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RESEARCH

Identification of Non-excitatory Amino Acids and Transporters Mediating the Irreversible Synaptic Silencing After Hypoxia

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Abstract

The contribution of excitatory amino acids (AA) to ischemic brain injury has been widely described. In addition, we reported that a mixture of non-excitatory AA at plasmatic concentrations turns irreversible the depression of synaptic transmission caused by hypoxia. Here, we describe that the presence of seven non-excitatory AA (L-alanine, L-glutamine, glycine, L-histidine, L-serine, taurine, and L-threonine) during hypoxia provokes an irreversible neuronal membrane depolarization, after an initial phase of hyperpolarization. The collapse of the membrane potential correlates with a great increase in fiber volley amplitude. Nevertheless, we show that the presence of all seven AA is not necessary to cause the irreversible loss of fEPSP after hypoxia and that the minimal combination of AA able to provoke a solid, replicable effect is the mixture of L-alanine, glycine, L-glutamine, and L-serine. Additionally, L-glutamine seems necessary but insufficient to induce these harmful effects. We also prove that the deleterious effects of the AA mixtures on field potentials during hypoxia depend on both the identity and concentration of the individual AA in the mixture. Furthermore, we find that the accumulation of AA in the whole slice does not determine the outcome caused by the AA mixtures on the synaptic transmission during hypoxia. Finally, results obtained using pharmacological inhibitors and specific substrates of AA transporters suggest that system N and the alanine-serine-cysteine transporter 2 (ASCT2) participate in the non-excitatory AA-mediated deleterious effects during hypoxia. Thus, these AA transporters might represent therapeutical targets for the treatment of brain ischemia.

Keywords Amino acid transporters · Hypoxia · ASCT2 · Glutamate receptors · Synaptic transmission · Hippocampus · Swelling

Introduction

In the brain, during pathological conditions such as ischemia and cerebral hemorrhage, the concentration of extracellular amino acids (AA) increases [5, 21, 49]. The participation of the excitatory AA glutamate and aspartate in excitotoxicity has been extensively studied [9, 40] but little is known about non-excitatory AA contribution to brain injury, especially in combination with the hypoxia produced when cerebral blood flow is reduced after stroke or brain trauma.

We found seven non-excitatory AA (L-alanine, glycine, L-histidine, L-glutamine, L-serine, L-threonine, and taurine), among a total of 19 AA, that increased extracellular field potentials amplitude in acute hippocampal slices when applied at high concentrations under normoxic conditions [26]. Additionally, we have recently shown that, during hypoxia, a mixture of these seven non-excitatory AA (AGHQSTU, as abbreviated by the one letter code) at plasmatic concentrations is intracellularly accumulated, causing an irreversible depression of field excitatory postsynaptic potentials (fEPSP), a potentiation of fiber volley (FV) amplitude, and the increase of slice electrical resistance [3], indicating a reduction of the extracellular volume [8, 44]. Furthermore, neuronal and astroglial swelling produced by four of these non-excitatory AA in hypoxia was subsequently probed using two-photon laser-scanning and electron microscopy [2].

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According to these data, we propose that intracellular accumulation of osmotically active AA is central to inducing cell swelling during ischemic conditions and that it might be attributed to the activity of plasma membrane AA transporters. Most of the AA causing these deleterious effects are substrates of Na^+-dependent neutral AA transporter systems, such as alanine-serine-cysteine transporters (ASCT, SLC1 family) [45], system A, and system N (SLC38 family) [6, 7]; and Na^+-independent AA transporters, such as system L (SLC7 family) [39]. Some of these AA transporters act as exchangers (ASCT and system L), meaning that they release substrates to the extracellular space with a 1:1 stoichiometry when taking up AA [6, 28]. In the case of ASCT, it releases endogenous D-serine, modulating N-methyl-D-aspartate receptors (NMDAR) [22], whose activity has previously been demonstrated to be involved in the harmful effects provoked by AGHQSTU in hypoxia [2, 3]. Additionally, we have shown that system A transporters are not involved in the irreversible effects caused by non-excitatory AA on synaptic transmission during hypoxia, indicating that the activity of system A is not necessary or sufficient for the intracellular accumulation of non-excitatory AA [3].

Another issue relevant to understanding the deleterious effects of non-excitatory AA is to reveal to what extent the amount, identity, and concentration of AA in the mixture are important in causing such effects. Using hippocampal slices and electrophysiological techniques combined with HPLC analysis, here we investigated whether all seven non-excitatory AA of the AGHQSTU mixture are necessary to provoke the irreversible outcomes during hypoxia or if a combination of fewer AA would also develop harmful effects. For this purpose, we used several artificial cerebrospinal fluids (ACSF) containing different combinations of non-excitatory AA with similar concentrations to the ones found in rat plasma. Specific substrates and pharmacological tools allowed a more detailed study of the possible AA transporters participating in the intracellular accumulation of the AA. The involvement of glutamate receptors that might be secondarily activated aggravating hypoxic damage was also addressed. These AA transporter systems may be implicated in ischemia-driven cell swelling, and the modulation of their activity might represent a new therapeutic target in the treatment of cerebral ischemia.

Materials and Methods

Hippocampal Slice Preparation and Solutions

All procedures followed the European Communities Council Directive (63/2010/ECC) and the Spanish legislation (R.D. 53/2013). Protocols and procedures were approved by the Animal Ethics Committee of Hospital Universitario Ramón y Cajal (ES-28079–00000-92). Sprague–Dawley rats were bred and housed in group cages in the certified animal facilities of Hospital Universitario Ramón y Cajal under pathogen-free conditions, in a 12-h light/dark cycle, with constant temperature (22 ± 1 °C), and provided with food and water ad libitum. A total of 163 young adult male rats (7–9 weeks old) were used.

Acute hippocampal slices (400 µm thickness) were obtained according to standard protocols [3]. Rats were decapitated under deep isoflurane anesthesia. The brain was quickly removed and placed into ice-cold standard ACSF solution containing (in mM): 119 NaCl, 26.2 $NaHCO_3$, 2.5 KCl, 1 KH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, and 11 glucose (osmolarity: 295.0 ± 0.5 mOsm; bubbled with 95% O_2 and 5% CO_2. Transverse dorsal hippocampal slices were cut using a manual tissue chopper (Stoelting, Wood Dale, IL). The whole procedure did not last more than 5 min. After sectioning, slices were allowed to recover in an interface holding chamber at room temperature for at least 3 h. After that, a single slice was transferred to an electrophysiological recording chamber. No more than two slices per rat were included in each experimental group.

Chemicals

Stock solutions of L-alanine, glycine, L- and D-histidine, L-serine, L- and D-threonine, and taurine were prepared in distilled water, stored at − 20 °C, and dissolved daily in standard ACSF solution at their final concentrations. L-glutamine was dissolved daily just before use in standard ACSF solution. Final plasmatic concentrations of these AA in the different mixtures, except when indicated, were 480 µM L-alanine, 733 µM L-glutamine, 172 µM glycine, 48 µM L-histidine, 177 µM L-serine, 263 µM taurine, and 234 µM L-threonine. D-2-amino-5-phosphopentanoic acid (D-AP5, 50 µM) was prepared as 25 mM concentrated stock solution in distilled water and stored at − 20 °C. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 µM) was prepared as 20 mM concentrated stock solution in distilled water and stored at − 20 °C. N-methyl-D-aspartate (NMDA, 20 µM) was prepared as 10 mM concentrated stock solution in distilled water and stored at − 20 °C. 2-Amino-2-norbornanecarboxylic acid (BCH, 1 mM) and L-glutamic acid γ-(p-nitroanilide) hydrochloride (GPNA, 1 mM) were prepared daily in standard ACSF at its final concentration. All drugs and chemicals were obtained from Sigma-Aldrich (Madrid, Spain). The osmolarity of ACSF was measured with a micro-osmometer (Advanced Instruments Mod.3MO, Norwood, MA). All drugs and AA were bath applied.

Electrophysiological Recordings

A single acute hippocampal slice was transferred into a submersion-type recording chamber. The slice was continuously superfused with oxygenated standard ACSF solution at a rate of 2 mL/min with a Cole Parmer pump (Vernon Hills, IL). The temperature was maintained at 32 ± 0.5 °C. Hypoxia was achieved by replacing O_2 with N_2.

Evoked fEPSP and presynaptic FV were evoked every 15 s by stimulating Schaffer collateral-commissural pathway with biphasic current pulses (100 μs per phase) delivered through monopolar tungsten insulated microelectrodes (0.5 MΩ, WPI, Sarasota, FL) at CA1 mid-stratum radiatum. Constant current pulses were provided by a pulse generator (AMPI Mod. Master 8, Jerusalem, Israel) connected to a biphasic stimulus isolator unit (Cibertec Mod. 200BIP, Madrid, Spain). The stimulus strength was adjusted to evoke a fEPSP around 40% of its maximal amplitude (10–30 μA). fEPSP and FV were recorded in CA1 stratum radiatum with a tungsten microelectrode (1 MΩ, WPI) connected to an AI-401 preamplifier (Molecular Devices, Sunnyvale, CA). Signals from CyberAmp 320 amplifier (Molecular Devices) were filtered at 3 kHz and digitized at 20 kHz with a Digidata 1440A interface board (Molecular Devices). Evoked synaptic responses in healthy slices had a sigmoidal input/output response function. The synaptic strength was calculated using the initial falling slope phase of the fEPSP to avoid contamination of the response by the propagated population spike. The FV amplitude was measured from the first positive shoulder after the stimulus artifact to the negative peak. The values of every four consecutive responses were averaged to give a mean per minute.

Some slices were used for the simultaneous electrophysiological recording of both intra- and extracellular evoked potentials (see diagram in Fig. 1H). Blind intracellular recordings of neurons in the CA1 stratum pyramidale were obtained with 2 M potassium methylsulfate-filled sharp micropipettes (80–100 MΩ) pulled from borosilicate glass (1.5 mm × 0.86 mm; A-M Systems, Sequim, WA) using a micropipette puller (Sutter Instruments, Mod. P87, Sarasota, FL). Micropipettes were driven by a micromanipulator with a motorized actuator (CMA25CC Newport, Irvine, CA) and were connected to an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA) used in continuous bridge mode. Signals were filtered at 10 kHz and digitized at 20 kHz. The input resistance (R_{in}) of the neurons was determined by applying hyperpolarizing current pulses (0.1–0.3 nA, 50 ms) through the micropipette 200 ms before applying the synaptic stimulation. Acceptance criteria for neurons were established as follows: (1) stable resting membrane potential (V_m) more hyperpolarized than − 60 mV and (2) R_{in} greater than 30 MΩ. Data analysis was performed with pCLAMP 11 software (Molecular Devices). Data were normalized to the mean values of the responses in the baseline period in standard ACSF.

HPLC Amino Acid Analysis

At the end of each electrophysiological experiment, the hippocampal slice was submerged in 200 μL of 5-sulphosalicylic acid (10%) and stored at − 80 °C until the homogenization procedure. The high-performance liquid chromatography (HPLC) procedure used for AA analysis was described by Perucho et al. [32]. Briefly, AA were separated by reverse-phase HPLC (Jasco, Tokyo, Japan) after pre-column derivatization with o-phthalaldehyde (OPA) on a Luna C18 column (150 × 4.6 mm, particle size 3 μm,Phenomenex, Torrance, CA) using gradient elution at 1 mL/min and quantified by fluorescence detection (Mod. FP2020/2025, Jasco). OPA reagent solution was composed of 32 mg OPA diluted in 0.8 mL of methanol with 7.140 mL of borate buffer 0.4 M (pH 9.5) and 60 μL of 3-mercaptopropionic acid. This solution was freshly prepared every week and light protected. A 6 μL volume of the slice sample was prepared for the derivatization procedure by adding 181 μL NaOH (0.01 M). Then, 20 μL of that mixture was allowed to react with 12 μL of OPA solution for 1 min before stopping the reaction with 5 μL of acetic acid (5%). The derivatization reaction of standards and samples was carried out via a programmable automatic injector (Gilson Mod. 231 XL, Middleton, WI). AA were identified by their retention times, and their concentrations were calculated by comparison to calibrated AA external standard solutions (1.5 μM). The following AA were regularly identified and separated in the extracts: aspartate, glutamate, serine, glutamine, histidine, glycine, threonine, taurine, alanine, and GABA. Measurement sensitivity was close to 1 pmol. Chromatograms were recorded and analyzed using the software ChromNAV (Jasco).

Statistical Analysis

Data are expressed as mean ± SEM. The number of experiments (n) corresponds to the number of individual hippocampal slices. Mean values throughout the text correspond to averages of 3- and 5-min periods for intra- and extracellular recordings, respectively. Representative traces correspond to averaged potentials recorded during 2 min. No data were excluded from statistical analyses. The Shapiro–Wilk test was used to evaluate the normal distribution of the data. To determine statistical significance between a pair of means that follow a normal distribution, t-tests were used. One-way ANOVA followed by Dunnett's or Tukey's multiple comparisons tests were performed to compare more than three data groups depending on whether the comparison was made against control or among different means, respectively. Pairs of samples that were not normally distributed were compared

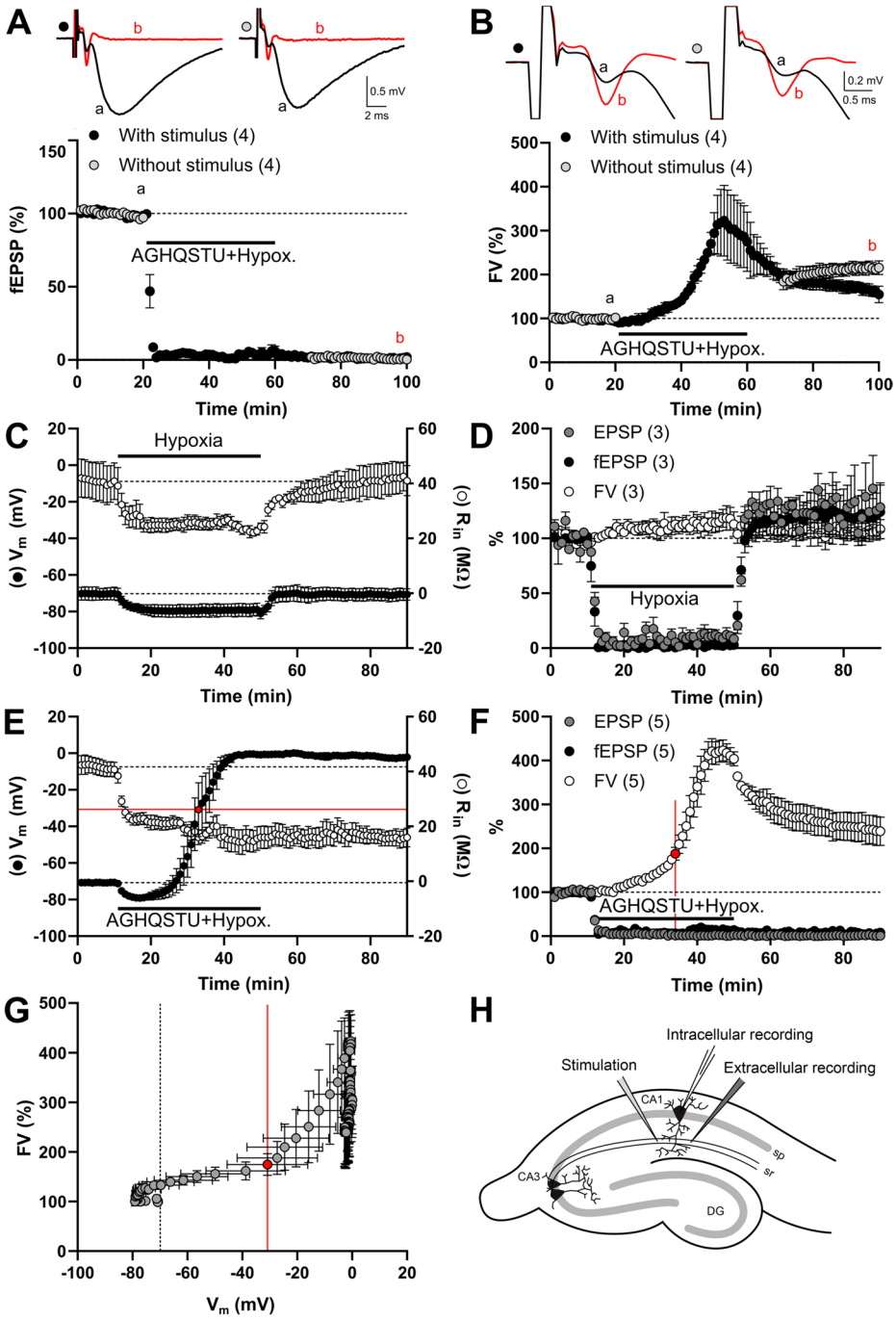

Fig. 1 Effects on field synaptic potentials provoked by the amino acid mixture AGHQSTU during hypoxia do not require synaptic stimulation. Time course of changes on fEPSP (**A**) and FV (**B**) induced by AGHQSTU solution application during 40-min of hypoxia (marked by the black horizontal bar) following the standard stimulation protocol (black circles) or without synaptic stimulation during hypoxia and the firsts 10 min of reoxygenation (gray circles). The traces correspond to averaged evoked field potentials recorded in a representative experiment at times indicated by the letters on the graphs. The stimulus artifact was truncated here and in the following figures for clarity. Changes on membrane potential (V_m; black circles) and inward resistance (R_{in}; white circles) induced by a period of hypoxia alone, $n = 3$ cells in different slices (**C**), or in the presence of AGHQSTU, $n = 5$ (**E**). The dashed lines indicate neuronal resting V_m and R_{in}. Time course of intracellular EPSP (gray circles), extracellular fEPSP (black circles), and FV (white circles) during 40-min hypoxia in the absence (**D**) and presence of AGHQSTU (**F**). **G** Correlation between V_m and FV in slices exposed to AGHQSTU and hypoxia. The dashed line indicates neuronal resting V_m. The red circle and line in **G** represent the inflection point in the slope. The V_m in which this turning point happens is indicated by a red circle and line in **E**. The time point in which that V_m is reached is indicated by a red circle and line in **F**. **H** Schematic drawing of a rat hippocampal slice showing the localization of stimulating and recording electrodes. In this and subsequent figures, numbers in parentheses indicate the number of experiments (slices) included in the mean

with the Mann–Whitney *U* test. When comparing three or more nonparametric data sets, the Kruskal–Wallis test was employed, followed by Dunn's test for multiple comparisons. GraphPad Prism 8 (GraphPad Software, San Diego, CA) was used for statistical analysis and graphing. Differences were statistically significant when $p < 0.05$.

Results

The Irreversible Effects of AGHQSTU Amino Acid Mixture During Hypoxia Are Not Dependent on Synaptic Stimulation

We have confirmed previously described data [3] showing that the application of a mixture of seven non-excitatory AA (AGHQSTU: L-alanine, glycine, L-histidine, L-glutamine, L-serine, L-threonine, and taurine at their plasmatic concentrations) during a 40-min period of hypoxia induces an irreversible loss of fEPSP (Fig. 1A, black circles) and a robust potentiation of FV amplitude (Fig. 1B, black circles). Here, we wanted to know whether the effect of AGHQSTU during hypoxia depended on synaptic stimulation. For this purpose, the hippocampal slices were not electrically stimulated during the 40-min period of AGHQSTU application and hypoxia and the firsts 10 min of the wash out and reoxygenation period. In these experimental conditions, the fEPSP also suffered an irreversible loss during reoxygenation, similar to that found under continuous stimulation ($n = 4$, $t_{(6)} = 2.149$, $p = 0.0752$, *t*-test) (Fig. 1A, gray circles). The FV potentiation caused by AGHQSTU and hypoxia was also observed in the absence of synaptic stimulation (190.8 ± 15.2% and 188.7 ± 17.5% increments with and without stimulation, respectively; $t_{(6)} = 0.08815$, $p = 0.9326$, *t*-test) (Fig. 1B). These results indicate that synaptic stimulation is not necessary for the development of the harmful effects induced by non-excitatory AA during hypoxia.

Basic Neuronal Membrane Properties Recorded During Hypoxia and AGHQSTU Application

To determine how the presence of the AGHQSTU mixture during hypoxia affects basic neuronal membrane properties, we performed intracellular recordings in the pyramidal layer of the hippocampal CA1 area simultaneously with the extracellular field recordings (see Fig. 1H for a schematic representation of the localization of electrodes and recording pipette) during a period of hypoxia without (Fig. 1C and D) and with the AGHQSTU mixture (Fig. 1E–G).

Forty minutes of hypoxia without AA induced a hyperpolarization of the neuronal V_m from − 70.2 ± 3.4 mV during the baseline to − 79.6 ± 3.2 mV ($n = 3$, $F_{(2,4)} = 44.72$, $p = 0.0019$, one-way repeated measures (RM) ANOVA, $p = 0.0019$, Dunnett's test), recovering baseline values upon reoxygenation (− 70.6 ± 3.3 mV, $p = 0.9193$, Dunnett's test). During hypoxia, the R_{in} of the membrane was reduced, although not significantly (baseline R_{in}: 40.0 ± 6.8 MΩ, R_{in} at the end of the hypoxic period: 23.0 ± 1.0 MΩ, R_{in} at the end of the reoxygenation: 41.9 ± 5.4 MΩ, $n = 3$; $F_{(2,4)} = 7.467$, $p = 0.0446$, one-way RM ANOVA; $p = 0.0574$ comparison between the end of hypoxia with baseline, $p = 0.9193$ comparison between the end of reoxygenation and baseline, Dunnett's test) (Fig. 1C). Hypoxia produced a reversible depression of extracellular fEPSP (fEPSP at the end of reoxygenation: 120.5 ± 20.4%, $t_{(2)} = 0.9728$, $p = 0.4333$, *t*-test in comparison with baseline values) and intracellular EPSP (132.3 ± 24.0%, $t_{(2)} = 1.513$, $p = 0.2694$, *t*-test), without affecting FV amplitude (FV at the end of reoxygenation: 106.8 ± 7.9%, $t_{(2)} = 1.097$, $p = 0.3871$, *t*-test) (Fig. 1D).

When hypoxia was accompanied by AGHQSTU, neuronal V_m hyperpolarized during the first 15 min of hypoxia from resting values of − 70.7 ± 0.8 mV to − 78.5 ± 1.6 mV ($n = 5$, $F_{(3,12)} = 1040.0$, $p < 0.0001$, one-way RM ANOVA, $p = 0.0032$, Dunnett's test). Afterwards, the V_m gradually depolarized until reaching − 2.4 ± 1.4 mV at the end of the reoxygenation period ($p < 0.0001$, Dunnett's test). The R_{in} was irreversibly reduced from 40.8 ± 2.7 MΩ during baseline to 15.6 ± 3.2 MΩ at the end of the experiments ($n = 5$, $F_{(2,8)} = 91.21$, $p < 0.0001$, one-way RM ANOVA, $p < 0.0001$, Dunnett's test) (Fig. 1E). As with the extracellular fEPSP, AGHQSTU caused an irreversible loss of intracellular EPSP after hypoxia (Fig. 1F).

We wondered if the intense increase in FV amplitude produced by the AA during hypoxia would be due to the progressive depolarization of the neuronal V_m. Figure 1G shows that both parameters are positively correlated ($r = 0.8833$, $p < 0.0001$, Pearson correlation). However, it is easily distinguishable that the first depolarizing phase from − 80 to − 30 mV (and displayed in the graph as the dots on the left side of the red line) was not accompanied by a great increase in FV amplitude. It is when the V_m continued depolarizing from − 30 to 0 mV (values on the right side of the red line) when the FV underwent a huge potentiation rising 423.0 ± 23.88% at the end of the hypoxic period. The V_m value corresponding to the turning point in the V_m/FV correlation (indicated as a red dot in Fig. 1G) could be used to calculate the time point in which an irreversible effect is reached under these experimental conditions (Fig. 1E and F). This result highlights that the striking increase in FV amplitude occurred after 24 min of hypoxia when the V_m completely depolarizes from − 30.9 ± 14.7 to − 2.4 ± 1.4 mV.

Are All Amino Acids in the AGHTQSTU Mixture Necessary to Induce Hypoxic Damage?

To answer this question, we applied reduced combinations of non-excitatory AA during the hypoxia period. We

began studying the effects of AGHSTU ($n=12$), a mixture without L-glutamine, as this is the most abundant AA in our mixture ([L-Gln] = 733 μM) and because L-glutamine can lead to the metabolic production of L-glutamate [4]. After 40 min of hypoxia, this combination of AA without L-glutamine caused a completely reversible loss of fEPSP that promptly recovered baseline levels with reoxygenation (83.5 ± 15.6% in the last 5 min of the wash out and reoxygenation period, $p=0.1782$, Mann–Whitney U test comparing with the last 5 min of baseline). In the presence of AGHSTU, the FV amplitude increased, although only reaching 151.5 ± 29.6% over baseline values (Fig. 2A). As removing L-glutamine from the mixture avoided the deleterious effects of AGHQSTU and hypoxia, we applied this AA alone at its plasmatic concentration (733 μM) during hypoxia (Q, $n=6$) (Fig. 2B). This showed that L-glutamine by its own does not cause an irreversible loss of fEPSP (84.9 ± 14.3%, $p=0.4848$, Mann–Whitney U test) nor a remarkable potentiation of FV (136.9 ± 18.8%, $t_{(10)}=2.085$, $p=0.0637$, t-test).

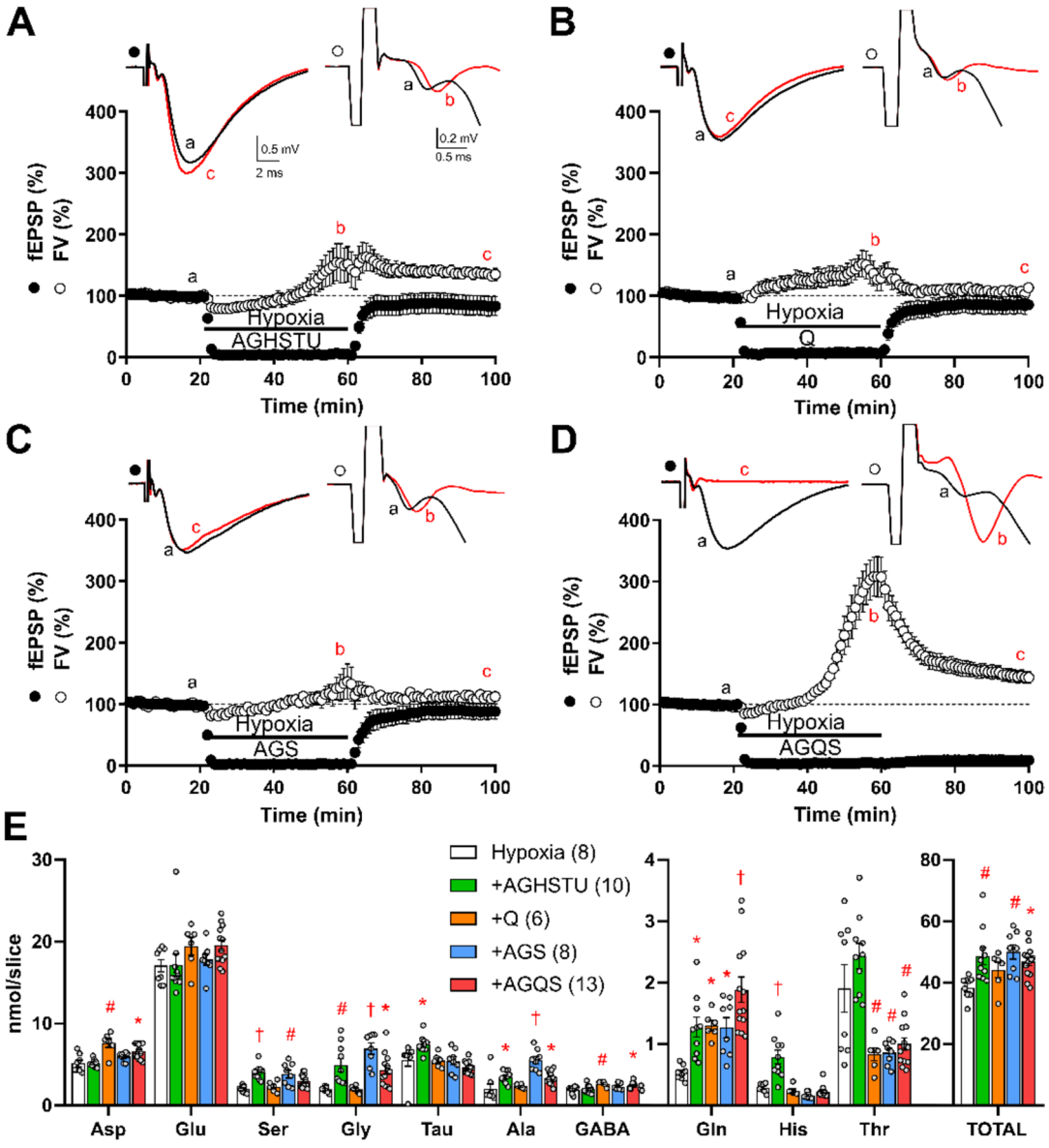

Fig. 2 Effects of AA solutions derived from AGHQSTU and applied during hypoxia on evoked field potentials and slice AA content. Time courses of changes on fEPSP (black circles) and FV (white circles) induced by AGHSTU, $n=12$ (**A**); Q, $n=6$ (**B**); AGS, $n=8$ (**C**); and AGQS, $n=15$ (**D**) during 40-min hypoxia (signaled by the black horizontal bar). **E** AA content of some of the slices used for the electrophysiological experiments. TOTAL denotes the sum of the different AA per slice shown in the graph. * $p<0.05$; # $p<0.01$; † $p<0.001$ represent significant differences compared to the control (hypoxia) group; one-way ANOVA followed by Dunnett's test for parametric data and Kruskal–Wallis followed by Dunn's test for non-parametric data

L-alanine, glycine, and L-serine are three potential co-agonists of NMDAR [30], whose activation is related to excitotoxicity. However, when those three AA were applied during hypoxia in a mixture called AGS ($n=8$), the fEPSP recovered baseline levels with reoxygenation (88.1 ± 12.8%, $t_{(14)}=0.8244$, $p=0.4235$, t-test), causing only a slight increase in FV amplitude (127.7 ± 22.2%, $p=0.6454$, Mann–Whitney U test) (Fig. 2C). Nevertheless, when L-glutamine was added to this AGS mixture (AGQS, $n=15$) during hypoxia, the fEPSP was irreversibly silenced (9.9 ± 4.6% at the end of the experiment) and the FV strongly potentiated (304.0 ± 32.0% at the end of the hypoxic period). These harmful effects on synaptic transmission during hypoxia are similar to those produced by the mixture of seven amino acids AGHQSTU (fEPSP: $p=0.1802$, Mann–Whitney U test comparing the last 5 min of experiment in the presence of AGQS and AGHQSTU; FV: $p=0.5910$, Mann–Whitney U test comparing the last 5 min of hypoxia in the presence of AGQS and AGHQSTU) (Fig. 2D).

Figure 2E shows the analysis of AA content of the slices used in the above electrophysiological experiments compared to slices exposed only to a 40-min hypoxia period. As expected, an increase in the levels of AA applied in each mixture was detected. In addition, the exposure of the slices to L-glutamine (either alone or as part of the AGQS mixture) caused an increment in the content of aspartate (Asp) and GABA, probably due to its metabolic conversion, although we did not detect significant increases in glutamate content. The total content of AA rose from 38.4 ± 1.3 nmol/slice after hypoxia without AA to 48.7 ± 2.8 nmol/slice in the tissue exposed to AGHSTU ($F_{(4,40)}=4.370$, $p=0.0050$, one-way ANOVA, $p=0.0049$, Dunnett's test), to 44.2 ± 2.5 nmol/slice with Q ($p=0.2706$, Dunnett's test), to 50.1 ± 2.4 nmol/slice with AGS ($p=0.0024$, Dunnett's test), and to 46.9 ± 1.4 nmol/slice with AGQS ($p=0.0163$, Dunnett's test). Moreover, no statistically significant differences were detected in the total content of AA when comparing slices exposed to AGHSTU, Q, and AGS (AA mixtures which did not cause an irreversible loss of fEPSP) versus AGQS-exposed slices ($p>0.05$, Dunnett's test). This result points out that an AA mixture with harmful consequences on synaptic potentials (AGQS) and mixtures with reversible effects (AGHSTU, AGS, and Q) induce akin changes in the total content of AA of the slices.

Then, we studied if a combination of AA with less components could cause an irreversible loss of fEPSP after hypoxia. For that purpose, experiments applying mixtures of three and two AA derived from AGQS (until now, the minimal combination of AA which impairs fEPSP recovery after hypoxia) and always containing L-glutamine were performed. Mixtures made with three AA (AQS, AGQ, and GQS) had a stronger effect avoiding fEPSP recovery and enhancing FV potentiation in comparison with combinations of two AA (AQ, GQ, and QS). AQS and AGQ caused a depression of fEPSP, recovering only 31.2 ± 19.9% ($n=7$, $t_{(12)}=3.441$, $p=0.0049$, t-test comparing the last 5 min of experiment with its baseline) and 27.6 ± 16.7% of basal values ($n=5$, $t_{(8)}=4.284$, $p=0.0027$, t-test) after reoxygenation, respectively (Fig. 3A and C), whereas GQS allowed the fEPSP to recover until 52.2 ± 24.9% ($n=5$, $t_{(8)}=1.854$, $p=0.1008$, t-test) (Fig. 3E). AQS, AGQ, and GQS caused a potentiation in FV amplitude at the end of the 40-min application and hypoxic period (AQS: 203.0 ± 36.0%, $n=7$, $p=0.0006$, Mann–Whitney U test comparing the last 5 min of hypoxia with its baseline; AGQ: 279.6 ± 76.5%, $n=5$, $t_{(8)}=2.397$, $p=0.0433$, t-test; and GQS: 218.0 ± 52.9%, $n=5$, $t_{(8)}=2.309$, $p=0.0497$, t-test).

Mixtures of two AA, L-glutamine one of them, did not cause deleterious effects on synaptic potentials during hypoxia. In the presence of AQ, the fEPSP recovered 89.9 ± 18.9% of basal values ($n=5$, $t_{(8)}=0.5389$, $p=0.6046$, t-test comparing the last 5 min of experiment with its baseline), and the FV was potentiated, reaching 120.2 ± 8.5% ($n=5$, $t_{(8)}=2.724$, $p=0.0261$, t-test comparing the last 5 min of hypoxia with its baseline) (Fig. 3B). After GQ application, the fEPSP reached 95.4 ± 16.4% ($n=5$, $t_{(8)}=0.2252$, $p=0.8275$, t-test), and FV amplitude was increased to 129.0 ± 13.9% ($n=5$, $t_{(8)}=2.366$, $p=0.0456$, t-test) (Fig. 3D). Finally, after QS and hypoxia treatment, the fEPSP recovered to 93.2 ± 3.6% ($n=5$, $p=0.1508$, Mann–Whitney U test) and the FV rose to 126.2 ± 7.2% ($n=5$, $t_{(8)}=3.898$, $p=0.0046$, t-test) (Fig. 3F).

As expected, analysis of the AA content of the slices revealed significant differences between the mean values of the experimental conditions for the applied AA (serine, glycine, alanine, and glutamine) (Fig. 3G). Nonetheless, some differences were also detected for AA which were not included in the mixtures (taurine and histidine). No differences were found in the total content of AA in these slices ($F_{(4,24)}=0.7591$, $p=0.5621$, one-way ANOVA), indicating again that different combinations of AA with opposite effects on synaptic transmission cause equal increments in the total content of AA of the tissue.

These results show that the seven AA of the AGHQSTU mixture are not necessary to cause an irreversible loss of fEPSP after hypoxia. The minimal combination of AA with hypoxia able to provoke a robust and reproducible loss of fEPSP and FV potentiation is AGQS. However, although L-glutamine seems to be important in inducing these harmful effects, its individual application reveals it is not sufficient for it.

Is the Presence of L-glutamine in AGQS Mixture Necessary to Induce Hypoxic Damage?

As L-glutamine seems to be an important component provoking an irreversible loss of fEPSP after hypoxia, we

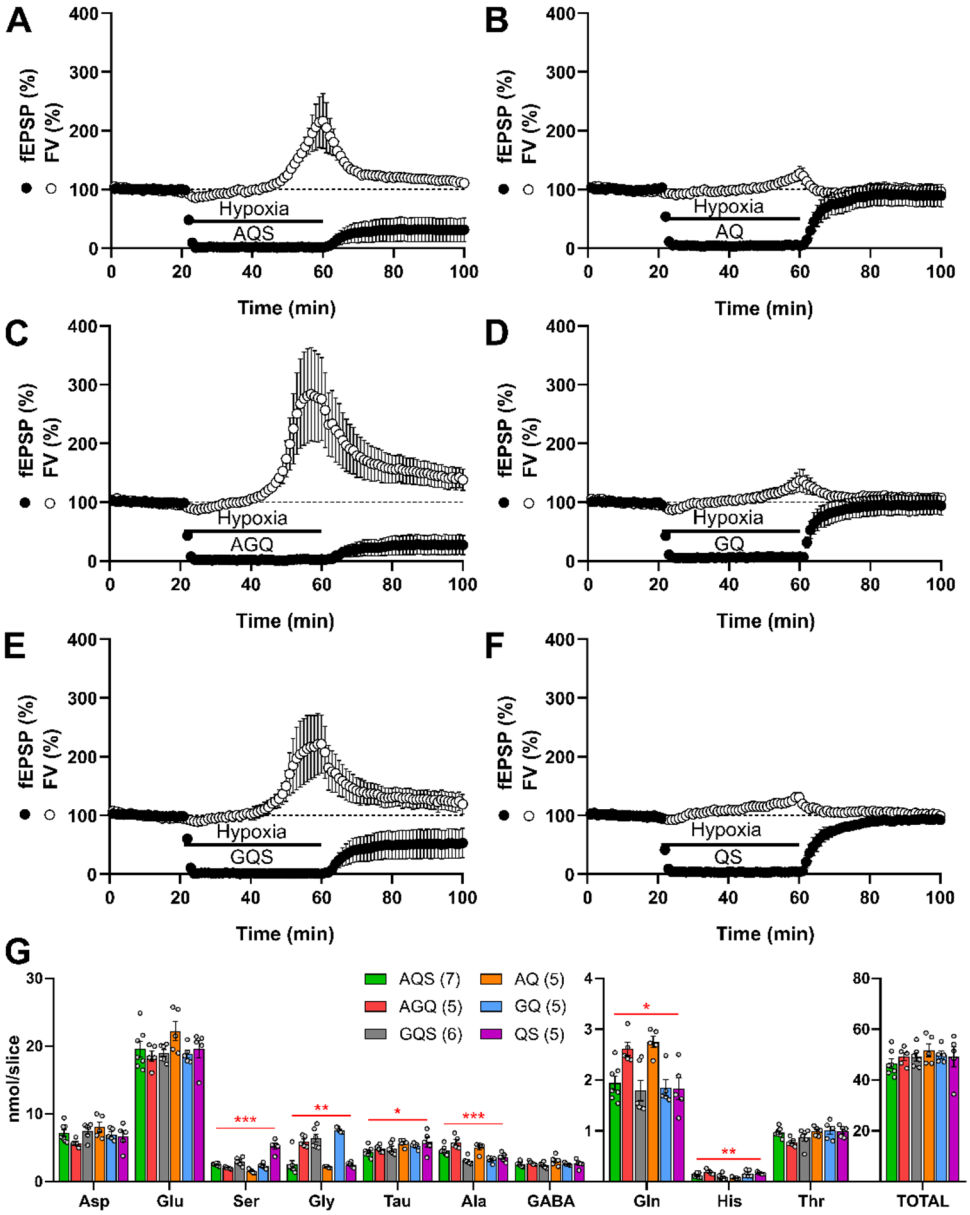

Fig. 3 Minimal combination of AA contained in the AGHQSTU solution necessary to provoke a strong loss of fEPSP and FV potentiation after hypoxia. Time courses of fEPSP (black circles) and FV (white circles) in the presence of AQS, $n=7$ (**A**); AQ, $n=5$ (**B**); AGQ, $n=5$ (**C**); GQ, $n=5$ (**D**); GQS, $n=5$ (**E**); and QS, $n=5$ (**F**) during hypoxia. **G** AA content of the slices used for the electrophysiological experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ represent significant differences between groups; one-way ANOVA and Kruskal–Wallis test for parametric and non-parametric data, respectively. To facilitate data interpretation, no post hoc test to compare between the six experimental conditions was performed

wonder if it was due to its identity or because it is the most abundant AA in the mixtures (733 µM). In the next experiments, L-glutamine was equimolarly substituted by another non-excitatory AA among the AGQS mixture. The substitution of L-glutamine by 733 µM L-histidine (AGS + L-His, $n=7$) (Fig. 4A) caused an irreversible loss of fEPSP (24.1 ± 10.7%) and a strong potentiation of FV amplitude during hypoxia (222.4 ± 34.1%), similarly to what occurred in the presence of AGQS (fEPSP: $p=0.2909$, Mann–Whitney *U* test; FV: $p=0.1417$, Mann–Whitney *U* test). When L-glutamine was substituted by 733 µM L-threonine (AGS + L-Thr, $n=11$) (Fig. 4C), the harmful effects on the synaptic transmission were again observed. AGS + L-Thr 733 µM prevented the recovery of the fEPSP during the reoxygenation (27.8 ± 13.4%, $p=0.5307$, Mann–Whitney *U* test comparing AGQS and AGS + L-Thr 733 µM conditions) and resulted in FV potentiation (286.1 ± 42.2%, $t_{(24)}=0.3443$, $p=0.7336$, *t*-test).

In another group of experiments, we tested whether the above effects of histidine and threonine were stereospecific. We performed experiments substituting L-glutamine with D-histidine or D-threonine. AGS + D-His 733 µM ($n=7$) was also able to impair fEPSP recovery during reoxygenation (19.2 ± 10.1%, $p>1.0$, Mann–Whitney *U* test comparing AGS + L-His 733 µM and AGS + D-His 733 µM) and FV was potentiated to similar levels as with L-histidine (270.9 ± 24.6%, $p=0.1649$, Mann–Whitney *U* test) (Fig. 4B). The substitution of L-glutamine by D-threonine (AGS + D-Thr 733 µM, $n=11$) instead of the L isomer also provoked detrimental effects on synaptic potentials after

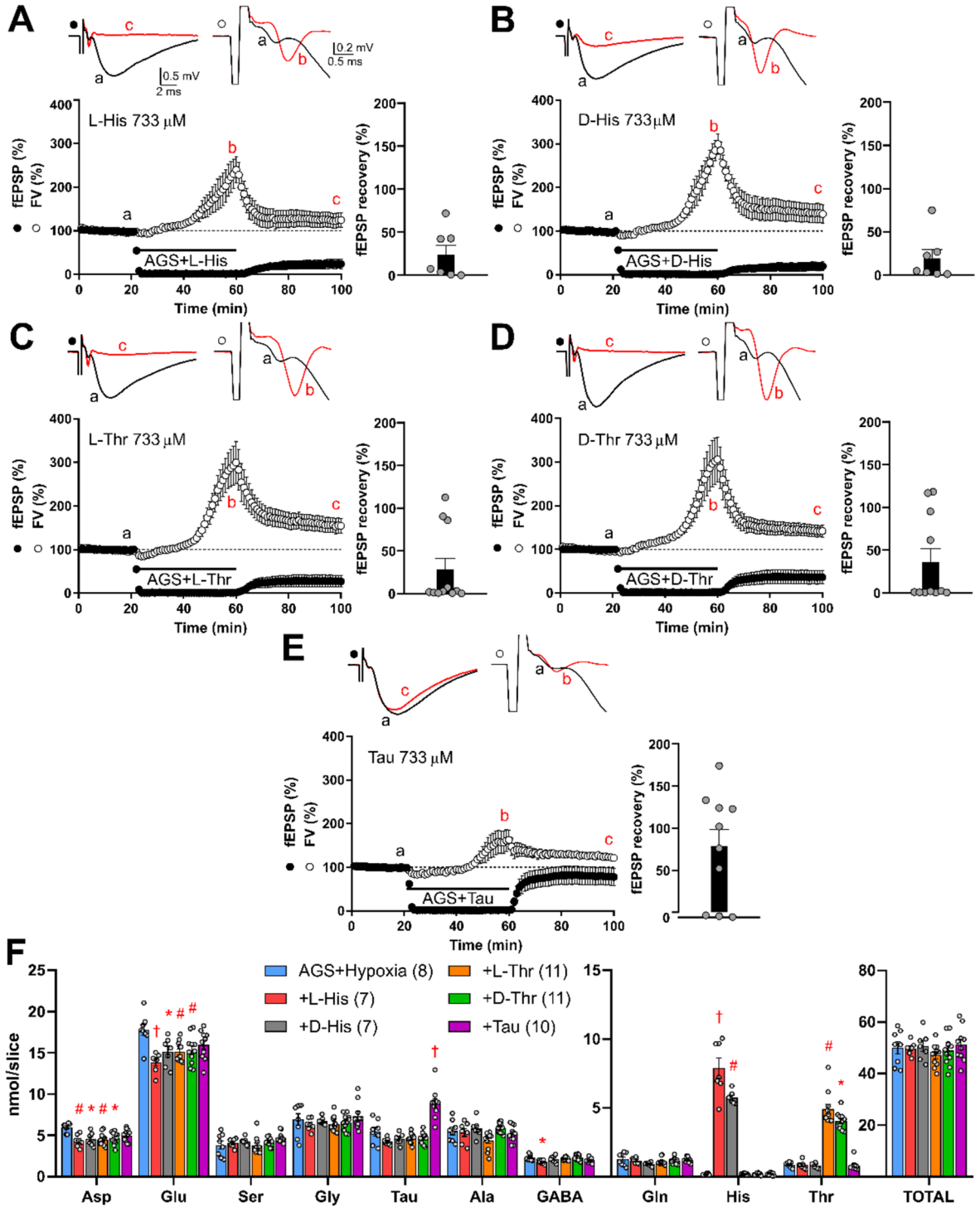

Fig. 4 Equimolar substitution of glutamine in the AGQS mixture for histidine or threonine, but not taurine, induced irreversible changes after hypoxia. Time course of changes on fEPSP (black circles) and FV (white circles), and percentage of fEPSP recovery in the last 5 min of reoxygenation (black bars and gray circles, which represent individual data values) when L-glutamine is equimolarly substituted for L-histidine (AGS + L-His 733 µM, $n = 7$) (**A**), D-histidine (AGS + D-His 733 µM, $n = 7$) (**B**), L-threonine (AGS + L-Thr 733 µM, $n = 11$) (**C**), D-threonine (AGS + D-Thr 733 µM, $n = 11$) (**D**), and taurine (AGS + Tau 733 µM, $n = 10$) (**E**) during hypoxia (signaled by the black horizontal bar). **F** AA content of the slices used in the electrophysiological experiments. * $p < 0.05$; # $p < 0.01$; † $p < 0.001$ represent significant differences compared to the control (AGS + hypoxia) group; one-way ANOVA followed by Dunnett's test for parametric data and Kruskal–Wallis followed by Dunn's test for non-parametric data

hypoxia (fEPSP: 36.2 ± 15.4%, $p = 0.8470$, Mann–Whitney *U* test comparing AGS + L-Thr 733 µM and AGS + D-Thr 733 µM; FV: 292.9 ± 51.9%, $p = 0.9487$, Mann–Whitney *U* test) (Fig. 4D).

Finally, we tested an AA combination in which L-glutamine was substituted by 733 µM taurine (AGS + Tau 733 µM, $n = 10$). In contrast with the results obtained with histidine or threonine, taurine was not able to mimic the deleterious effects produced by L-glutamine in the mixture during hypoxic conditions. At the end of the reoxygenation period, the fEPSP had recovered baseline levels (78.8 ± 19.8%, $t_{(18)} = 1.005$, $p = 0.3282$, *t*-test). The maximum potentiation of the FV was measured at the end of hypoxia, reaching only 159.4 ± 24.8% ($p < 0.0001$, Mann–Whitney *U* test) (Fig. 4E).

The analysis of the content of AA in these slices in comparison with slices exposed only to AGS during hypoxia showed, as expected, higher levels of histidine, threonine, and taurine, when 733 µM L- and D-histidine, L- and D-threonine, and taurine were added to the mixtures, respectively (His: $p < 0.0001$, Kruskal–Wallis test; Thr: $p < 0.0001$, Kruskal–Wallis test; Tau: $F_{(5,48)} = 25.23$, $p < 0.0001$, one-way ANOVA). Nevertheless, no significant differences in the total content of AA were detected between experimental conditions ($F_{(5,48)} = 0.7246$, $p = 0.6083$, one-way ANOVA) (Fig. 4F).

These results confirm that L-glutamine is not necessary to produce deleterious effects during hypoxia since histidine and threonine can mimic its outcomes. On the other hand, taurine, which is also accumulated in the slices, does not reproduce the irreversible effects on synaptic transmission. This result supports that the identity of the applied AA, and not only an increment in the number of particles accumulated in the tissue, contributes to the outcome. Nevertheless, it is meaningful to clarify whether the application of high non-physiological concentrations of individual AA provokes harmful effects.

Comparison of Synaptic Irreversibility Caused by Individual AA During Hypoxia

To continue studying if the harmful effect of non-excitatory AA during hypoxia is due to the concentration or the identity of the applied AA, we conducted experiments applying individual AA at concentrations higher than those found in plasma.

Firstly, the effect of the individual AA from the AGHQSTU mixture at the final concentration of the mixture (2.1 mM) on the synaptic potentials was tested under normoxic conditions (black circles in Fig. 5). The strongest effect was produced by 2.1 mM L-alanine ($n = 3$), reaching a fEPSP potentiation of 163.9 ± 8.5% ($t_{(4)} = 7.614$, $p = 0.0016$, *t*-test comparing with its baseline) and a FV increase of 146.7 ± 4.7% ($t_{(4)} = 10.25$, $p = 0.0005$, *t*-test) (Fig. 5E, black circles).

When individual AA were applied in the presence of 40-min hypoxia, the outcome allowed to differentiate between three groups of AA (white circles in Fig. 5). A first group, formed by L-serine, glycine, and L-histidine (Fig. 5A–C, white circles), caused an irreversible loss of fEPSP after hypoxia and a great potentiation of FV amplitude. The strongest FV potentiation was provoked by 2.1 mM L-serine, reaching 346.7 ± 37.8% ($n = 5$) (Fig. 5A), comparable to the potentiation caused by AGHQSTU ($t_{(11)} = 0.5421$, $p = 0.5986$, *t*-test). In a second group, L-glutamine and L-alanine ($n = 5$ each) caused only a partial loss of fEPSP after hypoxia, recovering 40.0 ± 20.6% ($t_{(8)} = 2.841$, $p = 0.0218$, *t*-test comparing with the baseline period) and 51.6 ± 25.9% ($t_{(8)} = 1.825$, $p = 0.1054$, *t*-test) from basal values, respectively (Fig. 5D–E). The third group, including taurine ($n = 5$) and L-threonine ($n = 6$), allowed a rapid recovery of fEPSP during reoxygenation, causing even a potentiation in the case of L-threonine (Tau: 102.2 ± 4.2%, L-Thr: 115.5 ± 4.7%, $t_{(10)} = 3.462$, $p = 0.0061$, *t*-test). Furthermore, the changes in FV amplitude produced by these two AA were minimal and not significant (Tau: 102.2 ± 4.2%, L-Thr: 115.5 ± 4.7%) (Fig. 5F–G). These results confirm that the deleterious effects of non-excitatory AA on field potentials after hypoxia depend on the specific AA and not only on their concentration.

ASCT2 Amino Acid Exchanger Blockade Impairs the Harmful Effects of AGQS and Hypoxia on Synaptic Transmission

In the next group of experiments, we sought to determine which neutral AA transporters contributed to the irreversible effect on synaptic transmission displayed by AA during hypoxia. Previously, we had described that the irreversible loss of fEPSP and the intense potentiation in FV amplitude caused by a combination of non-excitatory AA and hypoxia was not mediated by the AA transporters of system A (SLC38) [3]. Here, we have performed experiments to determine if the AA exchangers system L (SLC7) and ASCT2 (SLC1A5) are involved in the detrimental effects of AGQS on synaptic potentials during hypoxia. In a group of preliminary experiments, we also tested the participation of system N transporters using its pharmacological inhibitor L-glutamate-γ-hydroxamate [23, 47]. Unfortunately, no clear results were obtained due to the interaction of this inhibitor with NMDAR [18].

The inhibition of system L by 1 mM BCH [11, 47] did not avoid the irreversible loss of fEPSP provoked by AGQS and hypoxia (10.5 ± 2.3%, $n = 7$, and 5.6 ± 1.0%, $n = 3$, in the absence and presence of 1 mM BCH, respectively;

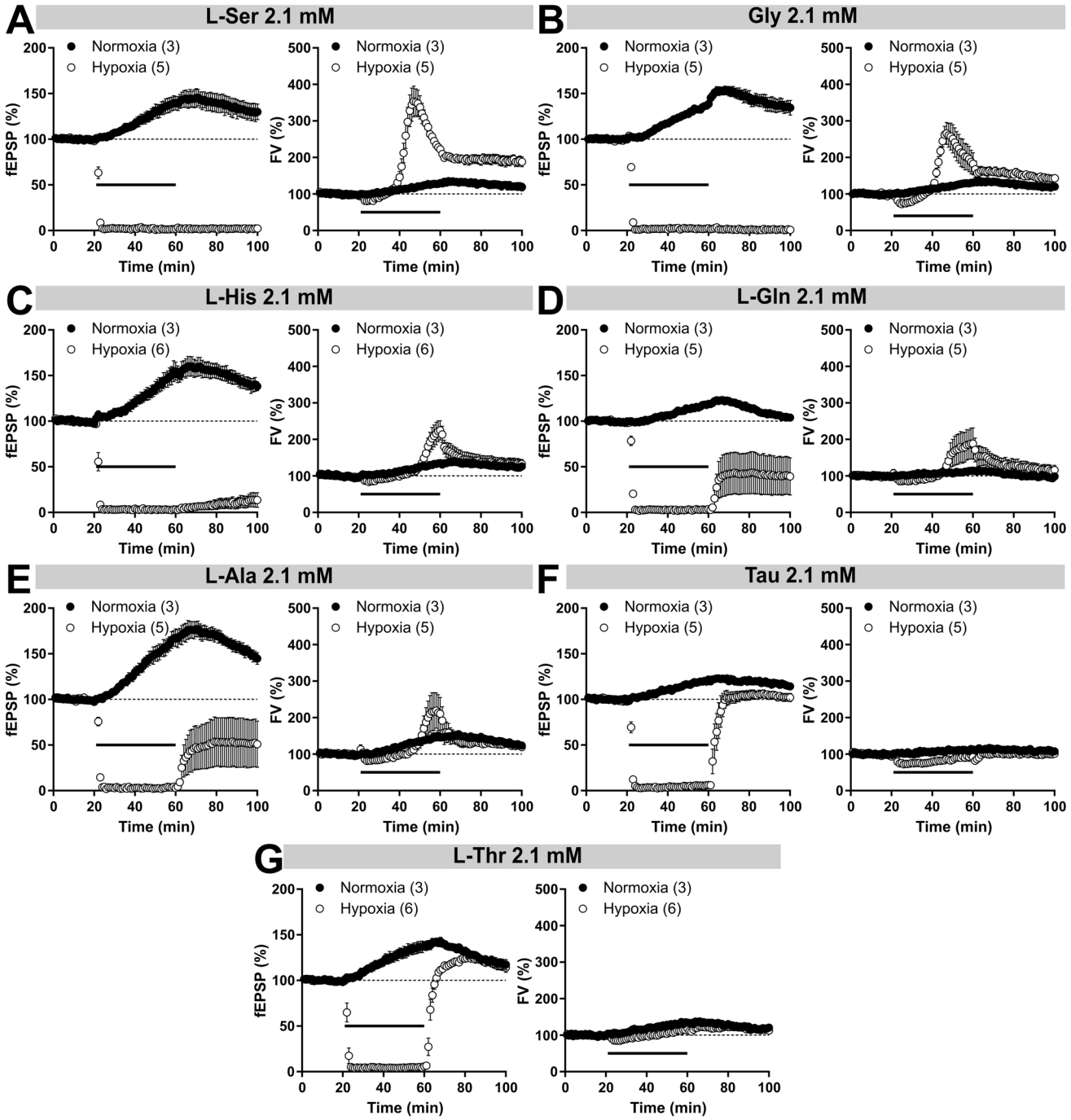

Fig. 5 Changes in evoked field potentials caused by the individual application of amino acids (2.1 mM) included in AGHQSTU during normoxic and hypoxic conditions. Graphs show time courses of fEPSP and FV in the presence of the seven AA of AGHQSTU applied individually at the final concentration of the mixture (2.1 mM) in normoxia (black circles) and hypoxia (white circles). Black horizontal bars indicate the perfusion of the AA during normoxia or hypoxia

$t_{(8)} = 1.329$, $p = 0.2206$, *t*-test) (Fig. 6A). No significant differences were neither detected in the FV potentiation in the absence ($342.8 \pm 26.0\%$, $n = 7$) and presence of 1 mM BCH ($269.3 \pm 26.0\%$, $n = 3$, $p = 0.5167$, Mann–Whitney *U* test) (Fig. 6B). However, the ASCT2 blocker GPNA [14, 16] prevented the detrimental effects of AGQS on synaptic potentials. Thereby, in the presence of the ASCT2 inhibitor, the fEPSP regained baseline levels with reoxygenation ($73.7 \pm 18.5\%$, $n = 4$, $t_{(6)} = 1.365$, $p = 0.2212$, *t*-test) (Fig. 6A). One millimolar GPNA also avoided the strong increment in FV amplitude, reaching only $125.5 \pm 14.8\%$ at the end of the hypoxic period ($n = 4$, $p = 0.0061$, Mann–Whitney *U* test

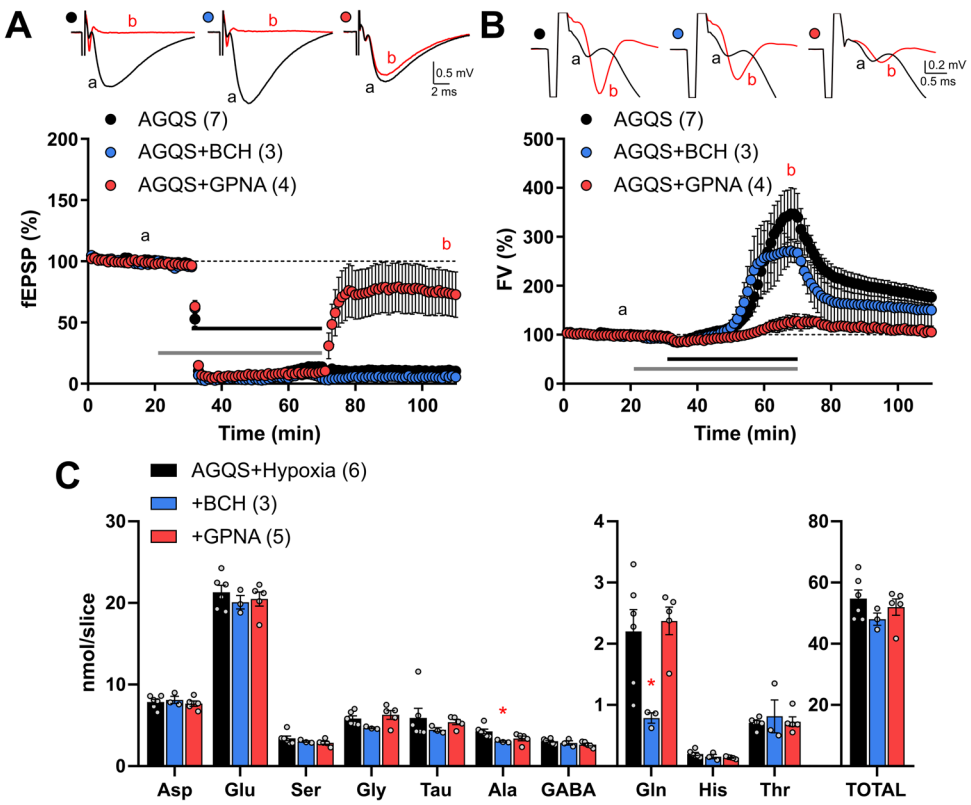

Fig. 6 Inhibition of ASCT2, but not of system L transporters, protects from the deleterious effects of AGQS and hypoxia on evoked field potentials. Changes of fEPSP (**A**) and FV (**B**) induced by AGQS and hypoxia (signaled by the black horizontal bars) in the absence (black circles) and presence of 1 mM BCH (blue circles) or 1 mM GPNA (red circles). The time period in which the blockers were applied is marked by the gray horizontal bars. **C** Amino acid content of some of the slices used for the electrophysiological experiments. * $p < 0.05$ represents significant differences compared to the control (AGQS + hypoxia) group; one-way ANOVA followed by Dunnett's test for parametric data and Kruskal–Wallis followed by Dunn's test for non-parametric data

comparing experiments with and without GPNA). Furthermore, the FV regained baseline values after reoxygenation ($t_{(6)} = 1.809$, $p = 0.1205$, *t*-test comparing the last 5 min of experiment with its baseline period) (Fig. 6B).

Nevertheless, the analysis of AA content in these slices revealed that GPNA ($n = 5$) did not impair either the accumulation of the AGQS amino acids or the increase in the total content of AA in the slice. On the other hand, 1 mM BCH induced a reduction of alanine and glutamine levels in the slices, but did not affect the total AA content. In the absence of BCH, AGQS caused an increase in alanine levels to 4.3 ± 0.3 nmol/slice, whereas in the presence of 1 mM BCH alanine levels were reduced to 3.0 ± 0.1 nmol/slice ($F_{(2,11)} = 4.757$, $p = 0.0325$, one-way ANOVA, $p = 0.0331$, Dunnett's test). Glutamine levels were diminished from 2.2 ± 0.4 to 0.8 ± 0.1 nmol/slice in the presence of BCH ($F_{(2,11)} = 6.014$, $p = 0.0172$, one-way ANOVA, $p = 0.0224$, Dunnett's test) (Fig. 6C).

These results show that the blockade of the ASCT2 exchanger prevents the deleterious effects caused by a mixture of four non-excitatory AA on synaptic potentials during hypoxia without affecting the slices' AA content. However, GPNA is a glutamine analog [14], with a region in its structure common to glutamate. Thus, we wondered if its protective actions against AGQS and hypoxia would be produced through the interaction with one of the key players involved in ischemic damage, the glutamate receptors.

We had previously described that the inhibition of NMDAR avoids the harmful effects of a mixture of non-excitatory AA during hypoxia [2, 3]. In the present study, we have also explored if AMPA receptors (AMPAR) are involved in the deleterious effects of AGQS during hypoxia. To this end, we used 10 μM CNQX, an AMPAR antagonist. Firstly, we tested the reversibility of CNQX depressant actions on the fEPSP. In the presence of this AMPAR antagonist, a fast silencing of fEPSP occurred ($9.6 \pm 1.4\%$, $n = 3$, $F_{(2,6)} = 231.6$, $p < 0.0001$, one-way ANOVA, $p < 0.0001$, Dunnett's test), partially recovering its basal amplitude after 40 min of wash out ($66.3 \pm 4.5\%$, $p = 0.0007$, Dunnett's test). The presence of CNQX did not interfere with the FV amplitude ($n = 2$, $F_{(2,3)} = 2.219$, $p = 0.2562$, one-way ANOVA) (Fig. 7A). In another group of experiments, the application of AGQS and hypoxia in the presence of 10 μM CNQX provoked an irreversible loss of fEPSP and a potentiation of FV similar to that caused in the absence of CNQX (fEPSP at the end of reoxygenation: $7.5 \pm 3.5\%$, $n = 5$, $p = 0.6594$, Mann–Whitney *U* test; FV at the end of hypoxia: $341.4 \pm 56.7\%$, $n = 3$, $t_{(16)} = 0.4883$, $p = 0.6319$, *t*-test) (Fig. 7B). This result indicates that AMPAR do not take part on the harmful outcomes caused by non-excitatory AA on synaptic potentials during hypoxia.

To clarify whether the effect of GPNA, the ASCT2 inhibitor, avoiding hypoxic damage in the presence of AGQS is mediated through NMDAR, we used the bioassay of

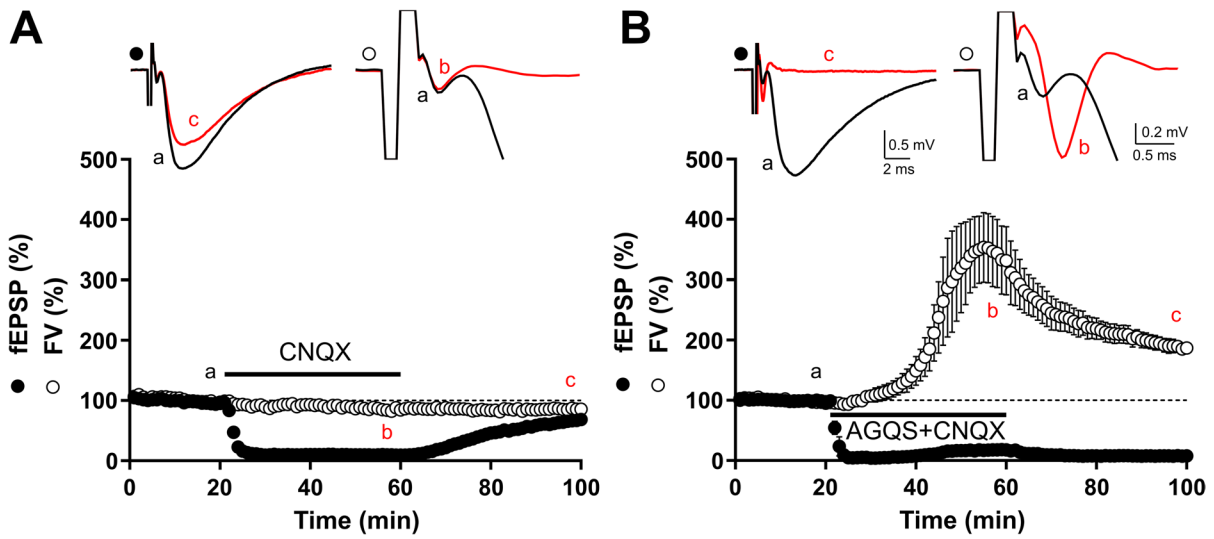

Fig. 7 AMPA receptors are not implicated in the deleterious effects produced by AGQS on evoked field potentials during hypoxia. **A** Time course of fEPSP, $n=3$ (black circles), and FV, $n=2$ (white circles) in the presence of 10 μM CNQX in normoxic conditions (indicated by the black horizontal bar). **B** Changes in fEPSP, $n=5$ (black circles), and FV, $n=3$ (white circles) induced by AGQS in the presence of 10 μM CNQX during 40-min hypoxia (marked by the black horizontal bar)

the NMDA-induced FV potentiation. In this bioassay, the application of NMDA on hippocampal slices increases the FV amplitude by a mechanism mediated by the activation of presynaptic NMDAR [41, 42]. In these experiments, the FV was isolated by blocking the fEPSP with 20 μM CNQX. Under this condition, a 30-min application of 20 μM NMDA caused a first depression in FV amplitude, promptly followed by an increment attaining 237.6 ± 13.1% after 10 min of perfusion ($n=3$) (Fig. 8B), as Suárez et al. [42] had formerly described. The FV potentiation provoked by NMDA was not altered in the presence of 1 mM GPNA (10 min application, $n=5$, $F_{(2,7)}=23.01$, $p=0.0008$, one-way ANOVA, $p>1.0$, Tukey's test comparing experiments in the absence and presence of GPNA). On the other hand, and as it was expected, the FV potentiation was abolished with 50 μM D-AP5, an NMDAR antagonist ($n=3$, $p=0.0015$, Tukey's test) (Fig. 8C). These results indicate firstly that GPNA does not affect presynaptic NMDAR involved in the modulation of Schaffer's collateral axons excitability. Furthermore, these results probe that the protective effect of GPNA against the irreversible silencing of synaptic transmission caused by non-excitatory AA and hypoxia is not due to its activity on NMDAR, but due to the inhibition of the ASCT2 amino acid transporters.

Discussion

In the present study, we extended the characterization of the deleterious effect promoted by a mixture of seven non-excitatory AA at plasmatic concentrations (AGHQSTU) during 40-min hypoxia on hippocampal synaptic transmission that we previously reported [3]. We found that this AA mixture during hypoxia initially hyperpolarized neuronal V_m, shifting to a progressive slow depolarization in the firsts 24 min of hypoxia. From that moment, V_m rapidly depolarized and irreversibly collapsed. This last period correlated with the large increase in FV amplitude caused by the AA during hypoxia. We also showed that the mixture formed by AGQS is the minimal combination of AA causing a reproducible and irreversible silencing of fEPSP after hypoxia. Furthermore, the presence of L-glutamine in this mixture seems necessary but insufficient to induce these harmful effects, which depend on both the identity and concentration of individual AA in the mixture. Contrary to our expectations, the total accumulation of AA in the slices after their application did not determine the outcome caused by different AA mixtures on synaptic transmission. Results obtained using substrates and inhibitors of AA transporters suggest that system N and ASCT2 transporters may participate in the deleterious effects of a mixture of non-excitatory AA during hypoxia.

The Concentration and Composition of the Amino Acid Mixture Determine the Detrimental Effects Observed in Hypoxia

Here, we have studied whether the formerly described irreversible loss of fEPSP and strong potentiation of FV amplitude caused by AGHQSTU during hypoxia [3] was determined solely by the final concentration of the AA in the mixture or whether their identity was

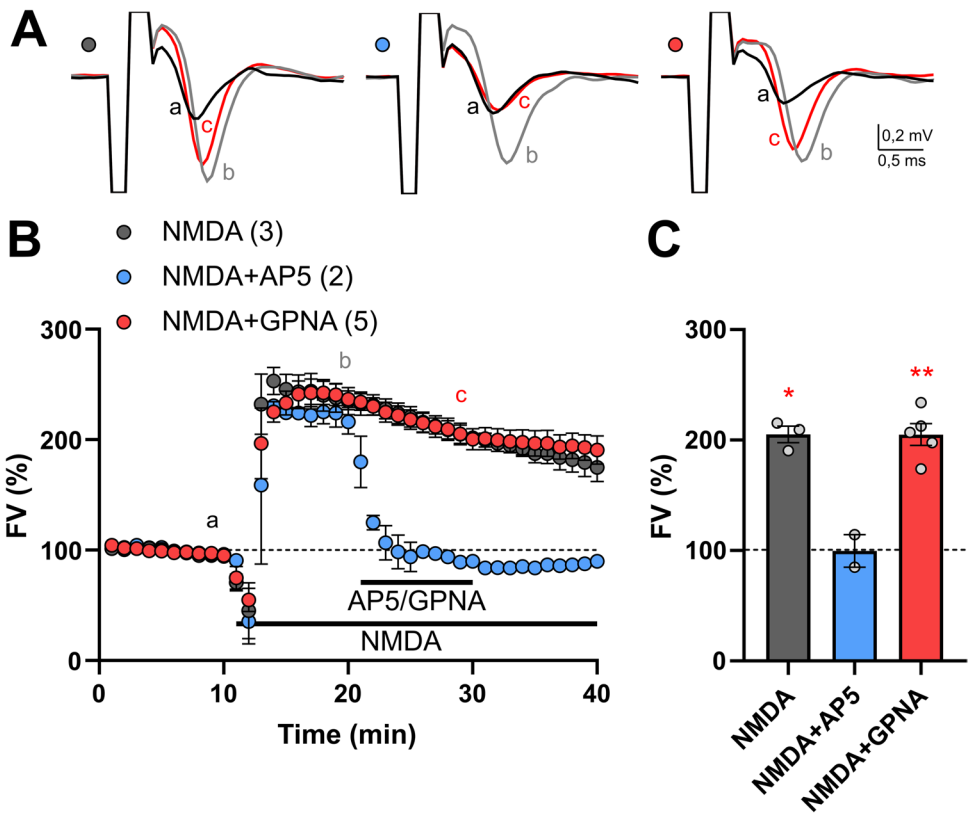

Fig. 8 GPNA does not affect the NMDAR-mediated FV potentiation. **A** FV representative traces for experimental conditions and times signaled in the graph shown in **B**. **B** Time course of FV changes induced by 20 μM NMDA alone (gray circles) and with either 50 μM D-AP5 (blue circles) or 1 mM GPNA (red circles). **C** FV amplitude in the last 3 min of D-AP5 (blue bar) and GPNA application (red bar) in the presence of NMDA and the same temporal window for the experiments in the presence of NMDA alone (gray bar). Twenty micromolar CNQX was perfused during the whole experiment. * $p = 0.0015$; ** $p = 0.0009$ represent significant differences compared to NMDA + AP5 group (blue bar); one-way ANOVA followed by Tukey's test

also of importance. Firstly, we focused on the effect of L-glutamine, because it is the most concentrated AA in the mixture (733 μM), and it can be a substrate for glutamate and GABA production [4]. In our experiments, the AGHSTU mixture (lacking L-glutamine) did not provoke the deleterious consequences on synaptic transmission observed when the slices were exposed to this mixture containing glutamine (AGHQSTU). Thus, the presence of L-glutamine during hypoxia seems necessary but insufficient to produce such harmful effects, as the individual application of L-glutamine did not provoke a synaptic silencing. The possibility that the simple reduction of the final concentration of the mixture (AGHSTU, whose final concentration is 1.4 mM vs. 2.1 mM of AGHQSTU) is the cause of the lack of effect was ruled out given the results obtained with various combinations formed by three AA, such as AGQ and AQS (final concentration of both mixtures: 1.4 mM), and by AGQS, the minimum combination of AA capable of eliciting an irreversible effect during hypoxia (final concentration: 1.6 mM).

Our results with different combinations of AA showed that the presence of L-glutamine in the mixtures is essential in order to cause the irreversible loss of synaptic potentials in hypoxia. This would align with the toxic effect that glutamine accumulation elicits in the brain [50] and in cultured astrocytes [20]. Moreover, such a specific requirement of glutamine is true if the concentrations of different AA in the mixture mimic plasmatic concentrations but not when the concentration of a specific AA, such as histidine and threonine, equals glutamine concentration. Indeed, we observed that equimolar replacement of L-glutamine in the AGQS mixture by histidine or threonine, but not taurine, induced deleterious effects during hypoxia in a non-stereospecific manner. Even more apparent are our results obtained with the individual application of each of the seven AA of the AGHQSTU mixture at the final concentration of the mixture (2.1 mM). In these experiments, and although such AA concentrations (2.1 mM) are far from their physiological plasmatic range, we probe that L-serine, glycine, and L-histidine are the most potent AA causing an irreversible loss of synaptic transmission in hypoxia. At the same time, L-alanine and L-glutamine allow a partial recovery of synaptic potentials, and the effect of taurine and L-threonine was fully reversible. Based on this set of results, we conclude that both identity and quantity of individual AA in a mixture determine the degree of synaptic transmission recovery after a hypoxic episode.

These two characteristics (identity and quantity) of the AA mixture-inducing deleterious effect with hypoxia could be related to the intracellular increase of osmotically active particles resulting from their uptake, a proposal we made in a previous work [3]. According to this model, we detected

an increase in the AA content in the slices exposed to each AA mixture. However, we did not observe differences in the total AA content in slices exposed to mixtures causing irreversible effects (e.g., AGQS, AQS, AGQ, or GQS) compared with those that allowed the recovery of synaptic responses (e.g., AGHSTU, AQ, GQ, or S). This result does not support the idea that the total intracellular accumulation of AA is the trigger of the deleterious process during hypoxia. In this respect, it is necessary to point out that the AA content in the slices corresponds to the AA accumulated at the end of the experiments. Still, part of the AA uptaked by the tissue may have been released into the extracellular medium and been lost in the bath fluid during the washout period. This could mask the differences in net AA accumulation under different experimental conditions. Moreover, it should be noted that the total content of AA analyzed in the slices by HPLC represents an average value resulting from the uptake of AA in different cell types and compartments, regardless of whether these elements are involved in the development of the deleterious effects or not. In this respect, we have recently shown that the AGQS mixture applied during normoxia induces astroglial swelling without modifying neuronal volume [2], probably due to the lack of aquaporins in neuronal membranes, which prevents neuronal swelling even if they accumulate osmotically active AA. An alternative possibility, unexplored in the present work, is that the accumulated AA did not act as osmolytes, but providing a toxic mechanism derived from their metabolism, similarly to the glutamine-induced oxidative stress and mitochondrial damage that causes astrocyte swelling during hyperammonemia [20]. Another possibility is that these AA share a common mechanism contributing to hypoxic injury. For example, if intracellular AA accumulation is mediated by sodium-dependent transporters, as we propose and show evidence here, this would lead to a rise in intracellular sodium levels, which would stay elevated due to the impairment of the sodium/potassium ATPase activity during ischemic energy failure. The rise in intracellular chloride concentration as a result of non-excitatory AA transporter activity could contribute to cell swelling and, therefore, to ischemic injury. This could be the case for the Na^+- and Cl^--dependent transporters TAUT and GLYT1 [25, 36], which mediate the transport of taurine and glycine. Also, the activation of some AA transporters by their substrates initiates an anionic conductance, as in the case of ASCT2 [6].

In summary, although the application of the AA mixtures produces an increase in the total AA content in the tissue, this is not sufficient to induce the deleterious effects caused during hypoxia on synaptic transmission. Thus, we wonder if this is related with the identity of the transported AA, the cellular type accumulating the AA (i.e., astrocytes or neurons), and with the transporter systems involved.

Amino Acid Transporter Systems Involved in the Harmful Effects Provoked by Non-excitatory Amino Acids and Hypoxia

In view of the AA that cause the most deleterious effects during hypoxia (alanine, histidine, glutamine, glycine, serine, and threonine), we can propose the involvement of specific transporter systems carrying these substrates: the Na^+-dependent systems A, L, N, and ASCT2 [45], Bröer, 2014). However, in one of our earlier works [3], and using MeAIB to inhibit system A, we did not find any experimental evidence that supports the involvement of system A in the detrimental effects of non-excitatory AA during hypoxia. Also in the same work, we considered unlikely that system L participates in the synaptic changes caused by non-excitatory AA, because a mixture containing the main substrates of system L (e.g., L-leucine, L-isoleucine, and L-valine) did not modify synaptic transmission. Nevertheless, because the four AA in the AGQS mixture are also substrates of system L [39], here we have used an inhibitor of system L, BCH [33, 47], to study its possible implication. We have shown that BCH did not prevent the irreversible loss of fEPSP and the FV potentiation caused by AGQS, indicating that system L is not involved in the effects elicited by these AA during hypoxia. The reduction of glutamine content observed in BCH-treated slices might be explained due to the ability of BCH to release glutamine, as found by brain microdialysis [12].

L-histidine is an effective activator of system N activity, among other AA contained in our mixtures, such as L-glutamine, L-alanine, and L-serine [13, 23]. We propose that system N transporters might contribute to the deleterious effect of AA mixtures, based on the results that show an irreversible loss of fEPSP, a robust FV potentiation, and a high intracellular accumulation of histidine observed when histidine substituted glutamine in the AGQS mixture (AGS + His 733 μM) or when applied alone at a supraphysiological concentration (2.1 mM).

ASCT1 and ASCT2 are sodium-dependent AA exchangers, mainly located in astrocytes and neurons, respectively [17, 22]. The following evidence supported the contribution of the ASCT2 transporter to the detrimental effects of AA during hypoxia. L-threonine is one of the preferred substrates of ASCT2, together with L-alanine, L-serine, and L-glutamine [45]. When L- or D-threonine substituted L-glutamine in the AGQS mixture (AGS + Thr 733 μM), a synaptic silencing was provoked after hypoxia. Additionally, GPNA, an inhibitor with more selectivity for ASCT2 than for ASCT1 [14, 16], impaired the irreversible fEPSP silencing and the great potentiation of FV amplitude caused by AGQS and hypoxia. This result confirmed those recently reported by us, showing that GPNA prevents the loss of fEPSP while impairing neuronal and astroglial swelling

after the application of AGQS during hypoxia [2]. The possibility that the inhibitory actions of GPNA were mediated by interacting with NMDAR due to the structural similarity between GPNA and glutamate [38] was rejected, as GPNA did not interfere with the known potentiating action of NMDA on FV amplitude [41]. However, it should be noted that ASCT1 and ASCT2 are sodium-dependent AA exchangers [45]. Thus, its activation will not modify the total content of intracellular AA, but it will rise the cytoplasmic concentration of sodium and, therefore, likely cause a gain of osmolytes that may contribute to cell swelling. Another feature of ASCT exchangers is that they regulate extracellular levels of D-serine [22]. Remarkably, it has been shown that ASCT2 can release D-serine in exchange with L-alanine and L-glutamine [15]. This opens an alternative possibility in which, during hypoxia, the AA mixture stimulates the release of D-serine from neuronal ASCT2 to co-activate NMDAR involved in neuronal soma swelling and dendritic beading [2].

Effect of Glutamate and its Receptors on Amino Acid and Hypoxia-Induced Injury

Pathological conditions such as hypoxia, ischemia, stroke, or TBI are characterized by the dysregulation of extracellular levels of excitatory AA and excitotoxicity [1]. The extracellular accumulation of glutamate activates NMDAR, causing a strong increase in intracellular calcium and initiating cell death cascades [43]. We have previously demonstrated that the activation of NMDAR is required for the deleterious effects provoked by non-excitatory AA in hypoxia on synaptic potentials [2, 3]. In the present work, we found that the activation of the other major group of inotropic glutamate receptors, the AMPA-kainate receptors, is unnecessary to produce such deleterious effects.

Additionally, we also presented experimental evidence probing that the activation of NMDAR during hypoxia does not require the glutamate that could be metabolically produced from the L-glutamine included in the AA mixtures, as it is the case when L-glutamine was applied alone or as part of the AGS mixture. Moreover, the presence of three potential NMDAR co-agonists: L-alanine, glycine, and L-serine [30] in the mixtures was also not sufficient to provoke the harmful effects here described on synaptic transmission during hypoxia. Furthermore, the exocytotic synaptic release of glutamate does not seem to be required for the harmful effects caused by non-excitatory AA during hypoxia, because in the absence of synaptic electrical stimulation the fEPSP was still irreversibly silenced and the FV strongly potentiated. In this respect, the intracellularly recorded EPSP was silenced during hypoxia. This can be explained by the already known effect of adenosine on presynaptic adenosine A1 receptors that causes a presynaptic inhibition on synaptic glutamate release [10, 19].

According to our previous works, non-excitatory AA mixtures at plasmatic concentrations caused cell swelling during hypoxia [2, 3], which could boost extracellular glutamate concentration in two ways. On one hand, glutamate could be released through volume regulated anion channels (VRAC) activated by swelling [46, 48]. On the other hand, the extracellular space volume reduction resulting from cellular swelling would increase the concentration of extracellular molecules, including glutamate. The time courses of V_m and FV changes observed in our experiments during the application of a mixture of non-excitatory AA during hypoxia provide new insights into the irreversible synaptic failure that occurs in brain ischemia. In the presence of an AA mixture, we found that the previously reported reversible hypoxia-induced 10-mV membrane hyperpolarization (Krnjević, 24) turned into a slow and progressive depolarization until reaching a V_m close to − 30 mV after 24 min of hypoxia. Afterward, and during the next 10 min, the V_m completely depolarized, probably inducing a massive calcium influx through NMDAR and L-type voltage-dependent calcium channels. The rise in intracellular calcium would finally trigger the irreversible cell damage [43]. This last period was accompanied by a massive increase in FV amplitude in our experiments. As the FV is an extracellular field potential, its strong potentiation may be due to the increase in the electrical resistance of the slice consequence of cell swelling [3, 8, 26]. These results set the turning point from which the loss of synaptic transmission becomes irreversible, corroborating our previous data showing that a 30-min hypoxia period in the presence of AA did not cause irreversible synaptic changes under our experimental conditions [3].

Conclusions

In this work, we have demonstrated that the irreversible silencing of synaptic transmission induced by some non-excitatory AA at plasmatic concentrations and during hypoxia is determined by the concentration and the identity of the applied AA. Using specific substrates and pharmacological inhibitors of AA transporters, we have obtained experimental evidence indicating that the deleterious effect caused by the non-excitatory AA in hypoxia might involve the activation of several transport systems (system N and ASCT2). These transporters would be responsible for the intracellular accumulation of AA, leading to cell swelling. This chain of events may generate a progressive increase in the extracellular concentration of glutamate and D-serine, which would boost NMDAR-mediated excitotoxicity during hypoxia.

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Data availability The data presented in this study are available on reasonable request from the corresponding author.

Declarations

Competing interests The authors declare no competing interests.

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