To assess whether the transient increase of extracellular glutamate induced by MK-801 (Figures 1B and 2B) is due to reduced uptake by EAAC1, we assessed the effects of MK-801 on membrane expression of EAAC1. Treatment of the

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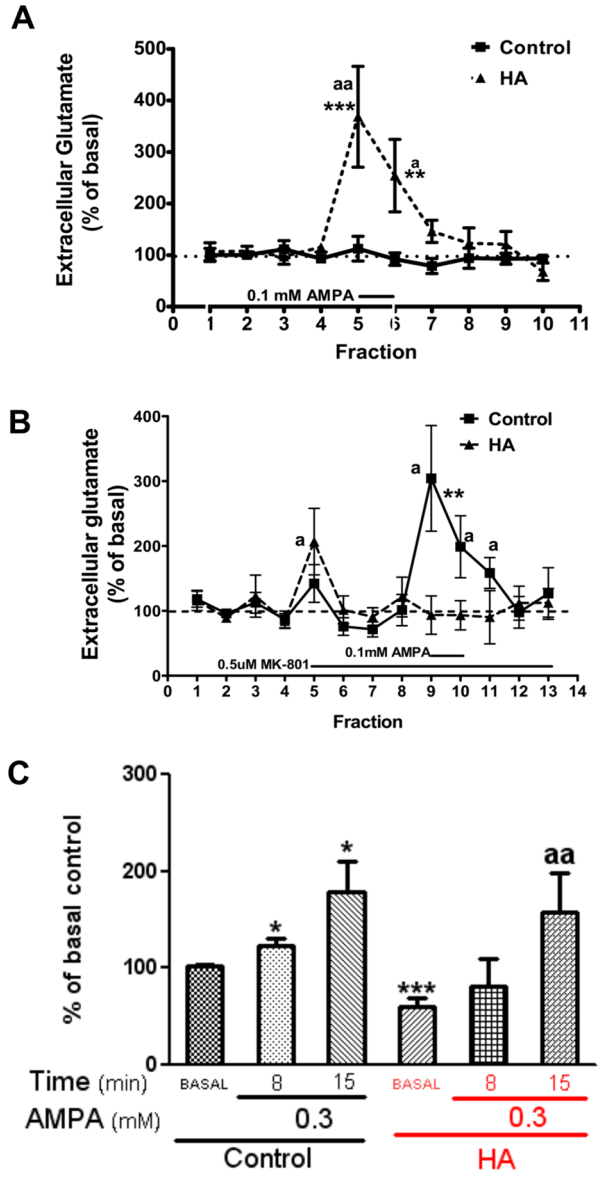

Figure 2. Effects of activation of high affinity AMPA receptors on extracellular glutamate and on membrane expression of EAAC1 in control and hyperammonemic rats. Experiments were performed as in Figure 1 but adding 0.1 mM instead of 0.3 mM AMPA in microdialysis experiments and 0.3 mM instead of 2 mM AMPA in slices. Values are the mean ± SEM of 6−13 rats per group. Values significantly different from basal values are indicated by a ($p < 0.05$) or aa ($p < 0.01$). Values significantly different from control values are indicated with asterisks: *($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

slices with MK-801 reduces ($p < 0.01$) membrane expression of EAAC1 in control rats to 62 ± 9% and 36 ± 13% of basal values at 15 and 30 min, respectively (Figures 1C and 3A). This would contribute to the transient peaks of glutamate shown in fraction 5 of Figures 1B and 2B following the addition of MK-801. However, the decrease of extracellular glutamate below basal levels shown in fraction 7 of the same Figures may not be explained by the change in EAAC1.

We therefore assessed the effects of MK-801 on membrane expression of another main glutamate transporter, GLT-1 (astrocytic). As shown in Figure 3B, MK-801 increases GLT-1 in the membrane of control rats to 183 ± 43% and 141 ± 15% of basal values at 15 and 30 min, respectively. This increase in GLT-1 would contribute to the decrease of extracellular glutamate in fraction 7 of Figures 1B and 2B.

In hyperammonemic rats, the amounts of EAAC1 and GLT-1 present in the membrane under basal conditions are significantly lower than those in control rats, reaching 61 ± 7% ($p < 0.05$) of controls for EAAC1 and 57 ± 8% ($p < 0.001$) for GLT-1. MK-801 did not affect membrane expression of EAAC1 at 15 min and increased it at 30 min to levels similar to basal levels in control rats (126 ± 25%). MK-801 also increased the membrane expression of GLT-1 to 108 ± 25% and 180 ± 28% of basal levels in controls at 15 and 30 min, respectively. These increases in EAAC1 and GLT-1 would contribute to the decrease of extracellular glutamate in fraction 7 of Figures 1B and 2B.

DISCUSSION

Mechanisms Involved in the Changes in Extracellular Glutamate Induced by MK-801. The results show that blocking NMDA receptors with MK-801 induces a transient increase in extracellular glutamate both in control and hyperammonemic rats. A similar increase in extracellular glutamate by NMDA receptor antagonists has been reported in the cortex, although the mechanisms involved may be different in the cerebellum and cortex.[32−35]

As summarized in Figure 4, in control rats the mechanisms by which blocking NMDA receptors enhance extracellular glutamate would involve reduction of glutamate uptake due to reduced surface expression of EAAC1 (Figure 3A), resulting in increased extracellular glutamate. This glutamate increase would be eliminated by reuptake through GLT-1 transporters, whose membrane expression is increased by MK-801 (Figure 3B), leading to the reduction of extracellular glutamate. The final equilibrium is reached at the same initial extracellular glutamate, indicating a tight control of the process (Figures 1B and 2B). It should be noted that NMDA receptors are mainly present in postsynaptic neurons but are also present in presynaptic neurons and in astrocytes.[36,37] This mechanism (Figure 4) would explain the changes induced by MK-801 in extracellular glutamate in normal rats. However, in hyperammonemic rats MK-801 did not decrease membrane expression of EAAC1 (Figure 3A). This suggests that in pathological situations such as hyperammonemia other mechanisms may also contribute to the modulation of extracellular glutamate. One possibility is that in hyperammonemia MK-801 could affect other glutamate transporters (e.g., GLAST) which may mediate the increase in extracellular glutamate. Another possibility is that in hyperammonemia MK-801 could stimulate glutamate release without affecting its uptake, thus resulting in increased extracellular levels.

As shown in Figure 4, EAAC1 is present mainly in neuronal membranes, while GLT-1 is present mainly in glial cells. This indicates that there is an interplay between different receptors (AMPA and NMDA) and transporters (EAAC1 and GLT-1) in different cell types (neurons and glia) to tightly control the extracellular concentration of glutamate. This is essential to allow rapid neurotransmission without inducing excitotoxic effects.

Mechanisms Involved in the Effects of Activation of High Affinity AMPA Receptors. *Effects in Control Rats.* AMPA receptor activation may induce glutamate release both by Na^+- and Ca^{2+}-mediated mechanisms.[38−40] In many systems, AMPA-induced glutamate release seems mainly due to Na^+-dependent mechanisms.[39−41] This is not surprising as most AMPA receptors are Ca^{2+}-impermeable.[42]

In control rats, 0.1 mM AMPA is enough to activate nearly completely Ca^{2+}-permeable AMPA receptors in the cerebellum

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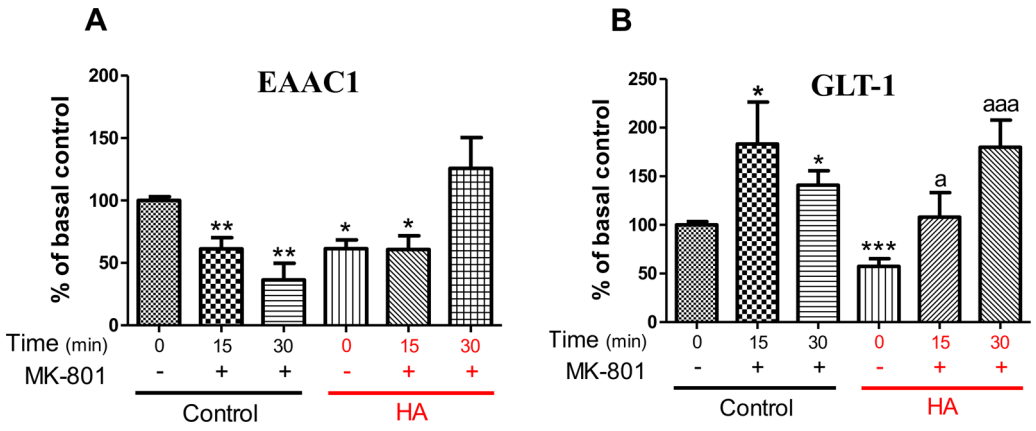

Figure 3. Time course of the effects of MK-801 on membrane expression of EAAC1 and GLT-1 transporters in cerebellar slices from control or hyperammonemic rats. Experiments were performed as in Figure 1C but adding 20 μM MK-801 during 15 and 30 min. Values are the mean ± SEM of 7−13 rats per group. Values significantly different from basal values are indicated by a ($p < 0.05$) or aaa ($p < 0.001$). Values significantly different from control values are indicated with asterisks: *($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

in vivo.[6] AMPA 0.1 mM induces a slight increase in the expression of EAAC1 in membranes (Figure 2C) but does not increase extracellular glutamate in control rats (Figure 2A), suggesting that the glutamate released by activation of Ca^{2+}-permeable AMPA receptors is compensated for by rapid glutamate uptake through EAAC1 (Figure 5A). Uptake of glutamate by EAAC1 would compensate for the release of glutamate induced by the activation by 0.1 mM AMPA of Ca^{2+}-permeable AMPA receptors, resulting in no net change in extracellular glutamate (Figure 5A).

In the presence of MK-801, EAAC1 expression in the membrane is reduced (Figure 3A). This would reduce glutamate uptake, which, under these conditions, would not be enough to compensate for the release induced by 0.1 mM AMPA, resulting in a net increase in extracellular glutamate in control rats (Figures 5B and 2B).

Effects in Hyperammonemic Rats. Chronic hyperammonemia increases the content of the GluR2 subunit of AMPA receptors in the cerebellum.[6] AMPA receptors containing the GluR2 subunit are calcium-impermeable, and its activation increases intracellular Na^+. Cabrera-Pastor et al.[6] suggest that in hyperammonemic rats activation of high affinity AMPA receptors with 0.1 mM AMPA induces only a mild calcium increase but a strong Na^+ increase.[6] This would potentiate NMDA receptor activation due both to reduced inhibition by calcium and enhanced activation by sodium.[7] In control rats, with lower levels of GluR2-containing AMPA receptors calcium influx reduces NMDA receptor activation by different mechanisms.[43−45] When the sodium increase is low, the inhibitory effect of calcium prevails leading to reduced NMDA receptor activation. This Ca^{2+}-induced inhibition of NMDA receptors is overcome by large increases in intracellular sodium, leading to increased NMDA receptor activation.[7] This would be the case in hyperammonemic rats; the larger amount of GluR2-containing AMPA receptors would lead to higher increase in sodium and lower increase in calcium than those in control rats, resulting in enhanced activation of NMDA receptors (Figure 5C).

Activation of NMDA-receptors leads to glutamate release.[9−12] Therefore, in hyperammonemic rats the sequence of events following perfusion of 0.1 mM AMPA would be a large increase in Na^+, NMDA receptor activation, and a large release of glutamate, which cannot be compensated for by uptake through EAAC1 (Figure 5C). According to this sequence, blocking

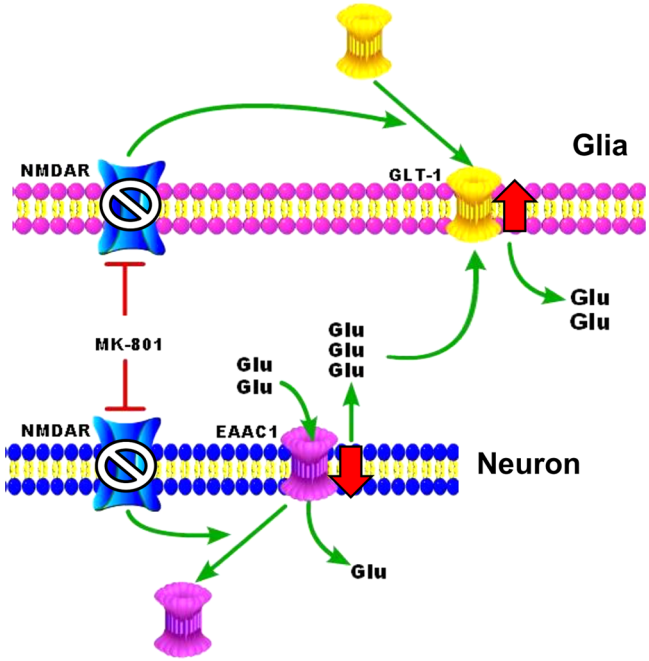

Figure 4. Possible mechanism of the changes in extracellular glutamate induced by MK-801. MK-801 induces a decrease in membrane expression of EAAC1 in neurons (see Figure 3C), increasing the extracellular glutamate followed by an increase in membrane expression of GLT-1 in glia which would mediate the decrease of extracellular glutamate.

NMDA receptors with MK-801 completely prevents the NMDA receptor-mediated increase of glutamate induced by 0.1 mM AMPA (Figure 2B and Figure 5D).

Mechanisms Involved in the Effects of Activation of Low Affinity AMPA Receptors. *Effects in Control Rats, without MK-801.* These effects are summarized in Figure 6A. In control rats, activation of low affinity AMPA receptors induces a rapid decrease in membrane expression of EAAC1 (Figure 1C, 8 min), reducing the uptake of glutamate and leading to increased extracellular glutamate (Figure 1A). The increased extracellular glutamate, together with the mild depolarization of neurons, activates NMDA receptors (Figure 6A) which in turn increases membrane expression of EAAC1 above basal levels (Figure 1C, 15 min). This results in a strong uptake of glutamate,

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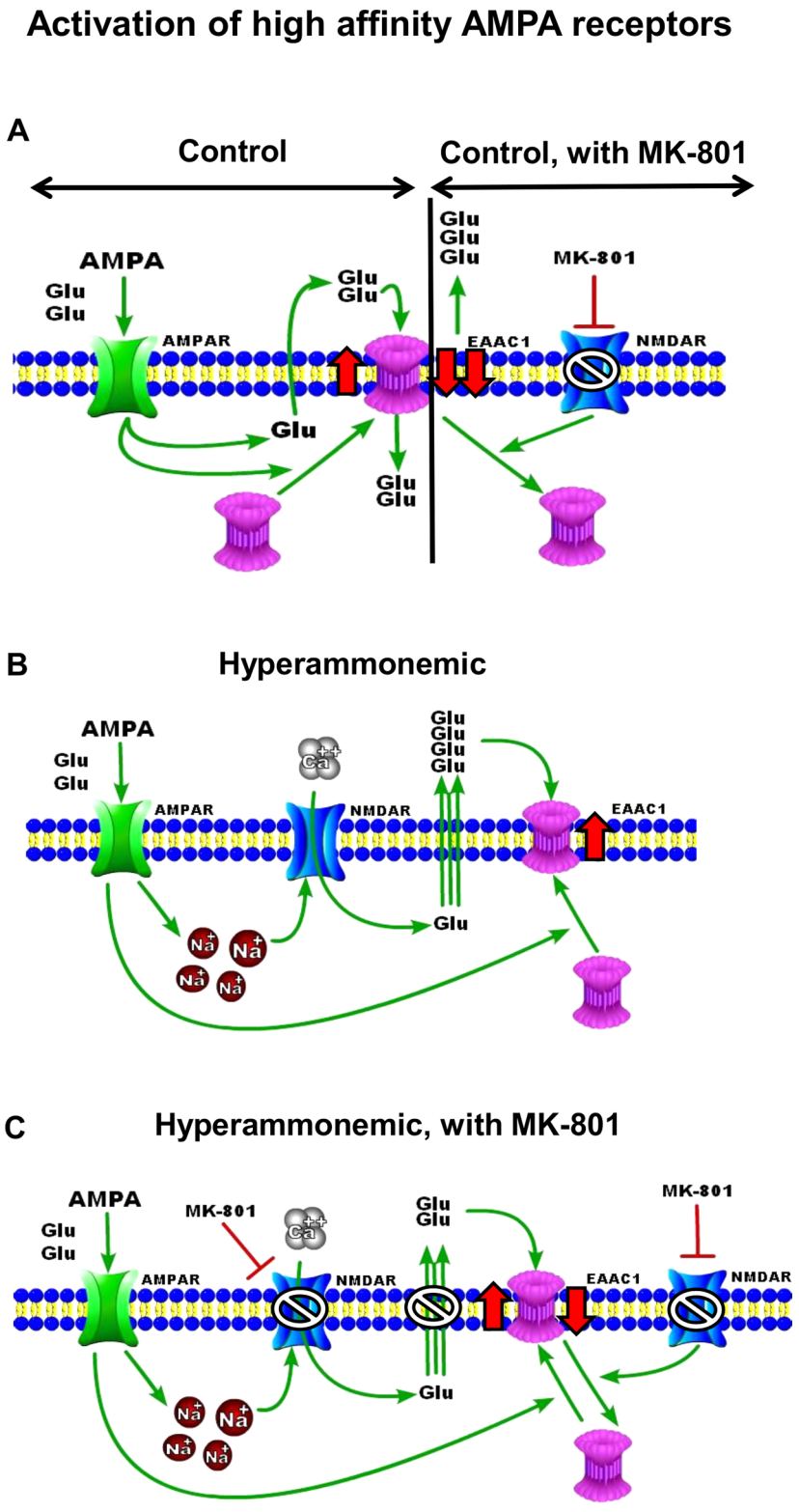

Figure 5. Possible mechanism of the changes in extracellular glutamate induced by activation of high affinity AMPA receptors in control and hyperammonemic rats. Role of NMDA receptors. (A) Control rats. AMPA (0.1 mM) induces a release of glutamate which is compensated by an increase in membrane expression of the EAAC1 transporter, resulting in no net change in extracellular glutamate. However, in the presence of MK-801, EAAC1 membrane expression is lower and is not enough to compensate for the release of glutamate induced by 0.1 mM AMPA, resulting in a net increase in extracellular glutamate. (B) Hyperammonemic rats, without MK-801. In hyperammonemic rats, perfusion of 0.1 mM AMPA would induce a large increase in Na^+ and activation of NMDA receptors, leading to a large release of glutamate which cannot be compensated for rapidly by the increase in membrane expression of EAAC1 also induced by 0.1 mM AMPA. (C) Hyperammonemic rats with MK-801. Blocking NMDA receptors with MK-801 prevents the NMDA receptor-mediated increase of glutamate by 0.1 mM AMPA, resulting in no net change in extracellular glutamate.

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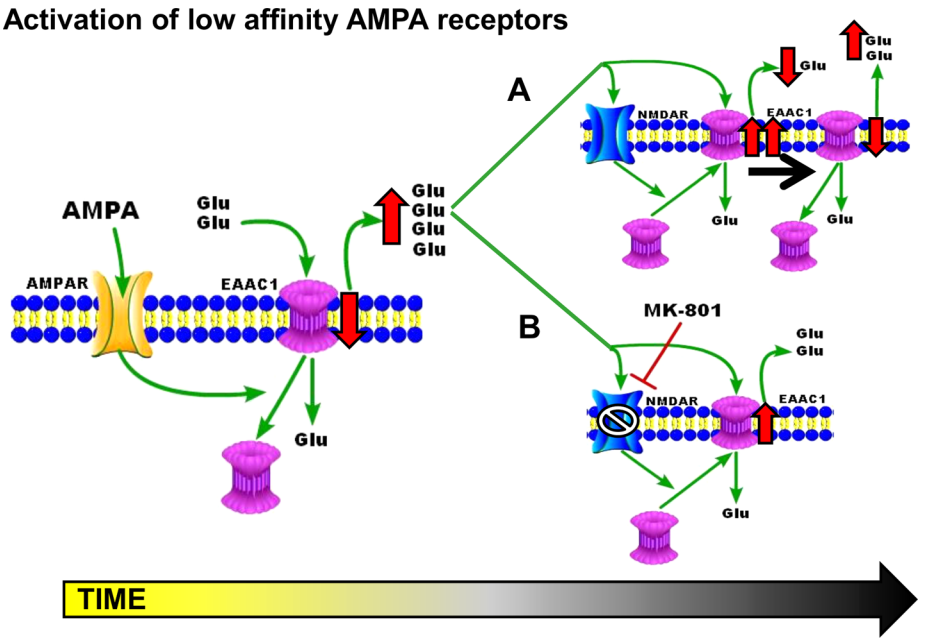

Figure 6. Possible mechanism of the changes in extracellular glutamate induced by activation of low affinity AMPA receptors in control and hyperammonemic rats. Role of NMDA receptors. AMPA (0.3 mM) induces a rapid reduction in membrane expression of EAAC1, resulting in increased extracellular glutamate which led to enhanced activation of NMDA receptors (A), thus enhancing membrane expression of EAAC1, which strongly reduces extracellular glutamate below basal levels. This reduces activation of NMDA receptors and membrane expression of EAAC1, restoring glutamate levels to basal levels. Blocking NMDA receptors with MK-801 (B) prevents this NMDA-induced increase of EAAC1 and extracellular glutamate decrease below basal levels.

which decreases below basal levels (Figure 1A), reducing activation of NMDA receptors and membrane expression of EAAC1 to basal levels (Figure 1C, 30 min). This in turn also restores extracellular glutamate to basal levels (Figures 1A and 6A)

Effects in Control Rats, with MK-801. According to this sequence of events, the release of glutamate induced by 0.3 mM AMPA does not depend on NMDA receptors, and it is therefore not affected by MK-801. However, the sustained reduction of glutamate observed after the third fraction following the addition of 0.3 mM AMPA in Figure 1A would be mediated by enhanced membrane expression of EACC1 and glutamate uptake as a consequence of NMDA receptor activation. Blocking NMDA receptors with MK-801 reduces this NMDA-induced increase of EAAC1 membrane expression, which is lower at 15 min in the presence than in the absence of MK-801 (Figure 1C). This reduces glutamate uptake, thus preventing the decrease in extracellular glutamate below basal levels and maintining it at basal levels (Figures 1B and 6B).

Effects in Hyperammonemic Rats, without or with MK-801. The effects of the activation of low affinity AMPA receptors are similar in hyperammonemic rats and in control rats. Chronic hyperammonemia does not affect the modulation of extracellular glutamate by low affinity AMPA receptors. However, it strongly alters its modulation by high affinity AMPA receptors (activated by 0.1 mM AMPA). The altered modulation of extracellular glutamate by high affinity AMPA receptors would contribute to the altered glutamatergic neurotransmission in chronic hyperammonemia, which in turn[21−26] is involved in the cognitive and motor alterations in chronic hyperammonemia and hepatic encephalopathy.

This report therefore shows that there is complex cross-talk between AMPA and NMDA receptors with EAAC1 and GLT-1 transporters to tightly modulate extracellular glutamate concentration to allow for proper function of neurotransmission and to rapidly return to lower levels to prevent excitotoxicity. Alterations of these processes in pathological situations such as hyperammonemia would lead to altered neurotransmission which may contribute to the associated cognitive and motor impairments. It should be noted that in the rat model of chronic hyperammonemia used in the present work the levels of ammonia in blood are similar to those present in patients with liver cirrhosis and that ammonia increase in the brain is also mild, reaching around 0.4 mM.[46,47] It is therefore very likely that alterations in the modulation of extracellular glutamate by high affinity AMPA receptors and by NMDA receptors similar to those reported here may also occur in the brain of cirrhotic patients with minimal hepatic encephalopathy. These alterations could contribute to the cognitive and motor alterations present in these patients.[24]

METHODS

Model of Chronic Hyperammonemia in Rats. Male Wistar rats (120−140 g, Charles River, France) were made hyperammonemic by feeding them an ammonium-containing diet for 4−6 weeks as in Felipo et al.[48] The experiments were approved by the Comité de Ética de Experimentación Animal of the Centro de Investigación Príncipe Felipe and carried out in accordance with the European Communities Council Directive (86/609/EEC).

***In Vivo* Microdialysis.** Rats were anesthetized using halothane, and a microdialysis guide (CMA, Stockholm, Sweden) was implanted in the right cerebellar lobule, in the molecular layer of the cerebellar cortex (coordinates AP −10.2, ML −1.6, and DV −1.2 according to Paxinos and Watson,[49] as previously reported in ref 50). The probes were placed in the external part of the cerebellum to minimize the damage produced by the microdialysis probe. After 48 h, a microdialysis probe (CMA/12; 3 mm long) was implanted in a freely moving rat. Probes (CMA, Stockholm, Sweden) were perfused (3 μL/min) with artificial cerebrospinal fluid (in mM): NaCl, 145; KCl, 3.0; $CaCl_2$, 2.26; buffered at pH 7.4 with 2 mM phosphate. After 2−3 h of stabilization period, five samples (30 min each) were collected to measure basal levels of glutamate. Then, the different compounds tested were perfused through the microdialysis probe for the times indicated in each Figure.

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The compounds used were the following: AMPA (100 μM) to activate high affinity AMPA receptors; AMPA (300 μM) to activate low affinity AMPA receptors; and MK-801 ((+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*] cyclohepten-5,10-imine maleate) (0.5 μM) to block NMDA receptors. Samples were stored at −80 °C until analysis.

We have shown that recovery of cGMP though the microdialysis probe is 11%.[50] A similar recovery has been used for AMPA and NMDA, for example, by Bhardwaj et al.[51] Taking this into account, the concentration of AMPA achieved in the extracellular fluid in the cerebellum would be around 10 and 30 μM when 0.1 or 0.3 mM AMPA are applied through the microdialysis probe.

Determination of Glutamate. Glutamate in the microdialysis samples were analyzed using a Waters reverse-phase HPLC system with fluorescence detection and precolumn *o*-phtalaldehyde derivatization (Waters), as previously described.[52]

Membrane Surface Expression of the EAAC1 and GLT-1 Transporters by Cross-Linking with BS_3. Membrane surface expression of the EAAC1 and GLT-1 transporters by cross-linking with BS_3 in cerebellar slices was analyzed as described by Boudreau and Wolf.[53] Control and hyperammonemic rats were decapitated and their brains transferred into ice-cold Krebs buffer (in mmol/L); NaCl 119, KCl 2.5, KH_2PO_4 1, $NaHCO_3$ 26.2, $CaCl_2$ 2.5, and glucose 11, aerated with 95% O_2 and 5% CO_2 at pH 7.4. Cerebellums were dissected and transverse slices (400 μm) were obtained using a vibrotome (LEICA, Vt1000s), transferred to incubation wells and incubated for 30 min at 35.5 °C in Krebs buffer. AMPA (0.3 or 2 mM) and MK-801 (20 μM) were added and the incubation continued for 20 min. The concentration used in slices was higher than that in vivo because our experience shows that there is a dilution effect in this system and that higher concentrations are required to induce similar effects (e.g., see ref 54). Slices were added to tubes containing ice-cold standard buffer with or without 2 mM BS_3 (Pierce, Rockford,IL) and incubated for 30 min at 4 °C. Cross-linking was terminated by adding 100 mM glycine (10 min, 4 °C). The slices were homogenized by sonication for 20 s. Samples treated or not with BS_3 were analyzed by Western blot using anti-EAAC1 (MAB1587; Millipore, dilution 1:250) and anti-GLT-1 (Tocris bioscience, Cat.No.2063, 1:4000). The surface expression of EAAC1 or GLT-1 was calculated as the difference between the intensity of the bands without BS3 (total protein) and with BS3 (nonmembrane protein).

Statistical Analysis. Results are expressed as the mean ± SEM. Data were analyzed by analysis of variance (ANOVA) followed by Dunnett's posthoc test. When only 2 values were compared, an unpaired Student's *t* test was used. Significance levels were set at $\alpha = 0.05$.

AUTHOR INFORMATION

Corresponding Author

*Tel: 34 963289680. Fax: 34 963289701. E-mail: vfelipo@cipf.es.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AMPA, (*R*,*S*)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; cGMP, cyclic guanosine monophosphate; GABA, aminobutyric acid; GluR, glutamate receptor subunit; mGluR, metabotropic glutamate receptor; MK-801, (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*- dibenzo[*a*,*d*]cyclohepten-5,10-imine; NMDA, *N*-methyl-daspartic acid; NO, nitric oxide; EAAC1, excitatory amino acid carrier 1; GLAST, glutamate/aspartate transporter; GLT-1, glutamate transporter-1; HPLC, high performance liquid chromatography

REFERENCES

(1) Liu, S. J., and Cull-Candy, S. G. (2005) Subunit interaction with PICK and GRIP controls Ca^{2+} permeability of AMPARs at cerebellar synapses. *Nat. Neurosci. 8*, 768−775.

(2) Geiger, J. R., Melcher, T., Koh, D. S., Sakmann, B., Seeburg, P. H., Jonas, P., and Monyer, H. (1995) Relative abundance of subunit mRNAs determines gating and Ca^{2+} permeability of AMPA receptors in principal neurons and interneurons in rat CNS. *Neuron 15*, 193−204.

(3) Hall, R. A., Kessler, M., and Lynch, G. (1999) Evidence that high- and low-affinity DL-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) binding sites reflect membrane-dependent states of a single receptor. *J. Neurochem. 59* (6), 1997−2004.

(4) Kessler, M., Suzuki, E., Montgomery, K., and Arai, A. C. (2008) Physiological significance of high- and low-affinity agonist binding to neuronal and recombinant AMPA receptors. *Neurochem. Int. 52* (8), 1383−93.

(5) Isaac, J. T., Ashby, M. C., and McBain, C. J. (2007) The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity. *Neuron 54* (6), 859−71.

(6) Cabrera-Pastor, A., Llansola, M., Reznikov, V., Boix, J., and Felipo, V. (2012) Differential effects of chronic hyperammonemia on modulation of the glutamate-nitric oxide-cGMP pathway by metabotropic glutamate receptor 5 and low and high affinity AMPA receptors in cerebellum in vivo. *Neurochem. Int. 61* (1), 63−71.

(7) Xin, W. K., Kwan, C. L., Zhao, X. H., Xu, J., Ellen, R. P., McCulloch, C. A., and Yu, X. M. (2005) A functional interaction of sodium and calcium in the regulation of NMDA receptor activity by remote NMDA receptors. *J. Neurosci. 25*, 139−148.

(8) Salonen, V., Kallinen, S., Lopez-Picon, F. R., Korpi, E. R., Holopainen, I. E., and Uusi-Oukari, M. (2006) AMPA/kainate receptor-mediated up-regulation of GABAA receptor delta subunit mRNA expression in cultured rat cerebellar granule cells is dependent on NMDA receptor activation. *Brain Res. 1087* (1), 33−40.

(9) Nei, K., Matsuyama, S., Shuntoh, H., and Tanaka, C. (1996) NMDA receptor activation induces glutamate release through nitric oxide synthesis in guinea pig dentate gyrus. *Brain Res. 728* (1), 105−110.

(10) Hamada, T., Sonoda, R., Watanabe, A., Ono, M., Shibata, S., and Watanabe, S. (1998) NMDA induced glutamate release from the suprachiasmatic nucleus: an in vitro study in the rat. *Neurosci. Lett. 256* (2), 93−96.

(11) Oh, S., Yun, B. S., Ryoo, I. J., McCaslin, P. P., and Yoo, I. D. (1999) Nitric oxide synthase inhibitor decreases NMDA-induced elevations of extracellular glutamate and intracellular Ca^{2+} levels via a cGMP-independent mechanism in cerebellar granule neurons. *Arch. Pharmacal Res. 22* (1), 48−54.

(12) Kretschmer, B. D., Goiny, M., and Herrera-Marschitz, M. (2000) Effect of intracerebral administration of NMDA and AMPA on dopamine and glutamate release in the ventral pallidum and on motor behavior. *J. Neurochem. 74* (5), 2049−2057.

(13) Barnes, J. M., Dev, K. K., and Henley, J. M. (1994) Cyclothiazide unmasks AMPA-evoked stimulation of [3H]-L-glutamate release from rat hippocampal synaptosomes. *Br. J. Pharmacol. 113* (2), 339−341.

(14) Patel, D. R., and Croucher, M. J. (1997) Evidence for a role of presynaptic AMPA receptors in the control of neuronal glutamate release in the rat forebrain. *Eur. J. Pharmacol. 332* (2), 143−151.

(15) Patel, D. R., Young, A. M., and Croucher, M. J. (2001) Presynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor-mediated stimulation of glutamate and GABA release in the rat striatum in vivo: a dual-label microdialysis study. *Neuroscience 102* (1), 101−111.

(16) Levenes, C., Daniel, H., and Crepel, F. (2001) Retrograde modulation of transmitter release by postsynaptic subtype 1 metabotropic glutamate receptors in the rat cerebellum. *J. Physiol. 537* (Pt 1), 125−140.

(17) Kakizawa, S., Miyazaki, T., Yanagihara, D., Iino, M., Watanabe, M., and Kano, M. (2005) Maintenance of presynaptic function by AMPA receptor-mediated excitatory postsynaptic activity in adult brain. *Proc. Natl. Acad. Sci. U. S. A. 102* (52), 19180−19185.

DOI: 10.1021/acschemneuro.5b00212
ACS Chem. Neurosci. 2015, 6, 1913−1921

(18) Diamond, J. S. (2001) Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. *J. Neurosci. 21* (21), 8328−8338.
(19) Waxman, E. A., Baconguis, I., Lynch, D. R., and Robinson, M. B. (2007) N-methyl-D-aspartate receptor-dependent regulation of the glutamate transporter excitatory amino acid carrier 1. *J. Biol. Chem. 282* (24), 17594−17607.
(20) Levenson, J., Weeber, E., Selcher, J. C., Kategaya, L. S., Sweatt, J. D., and Eskin, A. (2002) Long-term potentiation and contextual fear conditioning increase neuronal glutamate uptake. *Nat. Neurosci. 5* (2), 155−61.
(21) Monfort, P., Muñoz, M. D., ElAyadi, A., Kosenko, E., and Felipo, V. (2002) Effects of hyperammonemia and liver failure on glutamatergic neurotransmission. *Metab. Brain Dis. 17* (4), 237−250.
(22) Monfort, P., Kosenko, E., Erceg, S., Canales, J. J., and Felipo, V. (2002) Molecular mechanism of acute ammonia toxicity: role of NMDA receptors. *Neurochem. Int. 41* (2−3), 95−102.
(23) Felipo, V., Hermenegildo, C., Montoliu, C., Llansola, M., and Miñana, M. D. (1998) Neurotoxicity of ammonia and glutamate: molecular mechanisms and prevention. *Neurotoxicology 19* (4−5), 675−681.
(24) Felipo, V. (2013) Hepatic encephalopathy: effects of liver failure on brain function. *Nat. Rev. Neurosci. 14* (12), 851−858.
(25) Cauli, O., Rodrigo, R., Llansola, M., Montoliu, C., Monfort, P., Piedrafita, B., El Mlili, N., Boix, J., Agustí, A., and Felipo, V. (2009) Glutamatergic and GABAergic neurotransmission and neuronal circuits in hepatic encephalopathy. *Metab. Brain Dis. 24*, 69−80.
(26) Monfort, P., Cauli, O., Montoliu, C., Rodrigo, R., Llansola, M., Piedrafita, B., El Mlili, N., Boix, J., Agustí, A., and Felipo, V. (2009) Mechanisms of cognitive alterations in hyperammonemia and hepatic encephalopathy. Therapeutical implications. *Neurochem. Int. 55*, 106−112.
(27) Felipo, V., and Butterworth, R. F. (2002) Neurobiology of ammonia. *Prog. Neurobiol. 67* (4), 259−79.
(28) Erceg, S., Monfort, P., Hernández-Viadel, M., Rodrigo, R., Montoliu, C., and Felipo, V. (2005) Oral administration of sildenafil restores learning ability in rats with hyperammonemia and with portacaval shunt. *Hepatology 41* (2), 299−306.
(29) Erceg, S., Monfort, P., Hernandez-Viadel, M., Llansola, M., Montoliu, C., and Felipo, V. (2005) Restoration of learning ability in hyperammonemic rats by increasing extracellular cGMP in brain. *Brain Res. 1036*, 115−121.
(30) Cauli, O., Mansouri, M. T., Agusti, A., and Felipo, V. (2009) Hyperammonemia increases GABAergic tone in cerebellum but decreases it in rat cortex. *Gastroenterology 136* (4), 1359−1367.
(31) Boix, J., Llansola, M., Cabrera-Pastor, A., and Felipo, V. (2011) Metabotropic glutamate receptor 5 modulates the nitric oxide-cGMP pathway in cerebellum in vivo through activation of AMPA receptors. *Neurochem. Int. 58*, 599−604.
(32) Ceglia, I., Carli, M., Baviera, M., Renoldi, G., Calcagno, E., and Invernizzi, R. W. (2004) The 5-HT receptor antagonist M100,907 prevents extracellular glutamate rising in response to NMDA receptor blockade in the mPFC. *J. Neurochem. 91* (1), 189−199.
(33) Carli, M., Calcagno, E., Mainini, E., Arnt, J., and Invernizzi, R. W. (2011) Sertindole restores attentional performance and suppresses glutamate release induced by the NMDA receptor antagonist CPP. *Psychopharmacology (Berl) 214* (3), 625−367.
(34) Zuo, D. Y., Zhang, Y. H., Cao, Y., Wu, C. F., Tanaka, M., and Wu, Y. L. (2006) Effect of acute and chronic MK-801 administration on extracellular glutamate and ascorbic acid release in the prefrontal cortex of freely moving mice on line with open-field behavior. *Life Sci. 78* (19), 2172−8.
(35) López-Gil, X., Artigas, F., and Adell, A. (2009) Role of different monoamine receptors controlling MK-801-induced release of serotonin and glutamate in the medial prefrontal cortex: relevance for antipsychotic action. *Int. J. Neuropsychopharmacol. 12* (4), 487−99.
(36) Rossi, B., and Collin, T. (2013) Presynaptic NMDA receptors act as local high-gain glutamate detector in developing cerebellar molecular layer interneurons. *J. Neurochem. 126* (1), 47−57.
(37) Conti, F., DeBiasi, S., Minelli, A., and Melone, M. (1996) Expression of NR1 and NR2A/B subunits of the NMDA receptor in cortical astrocytes. *Glia. 17* (3), 254−8.
(38) Bezzi, P., Carmignoto, G., Pasti, L., Vesce, S., Rossi, D., Rizzini, B. L., Pozzan, T., and Volterra, A. (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature 391* (6664), 281−285.
(39) Smith, J. P., Cunningham, L. A., and Partridge, L. D. (2000) Coupling of AMPA receptors with the Na^+/Ca^{2+} exchanger in cultured rat astrocytes. *Brain Res. 887* (1), 98−109.
(40) Zamalloa, T., Bailey, C. P., and Pineda, J. (2009) Glutamate-induced post-activation inhibition of locus coeruleus neurons is mediated by AMPA/kainate receptors and sodium-dependent potassium currents. *Br. J. Pharmacol. 156* (4), 649−661.
(41) Dumuis, A., Sebben, M., Fagni, L., Prézeau, L., Manzoni, O., Cragoe, E. J., Jr., and Bockaert, J. (1993) Stimulation by glutamate receptors of arachidonic acid release depends on the Na^+/Ca^{2+} exchanger in neuronal cells. *Mol. Pharmacol. 43* (6), 976−981.
(42) Bennett, M. V., Pellegrini-Giampietro, D. E., Gorter, J. A., Aronica, E., Connor, J. A., and Zukin, R. S. (1996) The GluR2 hypothesis: Ca^{+2}-permeable AMPA receptors in delayed neurodegeneration. *Cold Spring Harb Symp. Quant Biol. 61*, 373−384.
(43) Lieberman, D. N., and Mody, I. (1994) Regulation of NMDA channel function by endogenous Ca^{2+}-dependent phosphatase. *Nature 369*, 235−239.
(44) Ehlers, M. D., Zhang, S., Bernhadt, J. P., and Huganir, R. L. (1996) Inactivation of NMDA by direct interaction of calmodulin with the NR1 subunit. *Cell 84*, 745−755.
(45) Krupp, J. J., Vissel, B., Thomas, C. G., Heinemann, S. F., and Westbrook, G. L. (1999) Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate Ca2+-dependent inactivation of NMDA receptors. *J. Neurosci. 19*, 1165−1178.
(46) Kosenko, E., Kaminsky, Y. G., Felipo, V., Miñana, M. D., and Grisolía, S. (1993) Chronic hyperammonemia prevents changes in brain energy and ammonia metabolites induced by acute ammonium intoxication. *Biochim. Biophys. Acta, Mol. Basis Dis. 1180*, 321−326.
(47) Rodrigo, R., Jover, R., Candela, A., Compañ, A., Sáez-Valero, J., Erceg, S., and Felipo, V. (2005) Bile duct ligation plus hyperammonemia in rats reproduces the alterations in the modulation of soluble guanylate cyclase by nitric oxide in brain of cirrhotic patients. *Neuroscience 130*, 435−443.
(48) Felipo, V., Miñana, M. D., and Grisolía, S. (1988) Long term ingestion of ammonium increases acetylglutamate and urea levels without affecting the amount of carbamyl phosphate synthase. *Eur. J. Biochem. 176*, 567−571.
(49) Paxinos, G., and Watson, C. (1996) *The Rat Brain in Stereotaxic Coordinates*, 4th ed., Academic Press, New York, NY.
(50) Hermenegildo, C., Montoliu, C., Llansola, M., Muñoz, M. D., Gaztelu, J. M., Miñana, M. D., and Felipo, V. (1998) Chronic hyperammonemia impairs the glutamate-nitric oxide-cyclic GMP pathway in cerebellar neurons in culture and in the rat in vivo. *Eur. J. Neurosci. 10*, 3201−3209.
(51) Bhardwaj, A., Northington, F. J., Ichord, R. N., Hanley, D. F., Traystman, R. J., and Koehler, R. C. (1997) Characterization of ionotropic glutamate receptor-mediated nitric oxide production in vivo in rats. *Stroke 28* (4), 850−7.
(52) Canales, J. J., Elayadi, A., Errami, M., Llansola, M., Cauli, O., and Felipo, V. (2003) Chronic hyperammonemia alters motor and neurochemical responses to activation of group I metabotropic glutamate receptors in the nucleus accumbens in rats in vivo. *Neurobiol. Dis. 14*, 380−390.
(53) Boudreau, A. C., and Wolf, M. E. (2005) Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. *J. Neurosci. 25* (40), 9144−51.
(54) ElMlili, N., Boix, J., Ahabrach, H., Rodrigo, R., Errami, M., and Felipo, V. (2010) Chronic hyperammonemia induces tonic activation of NMDA receptors in cerebellum. *J. Neurochem. 112*, 1005−1014.

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ACS Chem. Neurosci. 2015, 6, 1913−1921