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Extracellular mild acidosis decreases the Ca^{2+} permeability of the human NMDA receptors

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ABSTRACT

NMDA receptors (NMDARs) are glutamate-gated ion channels involved in excitatory synaptic transmission and in others physiological processes such as synaptic plasticity and development. The overload of Ca^{2+} ions through NMDARs, caused by an excessive activation of receptors, leads to excitotoxic neuronal cell death. For this reason, the reduction of Ca^{2+} flux through NMDARs has been a central focus in finding therapeutic strategies to prevent neuronal cell damage.

Extracellular H^+ are allosteric modulators of NMDARs. Starting from previous studies showing that extracellular mild acidosis reduces NMDA-evoked whole cell currents, we analyzed the effects of this condition on the NMDARs Ca^{2+} permeability, measured as "fractional calcium current" (P_f, i.e. the percentage of the total current carried by Ca^{2+} ions), of human NMDARs NR1/NR2A and NR1/NR2B transiently transfected in HeLa cells. Extracellular mild acidosis significantly reduces P_f of both human NR1/NR2A and NR1/NR2B NMDARs, also decreasing single channel conductance in outside out patches for NR1/NR2A receptor. Reduction of Ca^{2+} flux through NMDARs was also confirmed in cortical neurons in culture. A comparative analysis of both NMDA evoked Ca^{2+} transients and whole cell currents showed that extracellular H^+ differentially modulate the permeation of Na^+ and Ca^{2+} through NMDARs.

Our data highlight the synergy of two distinct neuroprotective mechanisms during acidosis: Ca^{2+} entry through NMDARs is lowered due to the modulation of both open probability and Ca^{2+} permeability. Furthermore, this study provides the proof of concept that it is possible to reduce Ca^{2+} overload in neurons modulating the NMDAR Ca^{2+} permeability.

1. Introduction

Neuronal damage and death represent the end point of several neurological diseases, with different aetiologies and molecular pathways, but usually sharing a common excitotoxic step: excessive glutamate-evoked Ca^{2+} entry [1]. In particular, the highly Ca^{2+} permeable N-Methyl-D-Aspartate glutamate receptor (NMDAR) is involved in glutamate excitotoxicity and has been identified as a pharmacological target in neurodegenerative pathologies and in brain ischemia [2–4]. NMDARs are a family of tetrameric channels with different subunit compositions, associated with different functional properties, pharmacology and subcellular localization for review see [5–7], allowing substantial Ca^{2+} entry if activated during depolarization, which eliminates the Mg^{2+}-dependent channel block [8]. Indeed, the selective NMDAR open channel blocker memantine is one of the very few pharmacological tools used in the therapy against Alzheimer disease [9]. However, the complete inhibition of NMDAR is not compatible with life, and even its partial block causes several adverse effects [10]. Thus, to find a way to reduce the glutamate-mediated Ca^{2+} overload, we propose the modulation of NMDAR Ca^{2+} permeability, as already shown possible with nicotinic acetylcholine receptors nAChRs [11,12]. We identified extracellular H^+ as possible modulators of Ca^{2+} flow through NMDARs for the following reasons: i) protons are well-described negative modulators of NMDAR kinetics [13]; ii) low extracellular pH (pH_e) reduces the Ca^{2+} permeability (measured as fractional Ca^{2+} current, Pf, i.e. the percentage of the total current carried by Ca^{2+} ions [14,15]) of other cationic channels [16]; iii) extracellular mild acidosis is neuroprotective [17,18]. In mild acidosis the pH_e can

Abbreviations: NMDA, N-Methyl-D-Aspartate; NMDAR, NMDA receptor; pH_e, extracellular pH; P_f, fractional Ca^{2+} current, i.e. the percentage of the total current carried by Ca^{2+} ions

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reach values of 6.8-6.5 [19], able to strongly reduce the open probability of NMDARs but not to significantly activate the Ca^{2+} permeable acid-sensing ion channels (ASICs), whose pH_{50} value is 5.8 [20]. Therefore, we measured the P_f of human NMDARs NR1/NR2 A and NR1/NR2B transiently transfected in HeLa cells in acidic conditions, finding for the first time, beside the well-described reduction of the open probability, a significant reduction of the Ca^{2+} permeability at pH_e 6.5. Furthermore, we describe for the first time a slight but

significant reduction of NMDA single-channel conductance in the same pH_e conditions. The pH_e-mediated modulation of NMDARs was confirmed in mouse cortical neurons in culture: extracellular acidosis decreased NMDA-mediated Ca^{2+} transients more than expected from the parallel reduction of whole-cell currents. Our data provide the proof of concept that Ca^{2+} entry through human NMDARs can be reduced by lowering the Ca^{2+}/Na^{+} ratio in the current flowing through these receptor-channels, highlighting the need to find other pharmacological modulators acting in the same way.

2. Methods

2.1. Whole-cell currents in transfected HeLa cells

Human epithelial HeLa cells were grown in Dulbecco's Modified Eagle Meadium (DMEM) supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin, at 37 °C in a 5% CO_2 humidified atmosphere. Cells were plated on cover slides (8 * 10^4 cells/ml) and transiently transfected 24 h later using Magnetofection (Neuromag; OZ Bioscences, Mairselle, France) according to the manufacturer's protocol, adding 0.5 μg human NR1, NR2 A or NR2B cDNA subtype per well. Recordings were carried out 24–36 h following transfection. Whole-cell currents were recorded at room temperature using borosilicate glass patch pipettes having a tip resistance of 3–5 MΩ filled with the internal solution: for P_f measurements, 140 mM CsCl, 10 mM HEPES and 0.5 mM Fura-2 (pH 7.3); otherwise, 140 mM CsCl, 10 mM HEPES, pH value was adjusted at 7.3 with CsOH 1 M. The patch series resistance was compensated by 80–95%, and measurements were performed at holding potential of −70 mV. Cell capacitance was routinely compensated using the amplifier function and value used to estimate cell surface. Membrane currents were filtered at 3KHz upon the acquisition with HEKA EPC 800 (HEKA Elektronik, Germany) and analyzed offline. During recording, cells were continuously superfused using a gravity-driven perfusion system consisting of independent tubes for normal and NMDA and Glycine containing external solutions: 140 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH values were adjusted with NaOH 1 M at 7.3, 6.8 and 6.5. Perfusion tubes were connected to a fast exchanger system (RSC-100; Bio-logic,Claix, France). Data sampling and analysis were performed using pClamp10 software (Molecular Devices, Sunnyvale, CA, USA).

2.2. Single channel recordings on transfected HeLa cells

Transfected HeLa cells, with 0.5 μM human NR1 and NR2 A cDNA as described above, were used for single channel recordings 36–48 h following transfection. Single channel recordings were performed from excised outside-out patches with HEKA EPC 800 (HEKA Elektronik, Germany) at room temperature using borosilicate glass patch pipettes having a tip resistance of 3–5 MΩ filled with the internal solution: 140 mM CsCl, 10 mM HEPES, pH value was adjusted at 7.3 with CsOH 1 M. The holding potential was −70 mV. External solution contained: 140 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH values were adjusted with NaOH 1 M at 6.5 and 7.3. NR1/NR2A channels in patches were activated by addition of NMDA 200 μ M and glycine 50 μM to the external solution. Responses were recorded during continuous perfusion of the agonist-containing external solutions at different pH values (7.3, 6.5) using a gravity-driven perfusion system consisting of independent tubes connected to a fast exchanger system (RSC-100; Bio-Logic, Claix, France). Voltage-clamp recordings were filtered at 5 kHz and digitized at 20 kHz (pClamp 10). Selected parts of the recordings were filtered at 1 kHz and used to find opening events, by means of a threshold-based routine (ClampFit 10, Molecular Devices). Distributions of event amplitudes were fitted to the sum of two Gaussian curves, and then chord conductance values were calculated.

2.3. Whole-cell currents in primary cortical neurons

Cortical neuronal cultures were prepared from the brain of newborn C57BL/6 mice (P0-P1). All experiments concerning animals strictly comply with the EU Directive 2010/63/EU for animal experiments. Cerebral cortices were chopped and digested in 2.5% (wt/vol) trypsin for 20 min at 37 °C. Cells (10×10^4 cells/cm^2) were seeded on dishes coated with poly-L-lysine (100 μg/ml) in MEM with Earle's BSS supplemented with 100 mM sodium pyruvate, 20% (wt/vol) Glucose, 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 200 mM Glutamine. After 4 hours, the medium was changed with Neurobasal medium supplemented with 200 mM glutamine, 1% B-27, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Twice a week, half Neurobasal medium was changed and replaced with fresh medium. Cell cultures were used after 10–11 days. Whole-cell patch-clamp recordings were performed on cortical neurons in culture at room temperature, at a holding potential of −70 mV, using borosilicate glass patch pipettes having a tip resistance of 3–5 MΩ filled with internal solution: 140 mM CsCl, 10 mM HEPES, pH value was adjusted with CsOH 1 M at pH 7.3. The patch series resistance was compensated by 10–30%. Cell capacitance was routinely compensated, membrane currents were filtered at 3KHz upon the acquisition with HEKA EPC 800 (HEKA Elektronik, Germany) and analyzed off-line. During recordings, cells were continuously superfused using a gravity-driven perfusion system consisting of independent tubes for normal and NMDA and glycine containing external solutions: 140 mM NaCl, 10 mM HEPES, 2.8 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, pH values were adjusted with NaOH at 6.5 and 7.3. Perfusion tubes were connected to a fast exchanger system (RSC-100; Bio-Logic, Claix, France). Data sampling and analysis were performed using pClamp 10 software (Molecular devices, Sunnyvale, CA, USA).

2.4. $[Ca^{2+}]_i$ measurements in cortical neurons in culture

Measures of $[Ca^{2+}]_i$ were obtained by time-resolved digital fluorescence microscopy using Ca^{2+} indicator Fura-2 (excitation 340 and 380 nm, emission 510 nm). Cortical neurons in culture were incubated with the cell-permeant Fura-2 acetoxylmethylester (2μM; Molecular probes, Life technologies) for 45 min at 37 °C in culture medium. Ca^{2+} transients were elicited by applying NMDA 200 μM and glycine 50 μM for 3 s with a preapplication of normal external solution at pH 6.5 or 7.3 for 10 s without agonist.

2.5. Fractional Ca^{2+} current (P_f)

The procedure used for P_f measurements follows the method proposed by [14], which has the advantage to be independent of any assumption on ion-permeation properties [15,21]. All measurements were performed in transfected HeLa cells, which have a membrane capacitance ranging between 10 and 20 pF and allow almost perfect voltage clamp, thus being an ideal preparation for determination of P_f, which requires that current flows uniquely through the channel under study. Cells were loaded with cell-impermeant Fura-2 through the patch pipette used to measure NMDA-evoked currents. Recordings of fluorescence signals and whole-cell membrane currents were synchronized, and images were acquired and analysed off-line. All optical parameters and digital camera settings were maintained throughout this study to avoid nonhomogeneous data. The changes of $[Ca^{2+}]I$ were expressed as

ΔF/F (*i.e.* basal fluorescence), using only one excitation wavelength, 380 nm, to increase the temporal resolution. Determinations were carried out after the basal fluorescence had reached a stable value. Cells displaying a low-basal F_{380} values were discarded. In order to evaluate P_f the F:Q ratio between the fluorescence increase (F) and total charge that had entered the cell at each fluorescence acquisition time (Q) was defined as F/Q = (ΔF/F)/ Q. For each cell, we used the F/Q points that, immediately after the onset of the NMDA evoked response, exhibited a linear relationship, indicating that the Ca^{2+}-buffering capability of Fura-2 was not saturated. The F/Q ratio value was then measured as the slope of the linear regression best fitting the F–Q plot. Finally, P_f was determined by normalizing the ratio obtained in standard medium (F/Q) to the calibration ratio, measured when Ca^{2+} ions were the only permeant species (F/Q_{Ca}): P_f = (F/Q) / (F/Q_{Ca}). Calibrations were performed on different days throughout the whole experimental period, using NMDA-evoked Ca^{2+} currents. NMDA was applied to each cell only once, to avoid possible variations of conditions upon repetitive applications, such as basal $[Ca^{2+}]_i$ increase.

2.6. Statisics

All data were expressed as means ± S.D. and analysed using one-way ANOVA or paired t-test, as appropriated. Data were tested for normality (Shapiro-Wilks) and equal variance. When necessary, the non parametric Kruskal-Wallis one way ANOVA on ranks was used. In case of significance, all pairwise multiple comparison procedure was used (Holm-Sidak, or Dunn's method for non parametric tests). Minimum power of statistical tests was set at 0.8. Significance for all tests was set at $p < 0.05$.

3. Results

3.1. Mild acidosis reduces the Ca^{2+} permeability of human NMDARs

To investigate the effect of acidosis on the NMDAR Ca^{2+} permeability we measured the fractional Ca^{2+} current (*i.e.* the percentage of the total current carried by Ca^{2+} ions; P_f) of human NR1/NR2 A NMDAR expressed in HeLa cells, simultaneously recording NMDA-evoked whole-cell currents and variations of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$; Fig. 1A). Selective agonist NMDA (200 μM) and glycine (50 μM) were co-applied for two seconds at three different pH_e values (6.5, 6.8, 7.3). At pH_e 7.3 the P_f value of NR1/NR2 A NMDAR was 11.3 ± 4.4% (n = 9), in agreement with previous studies [21]. Lowering pH_e from 7.3 to 6.8, the P_f value was not significantly different from the value at physiological pH_e (8.5 ± 3.1%; n = 9), whereas when pH_e was reduced to 6.5 the P_f was significantly decreased to 7.6 ± 1.9% (Fig. 1B,C; n = 12). The current density values at pH_e 7.3, 6.8 and 6.5 were measured in distinct transfected cells and did not show any significant difference, due to the high variability of expression levels (mean values 71 ± 59 pA/pF, n = 14; 63 ± 36 pA/pF, n = 17; 54 ± 23 pA/pF, n = 14, respectively). When pH_e was shifted from 7.3 to 6.5 during NMDA and glycine co-application on the same NR1/NR2 A transfected cells, the steady-state current amplitude was reduced to 52 ± 20% (n = 7, not shown), confirming that acidosis inhibits NMDAR-mediated currents.

To have a more general view about the effects of acidosis on the Ca^{2+} fluxes mediated by NMDARs, we extended our study to the human NR1/NR2B NMDAR subtype, mainly expressed at extrasynaptic locations and exhibiting distinct functional properties [22]. We analyzed the human NR1/NR2B NMDARs as above described for NR1/NR2 A receptor (Fig. 2A), again finding that low pH_e values could alter the Ca^{2+} permeability. In particular, P_f value of NR1/NR2B receptor was 9.1 ± 2.3% at pH_e 7.3 and 8.1 ± 2.5% at pH_e 6.8 P_f (not significantly different; n = 7 and n = 6, respectively), whereas the P_f value fell to 4.6 ± 1.2% at pH_e 6.5 (Fig. 2B,C; n = 9). Our data demonstrated that extracellular acidosis is able to reduce significantly and

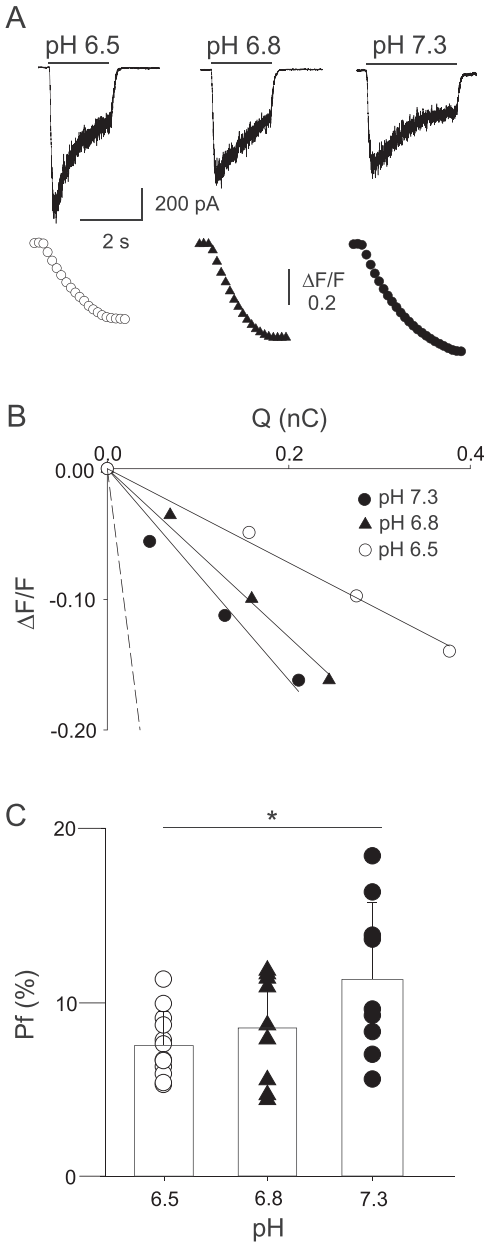


Fig. 1. Acid pH_e reduces the Ca^{2+} permeability of human NR1/NR2A NMDAR. A, typical traces obtained by simultaneous recordings of whole-cell currents (top) and Ca^{2+} transients (bottom) evoked by coapplication of NMDA and glycine (200 μM and 50 μM, respectively) at the indicated pH_e values in three distinct HeLa cells transiently transfected with human cDNA encoding for NR1 and NR2 A subunits. Please note that current amplitudes variability is due to different expression levels in distinct transfected cells, and hence do not reflect pH_e effects. Experiments were performed in Mg^{2+} free normal external solution to unmask NMDA currents at negative potentials. Holding potential, −70 mV. At excitation wavelength of 380 nm, $[Ca^{2+}]_i$ increase corresponds to a downward deflection of Fura-2 fluorescence emission. Traces are aligned and share the same temporal scale. B, linear relationships between ΔF/F and Q obtained from the same cells as in A. The dashed line represents the mean slope obtained from calibration experiments (5.54 nC^{-1}; n = 9). C, the P_f values was 7.6 ± 1.9% (n = 12), 8.5 ± 3.1% (n = 9), and 11.3 ± 4.4% (n = 9) for pH_e value of 6.5, 6.8 and 7.3, respectively. * P = 0.036 (ANOVA). Symbols represent the P_f value of the individual cells. Error bars represent SD.

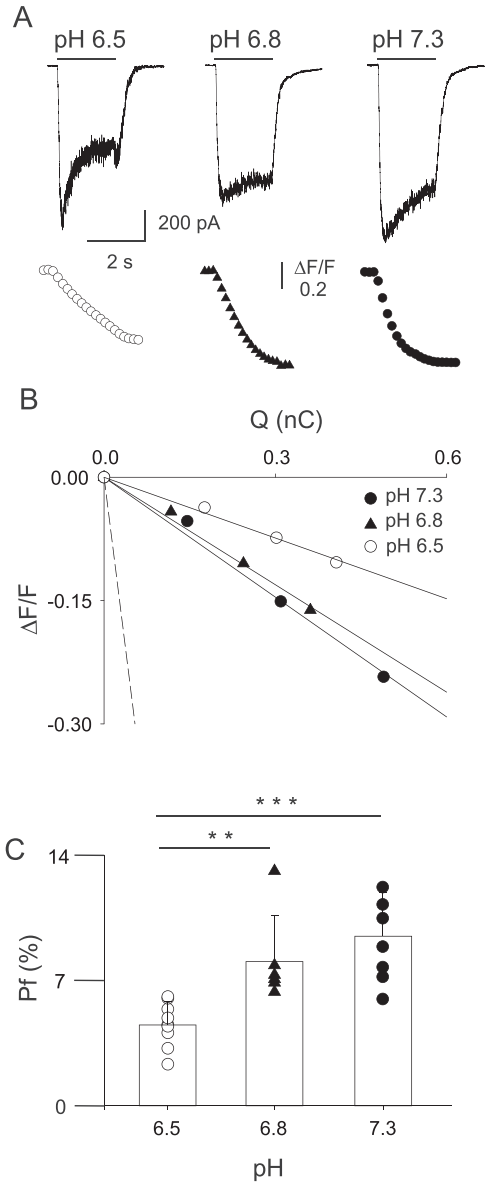
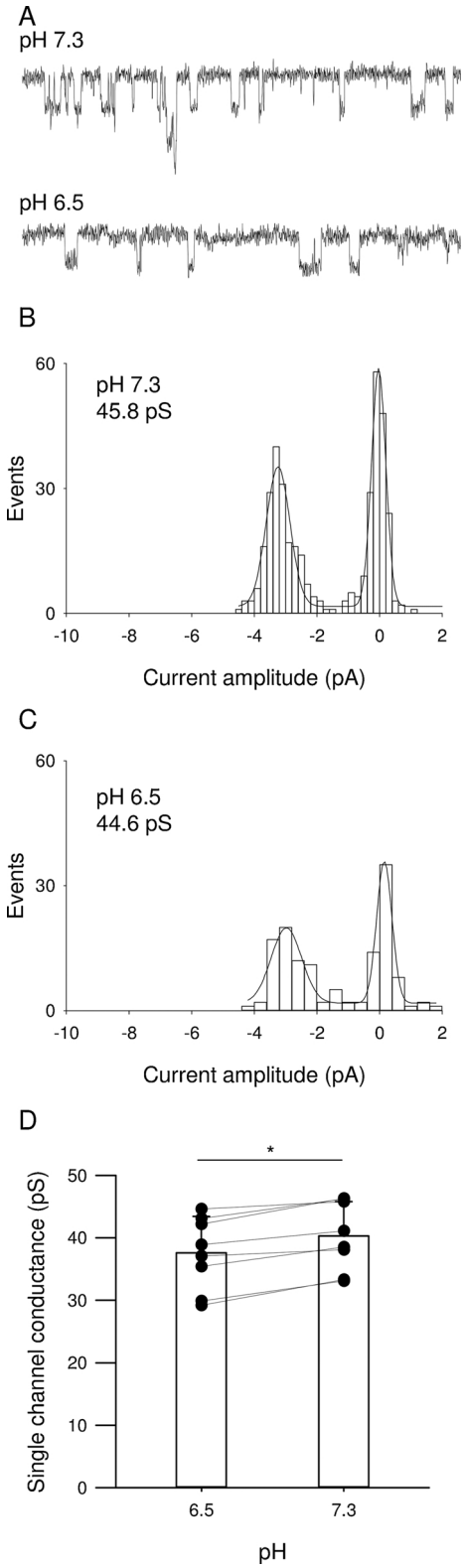


Fig. 2. Acid pH_e reduces the Ca^{2+} permeability of human NR1/NR2B NMDA receptor.

A, typical traces obtained by simultaneous recordings of whole-cell currents (top) and Ca^{2+} transients (bottom) evoked by coapplication of NMDA and glycine (200 μM and 50 μM, respectively) at the indicated pH_e values in three distinct HeLa cells transiently transfected with human cDNA encoding for NR1 and NR2B subunits. Experiments were performed in Mg^{2+} free normal external solution to unmask NMDA currents at negative potentials. Holding potential, −70 mV. At excitation wavelength of 380 nm, $[Ca^{2+}]_i$ increase corresponds to a downward deflection of Fura-2 fluorescence emission. Traces are aligned and share the same temporal scale. B, linear relationships between ΔF/F and Q obtained from the same cells as in A. The dashed line represents the mean slope obtained from calibration experiments (5.54 nC^{-1}; n = 9). C, the P_f values were 4.6 ± 1.2% (n = 9), 8.1 ± 2.5% (n = 6), and 9.1 ± 2.3% (n = 7) when measured at pH_e value of 6.5, 6.8 and 7.3, respectively. *** P < 0.001, **P = 0.006 (ANOVA). Error bars represent SD. Symbols represent the P_f value of the individual cells.



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selectively the Ca^{2+} flow through human NMDARs.

3.2. Mild acidosis reduces the single channel conductance of human NR1/NR2 A NMDARs

The above reported P_f data led us to hypothesize that the selective reduction, due to acidosis, of the Ca^{2+}/Na^+ ratio of the current flowing through NMDARs could affect the conductance of these receptor-channels, even though previous reports did not find significant variations of this parameter in similar conditions [13,23–26]. We recorded single channel activity in outside-out patches excised from HeLa cells transiently expressing human NR1/NR2 A NMDARs. To detect the slight

Fig. 3. Extracellular acidosis modulates single channel activity of human NR1/NR2 A NMDAR in outside-out patches.
A, Typical traces recorded from the same outside-out patch excised from a HeLa cell expressing human NR1/NR2 A NMDARs upon coapplication of NMDA and glycine (200 μM and 50 μM, respectively); pH_e values, 7.3 (top) and 6.5 (bottom). Holding potential, −70 mV. B, event amplitude histograms from the outside-out traces shown in A at physiological pHe. C, event amplitude histograms from the outside-out traces shown in A during acidosis. Distributions were obtained from the same outside-out patch and were best fitted by two Gaussian curves. The indicated chord conductances (−70 mV) were calculated from the Gaussian fit. D, mean (bar) and individual (symbols) single channel chord conductance values at two different values of pH_e 6.5, 7.3 (n = 8), * $P < 0.001$, paired t-test.

amplitude shifts expected from the observed Ca^{2+} permeability changes, the recording conditions were the same of P_f measurements. Furthermore, in these conditions (high extracellular Ca^{2+}, high agonist concentrations) the occurrence of subconductance levels is greatly reduced [27] (Banke and Traynelis, Nature Neuroscience 2003), allowing a more precise comparison between whole-cell and single-channel recordings, considering that subconductance states may have in principle different ion selectivity. Coapplication of NMDA and glycine (200 μM and 50μM, respectively) elicited a high number of channels openings, with a relatively high open probability, strongly reduced upon shifting the pH_e from 7.3 to 6.5 (Fig. 3A), as expected from previous studies [23,25,26].

The single channel chord conductance values were obtained by event histograms best fitted with two Gaussian distributions (Fig. 3B,C; −70 mV): all outside-out patch exhibited a reduction in chord conductance going from physiological pH_e to acidosis (pH_e 6.5). The mean chord conductance values were 40.3 ± 5.5 pS and 37.6 ± 5.8 pS for pH_e values of 7.3 and 6.5, respectively (Fig. 3D; $p < 0.001$; n = 8). Though the conductance value at physiological pH is similar to the values previously reported, the small but significant conductance reduction upon acidosis, to our knowledge, has never been recognized before [13,23,25,26].

3.3. *Extracellular acidosis differently modulates NMDAR-evoked whole-cell currents and Ca^{2+} entry in mouse cortical neurons in culture*

Basing on our findings, we hypothesized that also in native neurons an acid pH_e should reduce to a greater extent the Ca^{2+} entry due to NMDAR activation, rather than the NMDAR-mediated whole-cell currents. To investigate this hypothesis, considering that in neurons is not possible to reliably measure the P_f due to diffusion and clamp limitations, we decided to measure disjointedly Ca^{2+} transients and whole-cell currents evoked by NMDA application on cortical neurons in culture (Fig. 4A,B). Experiments were performed co-applying NMDA and glycine as above, at pH_e values of 7.3 and 6.5. As expected from previous studies [13,24–26], we observed a significant reduction of both NMDA-evoked Ca^{2+} transients (Fig. 4A) and NMDA-evoked whole-cell currents (Fig. 4B) when pH_e was decreased from 7.3 to 6.5. The extracellular acidosis did not affect $[Ca^{2+}]_i$ and currents at the same extent: the NMDA-evoked Ca^{2+} influx decreased significantly more than NMDA-evoked whole-cell currents (35.7 ± 19.7% vs 53.4 ± 10.0%; Fig. 4C; n = 318 and n = 15, respectively), confirming our hypothesis and suggesting that the proton-induced modulation of NMDAR P_f could exert significant effects on the accumulation of Ca^{2+} ions in native neurons. The reduction of whole-cell current amplitudes is very similar to what observed in HeLa cells transfected with human NR1/NR2 A NMDARs in the same kind of experiments (52 ± 20%; n = 7), suggesting that mouse and human receptors are modulated in the same way by acidosis.

Interestingly, in the absence of any agonist the application of external solution at pH 6.5 evoked small but evident Ca^{2+} transients in neurons, likely due to the opening of proton-gated ion channels

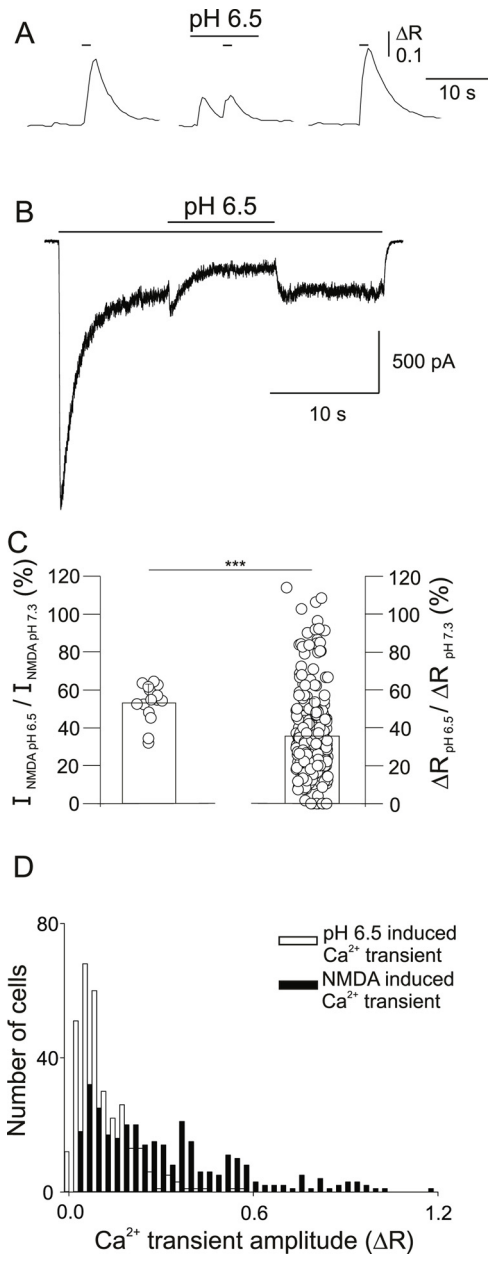

Fig. 4. Extracellular acidosis reduces both NMDA-evoked Ca^{2+} transients and NMDA-evoked whole-cell currents in cortical neurons in culture.
A, Ca^{2+} transients recorded from a mouse cortical neuron in culture elicited by coapplication of NMDA and glycine (200μM and 50μM, respectively; short horizontal bars). pH_e was transiently shifted during agonist application from 7.3 to 6.5 (3 s application, as indicated). Please note the Ca^{2+} transient elicited by the application of the external solution at pH 6.5 immediately before the application of agonists. B, whole-cell current elicited by coapplication of NMDA and glycine (200μM and 50 μM, respectively; long horizontal bar) recorded from a different cortical neuron. pH_e was transiently shifted during agonist application from 7.3 to 6.5, as indicated. Please note the small and transient inward current elicited by protons and the succeeding reduction of the NMDA-evoked current. C, percentage reduction of whole-cell currents (left, 53.4 ± 9.8%; n = 15) and Ca^{2+} transient amplitudes (right, 35.7 ± 19.7%; n = 318) recorded from individual mouse cortical neurons in culture when pH_e was shifted from 7.3 to 6.5. Please note that Ca^{2+} transient amplitudes were significantly more reduced than currents. *** $P < 0.001$ (ANOVA). D, distributions of the Ca^{2+} transient amplitudes elicited by application of an acid external solution (white bars, pH_e 6.5) or by the coapplication of NMDA and glycine (200 μM and 50 μM, respectively; black bars, pH_e 7.3).

Table 1
Effect of different extracellular cations on Pf value of human NR1/NR2 A NMDAR.

Cations	P_f (%)	Current amplitudes (pA)
- Mg^{2+}	11.3 ± 4.4, n = 9	1367 ± 920, n = 14
+ Mg^{2+} (2 mM)	9.9 ± 2.9, n = 11	183 ± 167, n = 13
Memantine (1 μM)	16.4 ± 2.8, n = 4	51 ± 17, n = 4
Spermine (100 μM)	8.6 ± 3.0, n = 14	147 ± 127, n = 19
IEM 1754 (1 μM)	9.3 ± 1.7, n = 4	121 ± 97, n = 6
IEM 1460 (100 μM)	9.2 ± 3.0, n = 11	150 ± 111, n = 15

All P_f values were measured at −70 mV holding potential. All measurements were done on distinct cells, and each drug was tested in the presence of Mg2+, to assess their ability to modulate Ca^{2+} permeability in the physiological presence of divalent cations. In the presence of Mg^2 the residual NMDA-evoked whole-cell currents were still adequate to allow reliable P_f determinations.

(Fig. 4A; [28]). However, neurons exhibited acid-evoked Ca^{2+} entry at pH_e 6.5 rather small and transient if compared with the Ca^{2+} entry through NMDARs, with a completely different distribution (Fig. 4D), suggesting a negligible excitotoxicity due to proton-gated ion channels at this level of pH_e.

3.4. In search of other cations able to modulate the P_f of human NMDARs

The reduction of the P_f of human NMDARs could be exploited to reduce glutamate neurotoxicity in pathological conditions. For this reason we looked for the action of different cations, starting with the effect of the presence of extracellular Mg^{2+} on the NMDAR Ca^{2+} permeability. Despite the strong reduction of the total current elicited in the presence of Mg^{2+} (2 mM, −70 mV) by NMDA in HeLa cells transfected with human NR1/NR2 A receptors, it was still possible to measure the P_f value: 9.9 ± 2.9% (n = 11, Table 1), not different from the same value in the absence of Mg^{2+}. Furthermore, we measured the P_f of the same receptor in the presence of other larger organic cations: memantine (1 μM), an open channel blocker of NMDAR used in patients with Alzheimer's disease [10]; spermine (100 μM), an intracellular polyammine known to block cation fluxes through ligand-gated channels [29]; IEM 1754 (1 μM) and IEM 1460 (100 μM), two open channel blocker of NMDARs [30,31]. None of these molecules was able to reduce the P_f value of human NMDA NR1/NR2 A receptors (Table 1), evidencing the need of future studies to identify different kinds of cationic modulators.

4. Discussion

Overactivation of NMDARs, with consequent Ca^{2+} overload, is a major cause of neuronal excitotoxicity [4]. Thus, NMDARs represent a suitable target for a pharmacological strategy against neuronal damage, but to date no drug appears to be useful for neuroprotection, considering that most molecules interfere with the normal neuronal function and cause substantial side effects at potentially therapeutic doses [32].

Here we report that mild extracellular acidosis (pH_e 6.5; [18]) reduces the Ca^{2+} permeability of human NMDARs, contributing to limit the Ca^{2+} entry through these receptors. Using simultaneous recordings of whole-cell currents and Ca^{2+} transients elicited by NMDA application, we showed that the fractional Ca^{2+} current (P_f, *i.e.* the percentage of total current carried by Ca^{2+}) of both NR1/NR2 A and NR1/NR2B human NMDARs is significantly reduced during extracellular acidosis compared to P_f measured at physiological pH_e. This reduction of Ca^{2+} flux has been confirmed also for NMDARs expressed in native mouse cortical neurons. Taken together, these results show a new molecular mechanism underlying the neuroprotective effect of extracellular mild acidosis [17,18], providing an important contribute in the search of a new strategy against glutamate-mediated neurotoxicity, mainly due to the an excess of Ca^{2+} ions flowing through NMDARs [4].

Acidification occurs in CNS during normal neuronal functions and modulates ligand-,voltage- and proton-gated ion channels [33], but is also involved in several brain acute pathologies and neurodegenerative disorders causing acidosis-mediated cell death [33,34]. Acidotoxicity and excitotoxicity are two major cell death mechanisms in ischemic brain, and for this reason several studies focused their attention to the modulation of rat NMDARs by extracellular mild acidosis [13,17,24,25]. These studies clearly showed that extracellular acidosis reduced NMDARs open probability with a consequent reduction of NMDA-evoked currents. Our results confirm the acidosis-induced reduction of NMDA activation also for human receptors, with a relevant novelty: extracellular acidosis significantly and selectively reduces the Ca^{2+} flux through open NMDA receptor-channels. It is important to note that the two pH-dependent mechanisms modulating open probability and Ca^{2+} permeability appear to be distinct, acting at different concentration ranges of H^+ and likely involving different H^+ binding sites.

The high Ca^{2+} permeability of NMDARs is due to several Ca^{2+} binding sites within the pore as well as in external vestibule, depends on subunit composition with receptors containing NR2 A and NR2B showing the highest Ca^{2+} permeability [5,21]. Previous studies demonstrated that the P_f of NMDARs and others receptor channels can be physiologically and pharmacologically modulated [11,35,36]. Our work reports a significant reduction of P_f of human NR1/NR2 A and NR1/NR2B NMDARs in extracellular acidosis, adding new evidence about the possibility to modulate the Ca^{2+} permeability of ligand-gated ion channels.

Previous studies stated that an acidification in pH_e strongly decreased the NMDA channel opening frequency without changing unitary conductance [24,25]. Thus, at least one of the H^+ binding sites on NMDARs is thought to be closely associated with the gating site, but the exact mechanism of action is still unclear [23,37]. Our analysis of single-channel activity of human NR1/NR2 A NMDARs in outside-out patches confirms a reduction in frequency of channel openings, highlighting a small but significant decrease of the single channel conductance. This finding is in good agreement with the reduction of ion fluxes expected from the observed reduction of P_f. In particular, combining our P_f and single channel data, it is possible to estimate that, upon shift of the pH_e from 7.3 to 6.5, the Na^+ flux through human NMDAR is almost unaffected (from 38.5 pS to 37.6 pS, 98%), while the Ca^{2+} flux is much more reduced (from 4.8 pS to 3.0 pS, 63%). The subtle but significant decrease of unitary conductance was not evidenced in previous studies analyzing the effects of acidification on the single-channel properties of NMDAR [13,25,26], likely because of different experimental conditions: lower extracellular concentration of Ca^{2+} ions, rat vs human NMDARs, different pH_e values. The observed Pf reduction of both NR1/NR2 A and NR1/NR2B receptors suggests that extracellular H^+ may modulate Ca^{2+} permeation binding negatively charged residues on NR1 subunit. A possible H^+ binding site could be a cluster of charged residues (DRPEER) located at the extracellular vestibule in NR1 subunit that represents a key determinant of the high Ca^{2+} permeability of NMDARs [38], but further studies will be necessary to identify the exact mechanism of the H^+-induced reduction the NMDAR Ca^{2+} permeability.

As expected from previous studies [13,24,25], our experiments with cultured cortical neurons show that both NMDA-evoked Ca^{2+} mobilization and whole-cell current amplitudes are strongly reduced upon mild acidification. However, the two reductions are not proportional, with NMDA Ca^{2+} mobilization significantly more reduced than NMDA currents, further supporting the hypothesis of a differential effect of protons on Ca^{2+} and Na^+ fluxes. All the experimental data show that mild acidosis exerts a dual action on NMDARs: a reduction of open probability plus a selective reduction of Ca^{2+} permeability. An important question raised from these results is whether this second mechanism may play a relevant pathophysiological role in neurons upon

acidosis. Indeed, the strong inhibition of the NMDAR open probability could suggest that a further reduction of the amount of Ca^{2+} ions flowing through these channels could hardly impact on the overall Ca^{2+} load. However, our data support a different conclusion. In fact, combining these two effects it is possible to quantitatively estimate the overall reduction, triggered by the pH_e shift from 7.3 to 6.5, of NMDAR-mediated Ca^{2+} influx: 35%, due to the decrease of both NMDA currents and P_f (to 52% and 67%, respectively, for human NMDA NR1/NR2 A receptor). Even considering a higher open probability inhibition, as reported by previous studies in rodents (Σ80% at pH_e 6.5; [25]), the total reduction of Ca^{2+} fluxes through NMDARs would be still affected by the P_f modulation, passing from 20% to 13%. The overall neuroprotective effect of this double downregulation of NMDAR-mediated Ca^{2+} entry can be esteemed considering that NMDARs are non-desensitizing channels and that previous study clearly demonstrated their involvement in acidotoxicity in combination with other mechanisms [39,40].

The extracellular acidosis is known to activate acid sensing ion channels ASICs [28], proton-gated channels expressed throughout the nervous system. To date seven isoforms have been identified, but only 1a, 2a and 2b subtypes are present in CNS neurons [41]. The homomeric ASIC1a channels are highly permeable to Ca^{2+}, whereas the other subtypes are only permeable to Na^+ [42]. Activation of ASICs1a is due to extracellular pH ranging from 6.9-5.0 with a pH_{50} of 5.8 [20] and increases the probability of action potential initiation [43]. A coupling between NMDARs and ASIC1a channels worsen the neuronal conditions during ischemic damage, leading to cell death [44]. In our experiments we show that in the absence of agonist, the pH_e 6.5 solution elicited small Ca^{2+} transients likely due to ASIC activation. These Ca^{2+} transients were much smaller than those evoked by NMDA at pH_e 7.3, suggesting a negligible contribution of ASICs to neuronal toxicity at pH_e 6.5, in agreement with their known pH_{50} values [20].

As expected, extracellular Mg^{2+}, present in all our experiments on neurons, is able to strongly decrease the channel open probability at hyperpolarized potentials [45], but we show here that it does not affect the Ca^{2+} permeability. Hence, the residual NMDA-evoked current, observed in neurons in the presence of Mg^{2+} at a holding potential −70 mV, is sufficient to mediate a relevant Ca^{2+} influx, which can contribute to neurotoxicity in the prolonged presence of extracellular glutamate. Besides Mg^{2+}, we tried to identify cationic molecules able to decrease the P_f of human NMDARs as new pharmacological tools against excitotoxity due to excessive glutamate-induced Ca^{2+} entry. Unfortunately, our effort was not successful. Further studies will be necessary to unveil possible novel pharmacological tools, as well as to explain the exact molecular mechanism by which extracellular H^+ modulate NMDARs ion selectivity.

5. Conclusions

Our data confirm the results of previous studies, according to which extracellular mild acidosis decreases the effect of glutamatergic neurotransmission reducing NMDA-evoked whole cell currents, and adds new evidence demonstrating that the neuroprotective action of mild acidosis is also due to a reduction of Ca^{2+} influx through NMDARs. Thus, this study provides a proof of concept of the hypothesis that, in different kind of neuropathologies, such as neurodegenerative diseases or ischaemic insults, it is possible to reduce Ca^{2+} overload in neurons, and hence excitotoxicity, modulating the NMDAR Ca^{2+} permeability, without blocking these receptor channels. Although to date several tested molecules have failed in attaining this goal, this therapeutic strategy will remain the focus of future studies, aiming to unveil the mechanisms and the sites of the H^+ modulation of human NMDARs, thus allowing a targeted pharmacological design.

Authors

All authors approved the final version of the manuscript. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. In this work S.P., M.S. and S.F contributed to the acquisition, analysis and interpretation of data and to the drafting and critical revision of the work. S.F. designed the study.

Declaration of interest

None.

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