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Variants of the electrogenic sodium bicarbonate cotransporter 1 (NBCe1) in mouse hippocampal neurons are regulated by extracellular pH changes: Evidence for a Rab8a-dependent mechanism

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ABSTRACT

Changes in extracellular pH are common events in both pathological conditions and during normal brain function. In organs other than the brain, cells may respond to pH changes by trafficking of acid–base transporters. However, regulation of neuronal acid–base transporters during pH shifts is not understood. The aim of this study was to investigate regulatory mechanisms of the variants of the electrogenic sodium/bicarbonate cotransporter 1, NBCe1-A and NBCe1-B/C, in neurons following changes of extracellular pH. Therefore, primary mouse hippocampal neurons were exposed to extracellular acidosis or alkalosis. We show that acid–base changes regulated trafficking and membrane expression of neuronal NBCe1 but the underlying molecular cues were distinct for individual NBCe1 variants. Following extracellular acidosis NBCe1-A was recruited from intracellular pools to the plasma membrane, followed by increased membrane expression, whereas NBCe1-B/C was retrieved from the membrane. Extracellular alkalosis had no impact on NBCe1-A, but caused translocation of NBCe1-B/C toward the dendrites. We also show that acidosis-induced NBCe1-A, but not NBCe1-B/C, trafficking is mediated by Rab8a. Rab8a is expressed in hippocampal neurons, co-localizes, and interacts with NBCe1-A. Loss-of-function of Rab8a using specific siRNA prevented acidosis-induced redistribution of NBCe1-A.

These data propose opposite recruitment pattern for NBCe1 variants in neurons following extracellular acid–base changes, implicating distinct physiological functions of individual NBCe1 variants, and introduce Rab8a as a novel molecular determinant and crucial mediator of acidosis-induced NBCe1 trafficking in neurons.

1. Introduction

Changes of extracellular pH (pHo) are events associated with both physiological conditions and pathological states in brain function (Chesler, 2003). Physiological processes in the brain are however mostly associated by transient pHo changes, while loss of pH homeostasis can lead to severe pathological conditions. Neuronal excitation is thought to cause a transient extracellular alkalosis and therefore synchronized neuronal firing is accompanied by an initial extracellular alkalinization followed by a more prolonged acidification (Chesler and Kaila, 1992). On the other hand, excessive acidosis in the CNS can result in coma while alkalosis can provoke seizures. Whether extracellular pH changes can influence intracellular pH (pHi) depends on the effectiveness of the mechanisms of pHi regulation, among them the membrane transport of acid–base equivalents. *Vice versa*, acid–base transporters may affect neuronal excitability and synaptic transmission by regulating the local pH in the brain (Chesler, 2003).

Neural cells are equipped with several acid–base transporters, among them the electrogenic Na^+/HCO_3^- cotransporter NBCe1, the product of the *Slc4a4* gene. Three variants of NBCe1 have been identified: the NH_2-terminal variants NBCe1-A (Romero et al., 1998) and NBCe1-B (Abuladze et al., 1998; Thévenod et al., 1999), and the COOH-terminal variant NBCe1-C (Bevensee et al., 2000). The expression of NBCe1-C is almost restricted to the brain, whereas NBCe1-A and especially NBCe1-B exhibit a broader expression profile, including the brain (Majumdar and Bevensee, 2010; Rickmann

Abbreviations: NBCe1, electrogenic sodium bicarbonate co-transporter 1; Rab, ras-related in brain; pHi, intracellular pH; pHo, extracellular pH.

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et al., 2007). Thus, in neural tissue all three NBCe1 variants that are expressed exhibit distinct functional properties, reveal differential cellular distribution, and therefore presumably serve different physiological roles. We have previously shown that neurons preferentially express NBCe1-A (Rickmann et al., 2007) and to a lesser extend NBCe1-B, and in a recent study hippocampal neurons respond to depolarization-induced alkalinization, at least in part, by the activation of neuronal NBCe1 (Svichar et al., 2011). In astroglial cells, electrogenic Na^+/HCO_3^- cotransport is mediated by NBCe1-B and is activated through depolarization of astrocytes due to neuronal activity, thereby serving to overcome extracellular alkalosis and to regulate synaptic pH (Deitmer and Rose, 1996; Ransom, 1992). Indeed, at the molecular and functional level, studies on NBCe1 in brain have mainly focussed on NBCe1-B and astrocytes. However, it is well established that NBCe1-A and NBCe1-B play a crucial role in regulating intracellular pH in other organs, and both are regulated by changes of systemic acid–base homeostasis (Brandes et al., 2007).

Therefore, in the present study we sought to investigate regulation mechanisms of NBCe1-A and NBCe1-B in neurons following changes of pHo *in vitro* and get insights on the underlying molecular mechanisms.

2. Materials and methods

2.1. Antibodies and reagents/chemicals

Generation of antipeptide antibodies against the NH_2-terminal NBCe1 variants NBCe1-A and NBCe1-B has been described earlier (Roussa et al., 2004). The mouse polyclonal antibody against Rab8a was purchased from Abnova (Taipei City, Taiwan), the mouse monoclonal against Golgi58K was from Sigma (Taufkirchen, Germany), against GAPDH was from Abcam (Cambridge, UK), against synaptophysin was from DAKO (Hamburg, Germany), and against Na^+/K^+-ATPase was from Developmental Studies Hybridoma Bank (Iowa, USA). Rabbit polyclonal antibody against GFAP was obtained from DAKO, and against β-III-tubulin from Merck Millipore (Darmstadt, Germany). Donkey anti-rabbit IgG coupled to Alexa 568 was obtained from Life Technologies (Darmstadt, Germany), and goat anti-mouse IgG coupled to FITC or to AMCA were from Jackson (Immunoresearch, Newmarket, UK) and were used as secondary antibodies for immunocytochemistry and immunohistochemistry. Donkey anti-rabbit or goat anti-mouse IgG coupled to HRP were purchased from GE Healthcare (Munich, Germany) and were used as secondary antibodies for immunoblots.

2.2. RT-PCR

Total RNA was isolated from mouse primary hippocampal cultures using the Qiagen RNeasy kit (Hilden, Germany) according to the manufacturer's instructions, as previously described (Brandes et al., 2007). For PCR, the following primers were used: Rab8a (Genbank accession number: NM_023126.2): Forward: 5′-ACGCCTTCAACTCCACATTCA-3′ (nt 251–271) and reverse: 5′-TTGGACACCTGTCTCTTGTCA-3′ (nt 557–537). For amplification of the housekeeping gene GAPDH (Genbank accession number NM_008084.2) the following primers were used: 5′-CGGCCGCATCTTCTTGTG-3′ (nt 6–23) as forward primer and 5′-TGACCAGGCGCCCAATAC-3′ (nt 99–82) as reverse primer. For detection of the transcripts, the following protocol was used: denaturation at 95 °C for 5 min followed by 35 cycles of PCR amplification performed under following conditions: denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s, and elongation at 72 °C for 45 s. Final extension at 72 °C for 10 min was terminated by rapid cooling to 4 °C. PCR products were analyzed by agarose gel electrophoresis.

2.3. Real-time PCR

Isolation of total RNA from mouse E18.5 primary hippocampal cultures and subsequent quantitative real time PCR was performed as described earlier (Rickmann et al., 2007). Primers for NBCe1-A were: 5′-GATAAAGGATGTCCACTGAAA-3′ as forward primer and 5′-AAACCCTGAGGAAAGTGGAG-3′ as reverse primer (the amplified product corresponds to nucleotides 16–105; GenBank accession number: AF004017; Romero et al., 1998). Primers for NBCe1-B were (corresponding to nucleotides 62–114; GenBank accession number AF210250; Giffard et al., 2000): 5′-TGGAGGATGAAGCTGTCC-3′ as forward primer and 5′-ACACACATGTTTAAGGAAGGAA-3′ as reverse primer. Primers for Rab8a were (corresponding to nucleotides 71–184; GenBank accession number: NM_023126.2): 5′-CAATAGGGGGCGGGATCTG-3′ as forward primer and 5′-ACAGGTAATCGTAGGTCTTCGC-3′ as reverse primer and for GAPDH: 5′-CGGCCGCATCTTCTTGTG-3′ as forward primer and 5′-TGACCAGGAGCCCAATAC-3′ as reverse primer.

2.4. Immunoblotting

Cell monolayers from mouse primary hippocampal cultures were processed for immunoblotting as described earlier (Oehlke et al., 2011, 2012). Primary antibodies were diluted as follows: Rab8a 1:2000, Na^+/K^+-ATPase 1:2000, NBCe1-A and NBCe1-B 1:500, β-III tubulin 1:2000, and GAPDH 1:10,000. Blots were developed in enhanced chemiluminescence reagents and signals were visualized on X-ray film. Subsequently, films were scanned and the signal ratio NBCe1-A:β-III tubulin, NBCe1-A:Na^+/K^+-ATPase, and Rab8a:GAPDH, was quantified densitometrically. Differences in signal ratio were tested for significance using Student's *t*-test. Results with levels of $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ were considered significant.

2.5. Dissociated mouse hippocampal neuronal cultures

Primary mouse hippocampal cultures were performed as previously described (Rickmann et al., 2007). Hippocampi were dissected from brains of mouse embryos at embryonic day 18.5 (E18.5) and dissociated cells were plated onto poly-ornithin/laminin (Sigma) coated 12 mm^2 glass coverslips at a density of 50,000 cells/coverslip. At day *in vitro* (DIV) 12, culture medium (pH 7.4) was replaced by medium adjusted to 6.8 or 7.8 by keeping the P_{CO_2} constant at 5% CO_2 while lowering [HCO_3^-] from 22 mM to 7.1 mM, or increasing [HCO_3^-] to 59.5 mM $NaHCO_3$, respectively, for 6 h. Treated cells and controls were then harvested and processed for RT- PCR, immunoblotting, or immunocytochemistry.

2.6. Immunocytochemistry

Immunofluorescence of cultures has been performed as previously described (Brandes et al., 2007). Primary antibodies (NBCe1-A 1:400, NBCe1-B 1:50, Golgi58K 1:75; Rab8a 1:200, GFAP 1:500, synaptophysin 1:200, β-III tubulin 1:200) were diluted in PBS. Cells were incubated with secondary antibodies, nuclei were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) and viewed with a Zeiss Axioplan2 fluorescence microscope with ApoTome module (Jena, Germany).

2.7. Transient transfection of mouse primary hippocampal cultures

Mouse primary hippocampal neurons grown on cover slips were transiently transfected either with Alexa 488-labeled siRNA