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ENaC γ-expressing astrocytes in the circumventricular organs, white matter, and ventral medullary surface: Sites for Na^+ regulation by glial cells

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ABSTRACT

Using a double immunofluorescence procedure, we report the discovery of a novel group of fibrous astrocytes that co-express epithelial sodium channel (ENaC) γ-subunit protein along with glial acidic fibrillary protein (GFAP). These cells are concentrated along the borders of the sensory circumventricular organs (CVOs), embedded in the white matter (*e.g.*, optic nerve/chiasm, anterior commissure, corpus callosum, pyramidal tract) and are components of the pia mater. In the CVOs, a compact collection of ENaC γ-immunoreactive glial fibers form the lamina terminalis immediately rostral to the organum vasculosum of the lamina terminalis (OVLT). Astrocyte processes can be traced into the median preoptic nucleus – a region implicated in regulation of sodium homeostasis. In the subfornical organ (SFO), ENaC γ-GFAP astrocytes lie in its lateral border, but not in the ventromedial core. In the area postrema (AP), a dense ENaC γ-GFAP glial fibers form the interface between the AP and nucleus tractus solitarius; this area is termed the subpostremal region. Antibodies against the ENaC α- or β-subunit proteins do not immunostain these regions. In contrast, the antibodies against the ENaC γ-subunit protein react weakly with neuronal cell bodies in the CVOs. Besides affecting glial-neural functions in the CVOs, the astrocytes found in the white matter may affect saltatory nerve conduction, serving as a sodium buffer. The ENaC γ-expressing astrocytes of the ventral medulla send processes into the raphe pallidus which intermingle with the serotoninergic (5-HT) neurons found in this region as well as with the other nearby 5-HT neurons distributed along ventral medullary surface.

1. Introduction

Astrocytes play a critical role in brain function by providing a functional interface between neurons and capillaries (Gourine and Kasparov, 2011), and they modulate numerous brain systems *via* what has been termed 'glial-neuronal vascular units' (Kirischuk et al., 2012). These units regulate the ionic and metabolic conditions of the brain environment (Magistretti, 2006), and this is accomplished by the release of a host of chemicals from astrocytes which include ATP/adenosine, glutamate, D-serine, and others which are now cataloged as 'gliotransmitters' (Haydon and Carmignoto, 2006).

From a neural systems perspective, the most compelling data suggesting that gliotransmitters modulate CNS functions has been the demonstration that astrocytes found on the ventral surface of the medulla oblongata function as central chemoreceptors. Small changes in the partial pressure of CO_2 or pH trigger the release ATP from these electrically nonexcitable cells which activates the local central chemorespiratory neurons of the ventral medulla, and in

Abbreviations: III, third ventricle; IV, fourth ventricle; XII, hypoglossal nucleus; AP, area postrema; CC, central canal; com, commissural subnucleus of the NTS; CVO, circumventricular organ; DMX, dorsal motor nucleus of vagus nerve; ENaC, epithelial sodium channel; GFAP, glial fibrillary acidic protein; Gr, gracile nucleus; is, interstitial subnucleus of the NTS; LT, lamina terminalis; med, medial subnucleus of the NTS; MnPO, median preoptic nucleus; NDB, nucleus of the diagonal band; NeuN, neuronal nuclear marker; NTS, nucleus tractus solitarius; or, optic recess; NA, nucleus ambiguus; OVLT, organum vasculosum of the lamina terminalis; OX, optic chiasm; Pe, periventricular hypothalamus; Psol, parasolitary subnucleus of the NTS; py, pyramidal tract; ROb, raphe obscurus nucleus; RPa, raphe pallidus nucleus; RVLM, rostral ventrolateral medulla; SFO, subfornical organ; SpV, Spinal trigeminal nucleus; SubP, subpostremal region of the NTS; t, solitary tract; v, ventral subnucleus of the NTS; VHC, ventral hippocampal commissure; vl, Ventrolateral subnucleus of the NTS; VMS, ventral medullary surface.

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turn, these neurons induce changes in the central respiratory network that affects rate and volume of breathing (Gourine et al., 2010).

Astrocyte excitability is largely dependent on increases in internal cytosolic concentration of calcium, but often overlooked is the fact that increases in the cytosolic Na^+ concentration – $[Na^+]_i$ is also an important factor that affects the regulation of synaptic transmission (Kirischuk et al., 2012). While much of the research on astroglia has focused on Ca^{2+} metabolism, there is a substantial literature supporting the idea that sodium channels and its related transporters play an important function of astrocytes (Kirischuk et al., 2012). In fact, one of the early discoveries in this area demonstrated the Müller glial cells of the retina express epithelial sodium channels (ENaCs) (Brockway et al., 2002). Thus, these workers provided the first anatomical evidence that astrocytes express the ENaC α-subunit, and also found that the inward Na^+ current recorded from these cells could be blocked by amiloride.

During a study of the patterns of c-Fos activation of ENaC-expressing neurons in the sensory circumventricular organs (CVOs) (Miller et al., 2013), we observed a unique group of astrocytes that lie in the border zones of the CVOs. These CVO areas were intensely immunostained by antibodies directed against the ENaC α-subunit antibody protein. In addition, the ENaC-expressing astrocytes of the pia mater were also strongly immunostained as well. The latter group of astrocytes sent fibrous processes into regions of the brainstem implicated in cardiovascular and respiratory functions. The present study describes the location of these ENaC γ-subunit expressing astrocytes and briefly discusses their potential role in Na^+ functions that occur in the brain.

ENaCs (Scnn1) are amiloride-sensitive, non-voltage dependent sodium channels that conduct Na^+ across the apical membrane of cells in salt-reabsorbing epithelia, such as in the distal nephron, airways, and distal colon. To date, the bulk of the research done on ENaCs has focused on the kidney and airways (Kashlan and Kleyman, 2012), but ENaCs are also present in the brain (Waldmann et al., 1995; Amin et al., 2005; Giraldez et al., 2007; Teruyama et al., 2012; Miller et al., 2013). ENaCs are expressed in astrocytes, ependymal cells of the choroid plexus, endothelial cells, and neurons in the brain (Amin et al., 2005), and due to this widespread expression, it is likely that these channels may affect a range of functions. Here, we describe a novel group of ENaC γ-subunit expressing astrocytes that have a highly specific distribution in the brain.

2. Experimental procedures

2.1. Animals and surgical procedure

The animal experiments described here were reviewed and approved by the Washington University School of Medicine Institutional Animal Care and Use Committee and followed NIH guidelines. Adult Sprague-Dawley rats (wt = 250–300 g, male and female, Charles River Laboratories, Wilmington, MA, USA) were provided with free access to tap water and standard rat chow (Pico Lab rodent #20, containing 0.33% sodium; Lab-Diet, Richmond, IN). They were housed in a room with an automated lighting system: 12/12 h light-dark schedule (lights on at 5:30 AM; lights off at 5:30 PM) and with a controlled temperature of 23 ◦C.

Between 7 and 10 AM, the rats were anesthetized with 3.5% chloral hydrate (1 ml/100 g body weight; intraperitoneal injection; Sigma, St. Louis, MO), and perfused through the heart with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH = 7.4). The brains were removed and stored in fixative for 3 days–2 weeks. The brains from these animals were used for immunohistochemistry. Sections were cut in the transverse or sagittal plane at 50 μm on a freezing microtome.

2.2. Immunohistochemisty

Free floating sections were processed by the indirect ABC immunofluorescence procedure given below. All solutions were made in a 5% donkey serum in a 0.1 M sodium phosphate buffer (pH = 7.4) containing 0.01% sodium azide. The detergent Triton-X 100 was omitted to prevent the solubilization of ENaCs. Histochemical reactions were carried out on a rotary shaker at room temperature. The immunoreactive reaction products were colorized with diaminobenzidine (Sigma) or Cy2 or Cy3 streptavidin (Jackson ImmunoReserach, West Grove, PA).

2.3. Primary antibodies

Antibodies to the rat ENaC-α subunit, ENaC-β subunit, and ENaC-γ subunit were purchased from StressMarq (Victoria, BC, Canada) and used to immunostain rat brain sections through the AP, SFO, and OVLT. These antibodies were originally made by Dr. Mark Knepper (NIH); they are highly specific as demonstrated in Western blots which show single bands between 75 and 82 kDa. These bands were blocked when the respective antibody was preadsorbed with the immunizing peptide (Masilamani et al., 1999; Hager et al., 2001). The same antibodies were used in our recent study on systemic sodium induced changes in the CVOs (Miller et al., 2013)

The ENaC α-subunit (Scnn1a) antibody (1:1000; SPC-403D) was produced against a synthetic peptide from the N-terminus of the ENaC α-subunit (amino acids 46–68, NP_113736; # 3560-2). Immunostaining of ENaC α-positive cell groups in the brainstem (*viz.*, hypoglossal and dorsal motor nuclei) was blocked when this antibody was preadsorbed with the ENaC α-subunit peptide with the following sequence: LGKGDKREEQGLGPEPSAPRQPTC-COOH (500 μg/ml; Thermo Fisher Scientific, Rockford, IL). The ENaC β-subunit antibody (1:250; SPC-404D) was produced against the C-terminal tail of rat ENaC β-subunit (amino acids 617–638; # 3755-2). Similarly, immunostaining of brainstem motor nucleus was blocked when this antibody was preadsorbed with the ENaC β-subunit peptide CNYDSLRLQPLDTMESDSEVEAI-COOH (500 μg/ml; Thermo Fisher Scientific, Rockford, IL). The ENaC γ-subunit antibody (1:750; SPC-405D) was produced against the C-terminal tail of rat ENaC γ-subunit (AA629-650; # L550). Immunostaining in the subpostremal region of the NTS was blocked when this antibody was preadsorbed with the ENaC γ-subunit peptide CNTLRLDRAFSSQLTDTQLTNEL-COOH (500 μg/ml); Thermo Fisher Scientific, Rockford, IL. No immunostaining was present in brainstem tissues when the primary antibodies had been omitted from this immunohistochemical staining procedure.

Three mouse monoclonal antibodies were used. One was made against glial fibrillary acidic protein monoclonal (1:1000; GFAP; MU020-UC; clone GA-5....Fremont, CA) the second was directed against tryptophan hydroxylase – the enzyme involved in the synthesis of serotonin (1:4000; MAB 5278, Millipore), and the third was directed against NeuN which is a generic marker for neurons (1:500; MAB377; Millipore). The specificity of all three of these antibodies has been described in previous studies that are listed in the Journal of Comparative Neurology database: http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291096-9861/homepage/jcn_antibody_database.htm.

2.4. Immunohistochemical staining procedures

2.4.1. DAB procedure

Free-floating sections were incubated overnight in rabbit antibodies that were made against either to ENaC α-subunit, ENaC β-subunit, or ENaC γ-subunit at concentrations described above. The sections were reacted at room temperature and gently agitated on a rotary shaker. All solutions were made up in 5% donkey serum in 0.1 M sodium phosphate (pH = 7.4), washed in potassium phosphate buffered saline (KPBS; 0.01 M, pH = 7.4). The sections were transferred to a biotinylated donkey anti-rabbit (1:250; Jackson ImmunoResearch, West Grove, PA) solution for 2.5 h, washed in KPBS, treated for 1 h in the avidin–biotin complex (ABC, Vectastain kit, Vector Labs, Burlingame, CA) solution for 2 h, washed in KPBS, and colorized in diaminobenzidine (DAB) solution (D-4418, Sigma, St. Louis). A single DAB tablet was dissolved in 20–25 ml of distilled water containing one tablet of urea. The sections were reacted for 15 min in this solution, washed three times in KPBS, mounted on gelatinized slides, and air dried. The sections were dehydrated and coverslipped directly without any counterstaining. Control sections were also reacted without primary antibodies and this resulted in no staining in the brain tissues.

2.4.2. Double indirect immunofluorescence procedure

Additional sections were immunostained by a double indirect immunofluorescence procedure. Free-floating sections were incubated in the ENaC γ-subunit antibody overnight, washed in KPBS, transferred to a solution of biotinylated donkey anti-rabbit (1:250; Jackson) for 2.5 h, washed in KPBS, transferred to the ABC complex (Vectastain kit, Vector Labs, Burlingame, CA) for 2 h, washed in KPBS, and transferred to Cy3-streptavidin (1:250; Jackson) for 3 h. The sections were transferred to a solution of mouse monoclonal antibodies against either GFAP, tryptophan hydroxylase, or NeuN (see above) overnight. The following morning, the sections were washed in KBPS buffer and transferred to a solution of biotinylated donkey anti-mouse (1:250; Jackson) for 2.5 h, washed in KPBS, transferred to the ABC complex (Vectastain kit, Vector Labs, Burlingame, CA) for 2 h, washed in KPBS, and transferred to Cy2-streptavidin (1:250; Jackson) for 3 h. The sections were washed in buffer, and then mounted on gelatin-coated glass slides. After drying, slides were coverslipped using a fade-retardant glycerol mounting solution containing sodium azide and n-propyl gallate, and secured around the edges with fingernail polish.

2.5. Digital images

Brightfield images were taken on a Nikon microscope using a CCD camera with Nikon ACT-1 software (v2.62). Image cropping, resizing and adjustments in