Brain, Behavior, and Immunity xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Down-sizing of neuronal network activity and density of presynaptic terminals by pathological acidosis are efficiently prevented by Diminazene Aceturate

Roberta de Ceglia [a], Linda Chaabane [a,d,2], Emilia Biffi [b,g,2], Andrea Bergamaschi [a], Giancarlo Ferrigno [b], Stefano Amadio [c], Ubaldo Del Carro [c], Nausicaa Mazzocchi [f], Giancarlo Comi [d], Veronica Bianchi [e], Stefano Taverna [g], Lia Forti [h], Patrizia D'Adamo [e], Gianvito Martino [a,*], Andrea Menegon [f,1], Luca Muzio [a,*,1]

[a] Neuroimmunolgy Unit, Institute of Experimental Neurology (INSPE), Division of Neuroscience, San Raffaele Scientific Institute, Italy
[b] Neuroengineering and Medical Robotics Laboratory, Department of Electronics, Information and Bioengineering, Politecnico di Milano, Italy
[c] Neurophysiology Unit, Division of Neuroscience, San Raffaele Scientific Institute, Italy
[d] Department of Neurology, Institute of Experimental Neurology (INSPE), Vita Salute San Raffaele University, Milan, Italy
[e] Dulbecco Telethon Institute at San Raffaele Scientific Institute, Division of Neuroscience, 20132 Milan, Italy
[f] Advanced Light and Electron Microscopy Bio-Imaging Centre, Experimental Imaging Centre, San Raffaele Scientific Institute, 20132 Milan, Italy
[g] Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, Genoa, Italy
[h] Center for Neuroscience and Dept. of Theoretical and Applied Sciences, Biomedical Division, University of Insubria, 21052 Busto Arsizio, Italy

ARTICLE INFO

Article history:
Received 29 July 2014
Received in revised form 2 December 2014
Accepted 2 December 2014
Available online xxxx

Keywords:
ASICs
Diminazene Aceturate
MRI spectroscopy
Multiple sclerosis
MEA

ABSTRACT

Local acidosis is associated with neuro-inflammation and can have significant effects in several neurological disorders, including multiple sclerosis, brain ischemia, spinal cord injury and epilepsy. Despite local acidosis has been implicated in numerous pathological functions, very little is known about the modulatory effects of pathological acidosis on the activity of neuronal networks and on synaptic structural properties. Using non-invasive MRI spectroscopy we revealed protracted extracellular acidosis in the CNS of Experimental Autoimmune Encephalomyelitis (EAE) affected mice. By multi-unit recording in cortical neurons, we established that acidosis affects network activity, down-sizing firing and bursting behaviors as well as amplitudes. Furthermore, a protracted acidosis reduced the number of presynaptic terminals, while it did not affect the postsynaptic compartment. Application of the diarylamidine Diminazene Aceturate (DA) during acidosis significantly reverted both the loss of neuronal firing and bursting and the reduction of presynaptic terminals. Finally, *in vivo* DA delivery ameliorated the clinical disease course of EAE mice, reducing demyelination and axonal damage. DA is known to block acid-sensing ion channels (ASICs), which are proton-gated, voltage-insensitive, Na^+ permeable channels principally expressed by peripheral and central nervous system neurons. Our data suggest that ASICs activation during acidosis modulates network electrical activity and exacerbates neuro-degeneration in EAE mice. Therefore pharmacological modulation of ASICs in neuroinflammatory diseases could represent a new promising strategy for future therapies aimed at neuro-protection.

1. Introduction

Extracellular acidosis accompanied the inflammatory process of the CNS, as recently shown in mice affected by Experimental Autoimmune Encephalomyelitis (EAE) (Friese et al., 2007). Moreover, CNS acidosis affects a large number of CNS diseases, in addition to autoimmune diseases. Indeed, the oxygen depletion, occurring in stroke, forces neurons to switch anaerobic glycolysis, and increases the generation of lactic acid and protons, thus causing the pH to fall in the ischemic and in the peri-ischemic tissue (Rehncrona et al., 1985). A substantial drop of pH has been also observed during seizures, in which the extracellular pH can fall below 6.8 (Somjen and Aitken, 1984). The increase of proton concentration directly activates Acid Sensing Ion channels (ASICs) in neurons. ASICs are voltage-independent, H^+-gated channels (Lingueglia et al., 1997) that belong to the NaC/DEG channel

* Corresponding authors at: San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy. Tel.: +39 02 2643 4958; fax: +39 02 2643 4855.
E-mail addresses: martino.gianvito@hsr.it (G. Martino), Muzio.luca@hsr.it (L. Muzio).
[1] Equally contributing senior authors.
[2] Equally contributing to the work.

http://dx.doi.org/10.1016/j.bbi.2014.12.003
0889-1591/

super-family, including epithelial Na^+ channels (ENaC) and degenerins (DEG) (Canessa et al., 1993, 1994). They are responsible for the proton-evoked inward currents in neurons, by fluxing Na^+ and, possibly, Ca^{2+} ions (Gruol et al., 1980). Four *Asic* genes (*Asic1–4*) and specific splice variants (a and b) for *Asic1* and *Asic2* have been described in mammals (Bianchi and Driscoll, 2002). Although ASICs isoforms are broadly expressed, CNS neurons preferentially express *Asic1a* and *Asic2a/2b* channels (Wemmie et al., 2002), which undergo random trimerization, forming both homotrimeric and heterotrimeric channels (Bartoi et al., 2014).

Pharmacological inhibition of ASICs in animal models of neurodegenerative disorders exerts beneficial effects in terms of neuronal survival. Indeed, amiloride-mediated inhibition of ASIC channels in EAE mice improves clinical outcomes and protects axons from degeneration (Friese et al., 2007; Vergo et al., 2010). In animal model of ischemia, the specific blockage of ASIC1a by Psalmotoxin-1 (PcTx-1) causes a significant reduction of the infarct volume (Xiong et al., 2004). Along the same lines, ASIC blockade with amiloride exerts neuroprotective effects in MS patients, reducing brain atrophy (Arun et al., 2013). Besides toxins derived from venoms, a new class of compounds, the diarylamidines, including Diminazene Aceturate (DA), 4′,6-diamidino-2-phenylindole (DAPI), hydroxystilbamidine (HSB), and Pentamidine, have been recently described as ASICs inhibitors (Chen et al., 2010). Among them, DA is the most potent small-molecular inhibitor of ASIC channels, being able to block ASIC1a/b, ASIC2a and ASIC3 in the low micromolar range (Chen et al., 2010). ASICs channel properties, including their pharmacological inhibition, have been extensively studied *in vitro*, often by single cell electrophysiology, while their functional roles in complex networks of neurons have not been yet investigated.

In this study we monitored pH fluctuations in inflammatory lesions of EAE mice by innovative, and non-invasive, Magnetic Resonance Spectroscopy (MRS) showing persistent acidosis in the inflamed CNS with pH levels ranging around pH 6.6. Furthermore, we demonstrated that persistent acidosis affects synchronized firing activity of neuronal networks by means of Micro Electrode Array (MEA) recordings. We showed that application of DA significantly reverted this phenotype, suggesting a key role of ASICs blockade. We also demonstrated that acidosis causes a substantial reduction of the number of pre-synaptic terminals in neurons, which was also rescued by DA administration. Finally, we show that DA efficiently reduced clinical disabilities, demyelination and axonal loss in EAE affected mice.

2. Material and methods

2.1. MR-spectroscopy of IEPA

All procedures involving animals were performed according to the guidelines of the Animal Ethical Committee of San Raffaele Scientific Institute (IACUC protocol number 427). Calibration of the pH dependence of the chemical shift of the H2 resonance were performed in 7 samples of saline water containing IEPA ((±)2-imidazole-1-yl-3-ethoxycarbonyl propionic acid, at 0.3 M (Soirem Research) with pH ranging from 5 to 8. ^1H NMR spectra were acquired on a 7T-MRI scanner (Biospec, Bruker Biospin) with a PRESS sequence (TR/TE = 2500/10 ms, average = 1, water suppression). The chemical shift of H2-IEPA peak was measured on spectrum acquired on each sample using Mnova tool (Mestrelab Research SL). The curve of pH as function of chemical shift (ppm) was fitted using a nonlinear regression with the Henderson–Hasselbalch equation (pH = pKa + Log((ppm − Acid)/(Base − ppm)), pKa, Acid and Base are constants) using Prism (GraphPad software). *In vivo* analysis of pH was done by MRI on EAE mice at 17 d.p.i. During MRI, animals were maintained under anesthesia with a mixture of 1–2% of flurane (IsoVect, Piramal Healthcare) in oxygen, respiration rate was constantly monitored and body temperature was maintained at 36–37 °C by warm water heating system. MR–Spectrums were acquired in a voxel selected the anatomic T2-MR images in CB and TH regions of the brain with the PRESS sequence (TR/TE = 1850/14 ms, average = 256, water suppression and voxel = $2 \times 3 \times 2$ or $1.3 \times 1.5 \times 2.2$ mm^3, respectively). Spectra were acquired before and after the slow infusion of IEPA (20 mmol/kg at 0.4 mL/h) through a catheter placed on the tail vein. Successively, BBB leakage was evaluated with T1 weighted images acquired post application of gadolinium contrast (1.2 mmol/kg of MultiHance, Bracco Imaging Spa). All spectrums were processed using Mnova program to determine the H2-IEPA chemical shift (between 8.8 and 7.67 ppm) using the creatine peak (Cr) as reference (3.02 ppm).

2.2. Primary cell cultures & MEA recording

Primary neuronal cell cultures were established from C57Bl/6/CD1 E17.5 embryos (Charles River). Brains were dissected in cold HBSS (Gibco) supplemented with glucose 0.6% and 5 mM Hepes pH 7.4 (Sigma). Cerebral cortices were mechanically dissociated in single cells and re-suspended in culture medium containing: 50% D-MEM (Lonza); 50% Ham's-F12 (Gibco); 5 mM HEPES pH 7.4 (Sigma); 0.6% glucose (Sigma); 0.5% glutamine (Gibco); 30 nM Na-Selenite (Sigma); 20 nM progesterone (Sigma); 60 nM putrescine (Sigma); 100 μg/ml apo-transferrin (Sigma); 0.025 mg/ml of bovine Insulin (TEBU-BIO) and 5% of FBS (EUROCLONE) in absence of antimitotic and antibiotic drugs. After 5 h, the medium was replaced completely with fresh culture medium without FBS. Cells were seeded onto coated MEA chip at the final density of 3×10^5 cells/MEA. For synapses analysis, neurons were plated onto 1 mg/ml poly-L-lysine (Sigma) coated glass coverslips at the density of 100 cells/mm^2. Neurons were maintained for 12–17 days in standard culture medium in a humidified 5% CO_2 atmosphere at 37 °C. We used MitTx (α and β subunits, Alomone) to activate ASIC1a channels and Diminazene Aceturate (DA, Sigma) to inhibit ASICs.

Standard 60 electrode Multi Electrodes Array (MEA) biochips with 200 μm electrode spacing and 30 μm electrodes diameter with an integrated reference electrode (Multichannel Systems GmbH) were employed for the study of multisite electrophysiology. Before plating the cells, MEAs were coated with poly-L-lysine (2 mg/ml) and laminin (10 μg/ml; Sigma). Upon plating, neurons were kept *in vitro* for 13/17 days and their electrical activity was recorded using a pre-amplifier stage (MEA-1060-Inv-BC-Standard, gain: 55, bandwidth: 200 Hz–8 kHz, MCS GmbH), an amplification and filtering stage (FA64S, gain 20, bandwidth: 10 Hz–3 kHz, MCS GmbH) and a data acquisition device. Raw data were detected using a sampling frequency equal to 25 kHz. Then, an off-line signal processing was performed and raw data were analyzed by MC_Rack Software (MCS GmbH). Spike detection was calculated by fixing in each channel a threshold equal to 5-folds the standard deviation of the average noise amplitude recorded in the first 500 ms. The analysis was implemented in Matlab (The Mathworks, Natick) as previously described (Rossi et al., 2010). Briefly, for each MEA culture we extracted parameters describing the spiking activity, such as the median number of active channels firing with a mean frequency > 0.03 Hz (Eytan and Marom, 2006) and the total number of spikes (Chiappalone et al., 2006), burst and network burst activities. Bursts were included in our study if they displayed a minimum number of spikes equal to 10/burst and a maximum Inter Spike Interval (ISI) of 100 ms (Martinoia et al., 2005). Network bursts (NBs), which are recurrent events of synchronized firing occurring in different electrodes, were identified when the product

Please cite this article in press as: de Ceglia, R., et al. Down-sizing of neuronal network activity and density of presynaptic terminals by pathological acidosis are efficiently prevented by Diminazene Aceturate. Brain Behav. Immun. (2014), http://dx.doi.org/10.1016/j.bbi.2014.12.003