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LOCALIZATION OF ELECTROGENIC Na/BICARBONATE COTRANSPORTER NBCe1 VARIANTS IN RAT BRAIN

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Abstract—The activity of HCO_3^- transporters contributes to the acid–base environment of the nervous system. In the present study, we used *in situ* hybridization, immunoblotting, immunohistochemistry, and immunogold electron microscopy to localize electrogenic Na/bicarbonate cotransporter NBCe1 splice variants (-A, -B, and -C) in rat brain. The *in situ* hybridization data are consistent with NBCe1-B and -C, but not -A, being the predominant NBCe1 variants in brain, particularly in the cerebellum, hippocampus, piriform cortex, and olfactory bulb. An antisense probe to the B and C variants strongly labeled granule neurons in the dentate gyrus of the hippocampus, and cells in the granule layer and Purkinje layer (e.g. Bergmann glia) of the cerebellum. Weaker labeling was observed in the pyramidal layer of the hippocampus and in astrocytes throughout the brain. Similar, but weaker labeling was obtained with an antisense probe to the A and B variants. In immunoblot studies, antibodies to the A and B variants (αA/B) and C variant (αC) labeled ~130-kDa proteins in various brain regions. From immunohistochemistry data, both αA/B and αC exhibited diffuse labeling throughout brain, but αA/B labeling was more intracellular and punctate. Based on co-localization studies with antibodies to neuronal or astrocytic markers, αA/B labeled neurons in the pyramidal layer and dentate gyrus of the hippocampus, as well as cortex. αC labeled glia surrounding neurons (and possibly neurons) in the neuropil of the Purkinje cell layer of the cerebellum, the pyramidal cell layer and dentate gyrus of the hippocampus, and the cortex. According to electron microscopy data from the cerebellum, αA/B primarily labeled neurons intracellularly and αC labeled astrocytes at the plasma membrane. In summary, the B and C variants are the predominant NBCe1 variants in rat brain and exhibit different localization profiles.

Key words: cerebellum, glia, hippocampus, neuron, pH, transporter.

It is well established that pH is an important cellular parameter because alterations in pH can affect a variety of cellular processes including the activity of enzymes and ion channels. In the nervous system, changes in pH can influence neuronal activity. In general, a decrease in extracellular pH (pH_o) inhibits neuronal activity, whereas an increase in pH stimulates activity (Chesler and Kaila, 1992; Ransom, 2000; McAlear and Bevensee, 2003; Chesler, 2003). Changes in pH are also seen with neuropathologic conditions. For example, decreases in brain pH are associated with ischemia, hypoxia, and epileptic events leading to neuronal necrosis and overall brain damage (Katsura and Siesjo, 1998).

The regulation of brain pH is complex because the movement of acid–base equivalents across the plasma membranes of neurons and glia (e.g. astrocytes and oligodendrocytes) changes both pH_o and intracellular pH (pH_i). Cells use a system of acid–base transporters in their plasma membranes to regulate pH_i, and subsequently pH_o. Some of the most powerful acid–base transporters in brain cells include HCO_3^--dependent ones, which are now classified as members of a bicarbonate transporter (BT) superfamily following their molecular identification over the past decade (Romero et al., 2004). The BT superfamily is composed of anion exchangers (AEs), electrogenic and electroneutral Na/bicarbonate cotransporters (NBCs), and Na-driven Cl-bicarbonate exchangers (NDCBEs).

The role of an electrogenic NBC in the nervous system has received considerable attention because the transporter couples changes in pH with neuronal activity (Chesler, 2003). In the nervous system, the activity of an electrogenic NBC was first characterized in invertebrate leech glial cells (Deitmer and Schlue, 1987, 1989; Deitmer, 1992), and subsequently identified in a number of mammalian astrocytes (see (Rose and Ransom, 1998; McAlear

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Abbreviations: BT, bicarbonate transporter; DIG, digoxigenin; ER, endoplasmic reticulum; HRP, horseradish peroxidase; K^+_o, extracellular K^+; MAP2, microtubule-associated protein; NBC, Na/bicarbonate cotransporter; NBCe1 (or 2), electrogenic Na/bicarbonate cotransporter 1 (or 2); NeuN, neuronal nuclei; OB, olfactory bulb; ORF, open reading frame; PBS, phosphate-buffered saline; PFA, paraformaldehyde; pH_i, intracellular pH; pH_o, extracellular pH; RT, room temperature; UAB, University of Alabama at Birmingham.

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and Bevensee, 2003)). In at least rat hippocampal astrocytes, the transporter has a stoichiometry of 1 Na^+:2 HCO_3^- (Bevensee et al., 1997a,b) and functions as an acid extruder. According to the model proposed by Chesler (1990) and Ransom (1992), astrocytes modulate neuronal excitability through NBC-mediated changes in pH_o. More specifically, an elevation in extracellular K^+ (K^+_o) during neuronal activity depolarizes astrocytes, thereby stimulating electrogenic NBC activity to transport Na^+, HCO_3^-, and net negative charge into the astrocytes. The ensuing decrease in pH_o will tend to dampen further neuronal activity. In support of this model, high K^+_o depolarizes astrocytes in the gliotic hippocampal slice and elicits a Na^+- and HCO_3^--dependent decrease in pH_o due to the activity of an NBC (Grichtchenko and Chesler, 1994a,b).

Our understanding of NBCs in the brain has advanced with the molecular identification of cDNAs encoding variants of electrogenic NBCs (NBCe1 and NBCe2) and electroneutral NBCs (NBCn1). NBCe1—the first NBC to be cloned (Romero et al., 1997)—has three splice variants: NBCe1-A, -B, and -C (Romero et al., 2004). In early cloning studies, the A variant was identified in rat and human kidney (Burnham et al., 1997; Romero et al., 1998), the B variant in human heart (Choi et al., 1999), human pancreas (Abuladze et al., 1998a), and rat brain (Bevensee et al., 2000), and the C variant in rat brain (Bevensee et al., 2000). These three variants are identical to one another except at the amino and/or carboxy termini. The amino-terminal 85 residues of the B and C variants replace 41 residues of the A variant, whereas the carboxy-terminal 61 residues of the C variant replace 46 residues of the A and B variants. This C variant—found predominantly in brain (Bevensee et al., 2000)—arises from a 97-base pair deletion near the 3′ open reading frame (ORF), thereby shifting the stop codon further 3′.

Shortly after NBCe1 was identified, two groups (Schmitt et al., 2000; Giffard et al., 2000) examined the localization of NBCe1 in rat brain primarily using polynucleotide probes and/or polyclonal antibodies that did not distinguish among the three splice variants. Somewhat expectedly, both groups found NBCe1 widely distributed throughout the brain and localized to glial cells. However, a surprise finding was the presence of NBCe1 in some neuronal populations, including granule cells of the hippocampus and neurons of the cortex (Schmitt et al., 2000).

Clearly, it would be informative to know which of the three NBCe1 variants are expressed in brain, and in what cell types. In the aforementioned Giffard et al. study on rat brain (Giffard et al., 2000), the authors noted that *in situ* hybridization with the nonspecific NBCe1 probe was mimicked by a more specific probe to the B variant (which should have also recognized the C variant), but not by a probe to the A variant, which displayed minimal labeling. Somewhat surprisingly, Rickmann et al. (2007) recently reported the expression of both NBCe1-A and NBCe1-B in mouse brain based on immunohistochemistry and immunoelectron microscopy. The authors found expression of the A variant throughout brain, particularly in neuronal subpopulations in the cerebellum, hippocampus, cerebral cortex, and olfactory bulb (OB). In contrast, the authors reported the expression of NBCe1-B in astroglia and Bergmann glia. However, it is important to point out that the NBCe1-B antibody is expected to recognize not only NBCe1-B, but also NBCe1-C, which is predominantly expressed in brain of at least rat. Therefore, the expression profiles of the B and C variants in brain have yet to be elucidated.

In the present study, we used mRNA and protein localization techniques to examine all three NBCe1 splice variants in rat brain. According to *in situ* hybridization data, NBCe1-A mRNA is minimal in brain, whereas NBCe1-B and possibly -C mRNAs are predominantly found in cerebellum (granule and Purkinje layers), hippocampus (dentate gyrus and pyramidal layers), piriform cortex, and OB. In immunoblot studies, we used previously characterized antibodies (Bevensee et al., 2000) that distinguish between the C terminus of either the C variant (αC) or the A/B variant (αA/B). Both antibodies recognized a ~130-kDa protein from all brain regions. Based on immunohistochemistry studies, αA/B displayed weak diffuse labeling throughout the brain and punctate labeling of pyramidal neurons in the hippocampus and neurons in the cortex. In contrast, αC displayed stronger diffuse labeling throughout the brain. αC appeared to label glia more so than the surrounding neurons in the cerebellum, hippocampus, and cortex. Immunoelectron microscopy data from the cerebellum support the plasma-membrane expression of NBCe1-C in astrocytes and intracellular expression of NBCe1-B in neurons. Portions of this work have been published in abstract form (Majumdar et al., 2006).

EXPERIMENTAL PROCEDURES

Animals

All animals were housed in approved animal care facilities at the University of Alabama at Birmingham (UAB). The animal facilities are under the direction of full-time veterinarians and are fully accredited by the American Association for Accreditation of Laboratory Animal Care, and meet all standards prescribed by the "Guide for the Care and Use of Laboratory Animals." All protocols involving animals were approved by the Institutional Animal Care and Use Committee at UAB. Efforts were made to minimize the required number of animals and their suffering in this study.

In situ hybridization

Designing NBC probes. Sense and antisense probes were created by PCR using primers that flanked the following regions: *i*) the unique 5′ ORF encoding the unique amino-terminal 41 residues of the A variant, *ii*) the different 5′ ORF encoding the different amino-terminal 85 residues of the B and C variants, and *iii*) the 97-bp region near the 3′ end of the ORF found exclusively in the A and B variants (Fig. 1). PCR products were purified and directionally subcloned into PCRII TOPO® vector (Invitrogen, Inc., Carlsbad, CA, USA). cRNA probes were generated by transcription using the T7 or SP6 promoter.

Performing in situ hybridization. Non-radioactive *in situ* hybridization was performed as previously described using digoxigenin (DIG) -labeled cRNA probes (Berger and Hediger, 2001). Frozen rat brain or kidney sections (10 μm), were captured onto Superfrost plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were fixed, acetylated, and incubated in the