Distribution of epithelial sodium channels and mineralocorticoid receptors in cardiovascular regulatory centers in rat brain

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Epithelial sodium channels (ENaC) are an important family of voltage-insensitive, amiloride-blockable cation channels. They are generally composed of three subunits (α, β, γ) of ~650–700 amino acids in length. ENaC are present in, e.g., the kidney, colon, lungs, and sweat and salivary gland ducts where expression at the apical membrane mediates unidirectional absorption of Na⁺ (11, 15, 35). In tongue fungiform papillae (28) and baroreceptor mechanotransducer of the aortic arch (10), ENaC contribute to the electrogenic movement of Na⁺. Expression of the subunits was also reported in the retina (3, 12), cochlea (6, 46), sensory nerve endings in the rat foot pad (8), as well as trigeminal mechanosensory neurons (14). Their function in these tissues has not yet been clarified. Other members of the degenerin/ENaC superfamily such as brain Na⁺ channels and acid sensing ion channels are highly expressed in the central nervous system (2, 27). So far, ENaC expression has not been demonstrated in the rat brain. A variety of functional studies suggest the presence of specific Na⁺ channels, presumably ENaC, in the brain that are activated by aldosterone or high-salt diet and blocked by amiloride or benzamil. In Wistar rats, intracerebroventricular infusion of aldosterone or Na⁺-rich artificial cerebrospinal fluid (CSF) increases blood pressure and renal sympathetic nerve activity (41, 42). In Dahl S (salt sensitive) but not R (salt resistant) rats, high-salt diet or intracerebroventricular infusion of aldosterone causes sympathetic excitation and hypertension. The blood-brain barrier in Dahl S rats is five to eight times more permeable to Na⁺ than that in Dahl R rats (40). Increases in CSF Na⁺ are observed in Dahl S but not Dahl R rats on high-salt diet and precede changes in blood pressure by 1–2 days (24). Importantly, the responses to aldosterone or Na⁺-rich artificial CSF in Wistar rats and to aldosterone or high-salt diet in Dahl S rats can all be prevented by intracerebroventricular infusion of benzamil or spironolactone (41–43). These findings suggest that mineralocorticoid receptor (MR)-mediated activation of sodium channels in the brain is responsible for mechanisms leading to increased sympathetic outflow and hypertension.

MR expression is well characterized in the brain (1, 21, 38). In contrast, ENaC expression in the brain has not yet been demonstrated. The objectives of the present studies were, therefore, 1) to evaluate expression of ENaC subunits and their relative abundance in different brain areas and nuclei and 2) to assess whether MR are present in the same areas as ENaC.

MATERIALS AND METHODS

Adult male Wistar rats (Charles River, Montreal, PQ, Canada) were housed under standard conditions (12-h light cycle, ambient temperature 23 ± 2°C) and received standard laboratory chow and tap water ad libitum for 5 days before entering the study. All procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care, which conform to National Institutes of Health guidelines and were approved by the University of Ottawa Animal Care Committee.

RT-PCR

RNA isolation and RT-PCR. Brains and kidneys from six rats were rapidly removed and quickly frozen in liquid nitrogen and stored at −80°C until use. Whole kidney and hypothalamus were homogenized in Trizol reagent (Invitrogen, Burlington, ON, Canada) using a polytron. Total RNA was isolated from tissues according to the manufacturer’s instructions. In an additional experiment, six rats were perfused with chilled diethyl pyrocarbonate-treated PBS (pH 7.4) under

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pentobarbital anesthesia. The brains were removed, immediately frozen in isopentane, placed on dry ice, and then stored at −80°C until use. Serial 80-μm-thick coronal slices were cryosectioned, and the brain punches of specific areas were taken with prechilled, 25-μl Drummond microdispensers (Drummond Scientific). The tissue pellet was homogenized in 0.2-ml Trizol reagent by using a pestle (Bel-Art-Products, Pequannock, NJ) driven by a pestle pestle motor, and 0.3 ml Trizol reagent was then added. To eliminate potential genomic DNA contamination, 20 μg of total RNA from the kidney, hypothalamus, or total RNA from the different punched brain areas were treated with DNase I (Ambion, Austin, TX) before the RT reaction.

cDNAs were synthesized by incubation with 200 U Superscript II RNase H− reverse transcriptase (Invitrogen) at 42°C for 50 min. Two microliters of the above RT reaction were subjected to PCR as follows: an initial denaturation step at 94°C for 2 min, then 40 cycles of 94°C for 50 s, 57°C for 45 s, and 72°C for 1 min. Specific primers for α-, β-, and γ-ENaC subunits, MR, and the housekeeping gene phosphoglycerate kinase (PGK) (Table 1) were designed based on published sequence information (GenBank accession nos. X70497, X77932, X77933, M36074, and NM053291, respectively). All PCR products of the ENaC subunits (α, β, and γ) were sequenced and analyzed for homology with the published rat kidney ENaC sequences.

**Real-time RT-PCR.** Real-time PCR amplifications were performed with a Roche light cyclser using fast-start DNA Master SYBR Green I (Roche Diagnostics). Two microliters of 1:10 diluted RT product from kidney or hypothalamus or undiluted RT product of different brain areas were used for the PCR reaction. The primers for α-, β-, and γ-ENaC, MR, and PGK were the same as the primers used in conventional RT-PCR. The real-time PCR conditions were as follows: 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 5 s, annealing the primers to the target for 5 s at 65°C for the α- and β-subunit and at 62°C for the γ-subunit, MR, and PGK. An extension step was performed at 72°C, and the extension time was determined by the formula of amplicon length/25 s. The specificity of real-time PCR products was documented with a melting curve analysis. In addition, a high-resolution gel electrophoresis was performed, which resulted in the amplification of a single product of the appropriate size (Table 1). Real-time RT-PCR analysis was performed in duplicate. Plasmids containing the cDNA of α-, β-, and γ-subunits, MR, and PGK were used to generate standard curves. All of the construct concentrations were quantified by absorbance at 260 nm. Serial 10-fold dilution (e.g., 100, 10, 1, 0.1, 0.01, 0.001 pg, etc.) of each subunit of ENaC, MR, and PGK plasmid clones were used to generate an external standard individually. Expression was normalized to PGK levels as an endogenous reference. Normalization was achieved by dividing the amount of cDNA of each ENaC subunit or MR by the PGK quantity. Real-time PCR efficiencies were calculated according to the equation $E = 10^{-1/slope}$, where $α = 1.98$, $β = 1.91$, $γ = 1.93$, $MR = 1.86$, and $PGK = 1.94$.

**Immunohistochemistry.** Under pentobarbital anesthesia, rats were perfused transcardially with chilled normal saline followed by 4% paraformaldehyde and 0.1% glutaraldehyde in PBS (pH 7.4). The brains were removed and kept in the perfusion solution for 5–6 h followed by cryoprotection with several changes of 20% sucrose in PBS over the next 48 h. Then the brains were rapidly frozen to −30°C in precooled isopentane and stored at −80°C until use.

Five-micrometer coronal sections of brain were cut in a rostral-to-caudal direction in a cryostat (−30°C) and thaw mounted on super-frost plus slides (VWR Scientific, West Chester, PA). Precautions were taken to maintain similar coronal planes while cutting the brain sections. Five to seven consecutive sections were collected on different slides in the hypothalamic areas (main area of interest) before proceeding to the next, which would be −50–70 μm. A distance of about 100 μm between two successive sections so that the same cells were not included in both. In more rostral or caudal areas of the brain, the distance between two sets of collections was −300–500 μm. Slides were then dried and stored in freezer at −20°C until further processing.

Just before staining, the slides were placed in the same fixative solution for 5–10 min followed by several washes in PBS. To quench endogenous peroxidase the slides were treated with 1% sodium borohydride (Sigma-Aldrich, Oakville, ON, Canada) in PBS for 30 min and then with 1% H2O2 in methanol for another 30 min. After several washes in PBS, the slides were incubated with blocking solution (1.5% normal sera from the secondary antibody species and 1% bovine serum albumin in PBS) for 2 h at room temperature. The sections were then incubated with the respective primary antibodies diluted appropriately in blocking solution (MR 2 μg/ml; α-ENaC 0.4 μg/ml; β-ENaC 0.5 μg/ml; γ-ENaC 0.7 μg/ml). The antibodies against α-, β-, and γ-ENaC (LL766AP, LL558AP, LL550AP, respectively) were a kind gift from Dr. Mark A. Knepper (National Institutes of Health, Bethesda, MD) and had been raised against synthetic peptides corresponding to predicted amino acid sequences in the rat (4, 5): α-ENaC (amino acids 46–68) NH2-LGKGDKREEQGLG-PEPSAPRPQPTC-COOH, β-ENaC (amino acids 617–638) NH2-CNYSDLRQLPDSLMDSEVEAL-COOH, and γ-ENaC (amino acids 629–650) NH2-CNTLRLDRAFSQILTDQTNLN-COOH. These antibodies detect specific bands at 85–90 kDa in membrane fractions of rat renal cortex (30). Use of different antibodies for α-ENaC (Chemicon, Temecula, CA) and β-ENaC (Alpha Diagnostic International, San Antonio, TX) showed a similar distribution in the brain. Antibodies for MR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and were directed against a 17-amino acid sequence at the NH2-terminus of rat MR (MCR N-17) or a 19-amino acid sequence at the COOH-terminus (MCR C-19). In preliminary studies, no significant differences in immunopositive signal distribu-

### Table 1. Primer sequences used to detect α-, β-, and γ-ENaC and MR mRNA transcript

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Amplicon Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ENaC</td>
<td>Forward 5'-GTCTTGTTGACATCCAGAAAAGGAG-3'</td>
<td>429 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GTTAGACAGCAGTAAAGTGTGATG-3'</td>
<td></td>
</tr>
<tr>
<td>β-ENaC</td>
<td>Forward 5'-ACCTCACACCTTTCTCAGAT-3'</td>
<td>220 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ACAGGAGGCACTAGCTTTGTA-3'</td>
<td></td>
</tr>
<tr>
<td>γ-ENaC</td>
<td>Forward 5'-GCAAGCAGCTCTCCGACACCCTG-3'</td>
<td>301 bp</td>
</tr>
<tr>
<td>MR</td>
<td>Forward 5'-GTCGCAACATATTGTCAGTCA-3'</td>
<td>260 bp</td>
</tr>
<tr>
<td>PGK</td>
<td>Forward 5'-GCTGCAAGAATGCGATCTC-3'</td>
<td>263 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCTGCAAGAATGCGATCTC-3'</td>
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</tr>
</tbody>
</table>

ENaC, epithelial Na channel; MR, mineralocorticoid receptors; PGK, phosphoglycerate kinase.
tion in the areas of interest were observed between the two antibodies, and experiments were continued with MCR N-17.

After overnight incubation with the primary antibody, the slides were processed using the Vectastain Elite ABC kit (Vector Laboratories) for rabbit (ENaC) or goat (MR) IgG or the ABC staining system for goat primary antibody (Santa Cruz Biotechnology) for MR. Briefly, these consisted of incubating with the biotinylated secondary antibody for 45 min, several washes in PBS, and incubation with biotinylated horseradish peroxidase for another 45 min. The antigens were visualized using either the Vector Nova red (red color) or Vector diaminobenzidine kit (black color). Some slides were counterstained with Vector hematoxylin, and all dehydrated in graded alcohol, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Ottawa, Ontario, Canada).

Several control experiments were performed to rule out nonspecific staining. For MR, the positive controls were paraffin-embedded kidney or heart sections of rats perfused with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.3), whereas for ENaC these were the frozen sections of kidney and colon from rats perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in cacodylate buffer. Prolonged freezing adversely affected immunoreactivity to the MR antibodies. Omission of the primary antibodies or incubation with the nonimmune IgG from goat (MR) or rabbit (ENaC), with all other steps left identical, were used as negative control in all cases. In addition, some sections were incubated with the primary antibody preadsorbed with excess of the immunizing peptide (MCR N-17). The peptide control experiments for the ENaC antibodies could not be performed due to lack of the specific immunizing peptides.

Double staining. Adjacent sections were studied to determine whether the ENaC or MR immunostaining was in neurons or glia and whether MR and ENaC were expressed in the same cells. Rabbit polyclonal antibodies to neuron-specific Enolase (Chemicon) or neuron-specific nuclear protein (Neu N, Alexa 488 conjugated, Chemicon) were used as neuronal markers and to glial fibrillary acidic protein (GFAP, Sigma) were used as glial cell marker. For double staining, slides were first incubated with anti-α- or β-ENaC primary antibody followed by Alexa red-conjugated goat anti rabbit secondary antibody (Vector Laboratories) and then with Alexa 488-conjugated Neu N antibody.

Microscopy and morphological analysis. The slides were studied with a high-resolution brightfield transmitted light microscope ( Olympus BX60 with 2×, 20×, and 40× objectives), and images were captured using Spot digital camera and Spot software. For fluorescent microscopy, the same microscope was used with appropriate emission spectra and filters. Complete labeling in immunoperoxidase staining was confirmed by the presence of uniform Nova Red or diaminobenzidine label intensity. All major nuclei and structures could readily be identified by morphological criteria based on labeling pattern and facilitated by the thinness of the sections. Neuroanatomical localization was confirmed by hematoxylin- or Nissl-counterstained sections and a rat brain atlas (33). Staining intensity was not assessed in the present study. Relative density of cellular staining was rated as “none” (−) if no staining was observed, “low” (+) if < 30% of cells in an area were stained, “moderate” (+++) if 30–70% of cells in an area were stained, and “high” (++++) if > 70% of cells in an area were stained.

RESULTS

Transcripts Encoding ENaC and MR in Brain

Figure 1 shows various lengths of real-time PCR products (α = 429 bp; β = 220 bp; γ = 301 bp), which were amplified in different brain areas [organum vasculosum of lamina terminalis (OVLT), subfornical organ (SFO), supraoptic nucleus (SON), paraventricular nucleus (PVN), median preoptic nucleus (MnPO), choroid plexus, and hippocampus], were of comparable size to the PCR amplified transcripts of each subunit predicted from the kidney. There was no amplification when reverse transcriptase was absent. The putative ENaC subunit transcripts were sequenced by PCR, and the DNA sequences of α-, β-, and γ-ENaC from Wistar rat brain were identical to the published ENaC subunit nucleotide sequences (data not shown).

Figure 2 shows the relative abundance of α-, β-, and γ-ENaC mRNA in the whole kidney, hypothalamus, and specific brain areas. In the kidney, mRNA levels of the α-ENaC subunit were ~6-fold higher than β and ~10-fold higher than γ, and in the hypothalamus ~30-fold higher than β and ~60-fold higher than γ. However, mRNA levels of the ENaC subunits in specific brain areas exhibited different patterns from those in the whole hypothalamus. For α, mRNA levels were similar to or higher than in the whole hypothalamus, particularly in the OVLT, SFO, and PVN. For β, levels in the OVLT, SON, and hippocampus were lower than in the whole hypothalamus, but mRNA levels in MnPO and choroid plexus were much higher. For γ, mRNA levels in most brain areas were higher, and in the SFO and MnPO were up to ~40-fold higher than in the whole hypothalamus (Fig. 2).

Figure 3 shows the relative MR mRNA levels in the whole kidney, hypothalamus, and different brain areas. The mRNA...
levels were approximately twofold less in the whole hypothalamus than in the kidney. Most of the examined brain areas showed similar mRNA levels of MR to the whole hypothalamus except the SFO and hippocampus. In the hippocampus, mRNA levels of MR were 3.5-fold higher than in the whole hypothalamus and 1.5-fold than in the kidney. mRNA levels in the SFO were approximately twofold higher than in the hypothalamus and similar to levels in the kidney.

**Distribution of α-, β-, and γ-ENaC**

In rat kidney, staining for all ENaC subunits (Fig. 4, A–C) was prominent in the distal tubules and collecting ducts in cortex and outer medulla, with no labeling in the glomeruli. The expression was diffusely cytoplasmic. No labeling was seen with omission of the primary antibodies (Fig. 4E). In the colon, all three subunits showed prominent immunoreactivity along the brush border of the enterocytes (arrows in Fig. 4, F–H). Distribution in kidney and colon was consistent with previous studies (11, 20, 35). Immunohistochemistry of frozen coronal sections of rat brain demonstrated specific immunoreactivity with negligible background to all three ENaC subunits. Omission of the primary antibodies abolished the entire specific staining (see Fig. 7, A and F). Incubation with normal IgG did show high-background, nonspecific nuclear staining in some areas, but these were clearly different from the staining obtained with the specific antibodies. Distinct morphological appearance and double staining with Neu N or GFAP demonstrated that ENaC immunostaining in most parts of the brain was neuronal (Fig. 5) except for the epithelia of the choroid plexus, ventricular ependyma, and blood vessels. Some Neu N- and GFAP-negative cells were noted that were α- and β-ENaC positive (arrows in Fig. 5, C and F). These included the magnocellular cells of the SON and PVN and other dispersed groups of cells.

In the brain, both α- and β-ENaC showed similar widespread distribution. However, γ-ENaC signal was weak in most parts. The weak γ-signal was not due to antibody concentrations, since similar concentrations in other tissues resulted in prominent immunostaining and changing the antibody concentrations in the brain did not affect regional distribution. The clear nuclear halos in magnified views of most cells suggest a cytoplasmic and/or membranous distribution of ENaC subunits (insets in figures). Nuclear immunoreactivity was noted in some cells consistent with previous studies (3).

Table 2 shows relative distribution of the ENaC subunits and MR in the areas of the brain that were examined. In the cerebral cortex (Fig. 6A), β-ENaC immunopositive neurons were noted in all parts of the cerebral cortex. γ-ENaC
immunopositivity (Fig. 6C) was restricted to parts of the cingular and piriform cortex. Immunoreactivity was usually restricted to the layers II to VI in the soma of the pyramidal neurons and some of their processes.

Hippocampus. In the hippocampus, immunoreactivity to \( \alpha \)-ENaC (Fig. 6E) and \( \beta \)-ENaC (Fig. 6F) and weaker immunoreactivity to \( \gamma \)-ENaC (Fig. 6G) was noted in the pyramidal layers of all the cornu ammonis subfields and the dentate gyrus. Immunoreactivity was usually limited to the soma and some proximal processes of the neurons.

Hypothalamus. Immunoreactivity to all three ENaC subunits was present in the MnPO (Fig. 6, I–K), SON (Fig. 7, B–D), and magnocellular PVN (Fig. 7, L–N). Studies of adjacent sections suggested expression in the same cells. Weaker expression of \( \alpha \)- and \( \beta \)-ENaC and undetectable expression of \( \gamma \)-ENaC was noted in the parvocellular PVN. Other parts of the hypothalamus, such as the suprachiasmatic nucleus, periventricular nucleus (Fig. 7, G–I), preoptic areas, and anterior and posterior hypothalamic areas, showed specific staining only for \( \alpha \)- and \( \beta \)-ENaC.

Other areas. Prominent immunoreactivity to \( \alpha \)- and \( \beta \)-ENaC was noted in the amygdala (Figs. 5, D–F, and 8, A and B), in the nucleus of tractus solitarius (Fig. 8C), and in the circumventricular organs such as the SFO (Fig. 8, E and F) and OVLT (Fig. 8G). \( \gamma \)-Immunoreactivity in these areas was low or undetectable.

Brain epithelial components and blood vessels. The cuboidal epithelium of the choroid plexus and the ventricular ependyma expressed all three ENaC subunits (Fig. 8, I–K). Notable was expression of all three subunits along the ependyma on a single layer of cells lining the cavity (Figs. 7, G–I, and 8, I–K) and only \( \beta \)-ENaC in the subependymal zone. The pia-arachnoid membrane surrounding the brain also showed very dense ENaC staining. ENaC immunopositivity was also present in the endothelia and smooth muscle cells of blood vessels (Fig. 8, M–O).

Colocalization of \( \alpha \) and \( \beta \) ENaC with NeuN

Fig. 5. Colocalization of \( \alpha \)- and \( \beta \)-ENaC with Neu N: double staining with anti-\( \alpha \)- or -\( \beta \)-ENaC and Neu N demonstrates neuronal expression of ENaC. \( \alpha \)-ENaC (A; red) and Neu N (B; green) in cerebral cortex. C: overlay by exciting both dyes (Texas Red and Alexa Fluor 488) demonstrates double staining (light green to yellow). Scale bar = 37 \( \mu \)m. \( \beta \)-ENaC (D; red) and Neu N (E; green) in the amygdala. F: overlay of D and E showing double staining (yellow). Scale bar = 37 \( \mu \)m. Arrows in C and F, Neu N- and GFAP-negative cells noted to be \( \alpha \)- and \( \beta \)-ENAC positive.
Table 2. Relative abundance of α-, β-, and γ-ENaC and MR immunopositive cells in selected brain areas

<table>
<thead>
<tr>
<th>Region of Brain Studied</th>
<th>α-ENaC</th>
<th>β-ENaC</th>
<th>γ-ENaC</th>
<th>MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>++</td>
<td>++</td>
<td>+/-+</td>
<td>++</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cornu ammonis pyramidal</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Amygdala</td>
<td>++</td>
<td>++</td>
<td>-/+</td>
<td>++</td>
</tr>
<tr>
<td>Medial amygdaloid nucleus</td>
<td>++</td>
<td>++++</td>
<td>-/+</td>
<td>+</td>
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<tr>
<td>Lateral amygdaloid nucleus</td>
<td>++</td>
<td>++++</td>
<td>-/+</td>
<td>+</td>
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<tr>
<td>Hypothalamus</td>
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<td>++++</td>
<td>++++</td>
<td>+</td>
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<tr>
<td>Suprapoventricular nucleus</td>
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<td>Medial preoptic nucleus</td>
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<td>Periventricular nucleus</td>
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<td>+</td>
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<td>Parvocellular division</td>
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<td>+</td>
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<tr>
<td>Nucleus of tractus solitarius</td>
<td>++</td>
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<td>+</td>
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<tr>
<td>Circumventricular organs</td>
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<td>Organum vasculosum of lamina terminis</td>
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<tr>
<td>Ventricular ependyma</td>
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<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Blood vessels (small- and medium-sized vessels and capillaries)</td>
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<td>+/++</td>
<td>-/+</td>
<td>+</td>
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<tr>
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<td>+</td>
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<tr>
<td>Vascular smooth muscle cells</td>
<td>++</td>
<td>++</td>
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Relative density of cellular staining was rated as “none” (−) if no staining was observed, “low” (+) if < 30% of cells in an area were stained, “moderate” (+ +) if 30–70% of cells in an area were stained, and “high” (+++ + +) if > 70% of cells in an area were stained.

Distribution of MR

MR immunoreactivity was specific with no staining when the primary antibody was omitted. Incubation with normal IgG or preadsorption with the immunizing peptide abolished all the primary antibody was omitted. Incubation with normal IgG.

Coexpression of α-, β-, and γ-ENaC with MR

In the kidney, expression of both MR and ENaC subunits was found on the known aldosterone-responsive epithelia. In nonneural components of the brain, such as the choroid plexus, ependyma, the pia-arachnoid, endothelia, and VSMC, all three ENaC subunits were expressed in parallel with MR. In brain tissue itself, ENaC expression was mainly observed on neurons, i.e., GFAP-negative but Neu N-positive cells, as assessed by double-stained sections (Figs. 5 and 7K) and morphological appearance. Expression of all three ENaC subunits and MR were abundant in the SON, MnPO and magnocellular PVN, cingular and piriform cortex, and the hippocampus. Double labeling did not yield fine-resolution images with peroxidase staining. Study of adjacent sections, however, suggested that all three ENaC subunits and MR were expressed in the same groups of cells in the different regions. In areas such as the suprapoventricular nucleus, arcuate nucleus, most cortical areas, amygdala, and the SFO, immunoreactivity was prominent only for the α- and β-ENaC and MR but undetectable for γ-ENaC. Immunoreactivity to α- and β-ENaC subunits usually paralleled MR positivity.

DISCUSSION

This study demonstrates for the first time that α-, β-, and γ-subunits of ENaC are all expressed in the rat brain. By using RT-PCR, we showed the presence of mRNA transcripts of all three ENaC subunits and MR in regional extracts from the adult rat brain. By using immunohistochemistry, we mapped the relative distribution of α-, β-, and γ-ENaC and showed their parallel localization with MR.

Canessa et al. (4, 5) cloned ENaC subunit cDNA from rat kidney, distal colon, and lung. No transcripts were detected in rat brain. Recently, α-ENaC gene expression was reported in the cortex and cerebellum of mice (12). mRNA transcript levels in the whole hypothalamus are ~100-fold lower than the kidney, which explains why ENaC transcripts cannot be detected in the brain by Northern blot. Sequence analysis shows that the DNA sequences are the same in the brain and kidney.

The topology of each of the subunits includes two membrane-spanning segments, cytoplasmic NH2 and COOH termini, and a large glycosylated extracellular domain (4, 27). A low level of amiloride-sensitive current can be observed after expression of α-ENaC alone in oocytes, but expression of β- or γ-subunits individually does not induce an amiloride-sensitive current (4, 5). Expression of combinations of α-β- or α-γ-ENaC generates a higher level of current than after expression of α-ENaC alone, but still only 5% of the maximal current observed after coexpression of the three subunits in oocytes (4, 31). In different tissues, the three subunits may be synthesized in a differential fashion, with one or two subunits expressed constitutively, whereas the other(s) are induced by different physiological stimuli in parallel with increased channel activity (44). Different assemblies of the subunits, alone or with other degenerin/ENaC family members, may also be responsible for different functions (27). β- and γ-ENaC appear to form mechanosensitive complexes in baroreceptor nerve endings (10) and smooth muscle cells of cerebral (9) and renal (26) blood vessels.

AJP-Regul Integr Comp Physiol • VOL 289 • DECEMBER 2005 • www.ajpregu.org
In the whole hypothalamus of Wistar rats, α-ENaC mRNA was found to be ~30-fold more abundant than β- and γ-ENaC. In individual brain nuclei, higher levels of β- and γ-ENaC mRNA were detected. Interestingly, γ-ENaC mRNA levels in some areas were of comparable values to α-ENaC, whereas β-ENaC levels were still ~10-fold lower (Fig. 2). Whereas levels of ENaC mRNA in the brain are low compared with the kidney, immunohistochemistry demonstrated that all three ENaC subunits are abundantly expressed in areas such as the MnPO, SON, PVN, hippocampus, choroid plexus, and ependyma. The different relation between mRNA and protein levels in different brain areas compared with the kidneys may reflect higher turnover of mRNA and/or lower protein turnover. Immunoreactivity for γ-ENaC was undetectable in the SFO and OVLT, where an appreciable amount of γ-ENaC mRNA was present. This absence may relate to lack of translation of the γ-ENaC mRNA, high protein turnover, or posttranslational modifications affecting detection of the protein.

The combined approach of RT-PCR and immunohistochemistry establishes not only that ENaC mRNA transcripts are present in the brain but that they also are functional, i.e., lead to abundant presence of channel protein recognized by appropriate antibodies. However, this approach does not establish whether active channels are present. Some argue against the presence of truly silent or nonconductive channels at the cell membrane (13); others indicate that distinct pools of mature and immature channels can be present (25). In the context of the present study, it is difficult to comment on this issue. In the peripheral nervous system, ENaC expression has been noted in a variety of tissues, e.g., retina (3, 12), cochlea (6, 46), tongue (28), skin (8), and baroreceptors (10). Functionally active ENaC have so far been demonstrated only in the tongue (28).

Fig. 6. ENaC and MR staining in different areas in the brain. Red staining was developed by Nova red and brown-black with nickel-enhanced DAB. A–D: cerebral cortex showing staining for α- (A; red), β- (B; brown-black), and γ- (C; red) ENaC subunits and MR (D; red). Insets show magnified cells. Note staining along some neuronal processes and the clear nuclear halos. Scale bar = 37 μm. E–H: pyramidal neurons in the cornu ammonis subfields of hippocampus showing α- (E; brown-black), β- (F; brown-black) and γ- (G; red) ENaC and MR (H; red). Note some nuclear staining for MR. Scale bar = 37 μm. I–L: median preoptic nucleus dorsal part showing α- (I; brown-black), β- (J; black) and γ- (K; red) ENaC and MR (L; red). Scale bar = 37 μm.
and baroreceptors (10). Benzamil, a specific blocker of ENaC, had no effect on hypertonicity-induced depolarization in MnPO neurons of normal Wistar rats (19). Intracerebroventricular infusion of benzamil also has no demonstrable effects in rats on regular-salt diet but prevents sympathetic hyperactivity and hypertension in Dahl S rats on high-salt diet, suggesting that ENaC in the brain can become functionally active (18, 42).

Fig. 7. ENaC and MR staining in hypothalamic areas. Red staining was developed by Nova Red and brown-black with nickel-enhanced DAB. A–E: SON omission of the primary antibodies abolished any specific staining (A), whereas inclusion of the specific antibodies shows immunoreactivity to α- (B; red), β- (C; black), and γ- (D; red) ENaC subunits and MR (E; red) in adjacent sections. Staining appears to be on magnocellular cells by morphological criteria. Scale bar = 37 μm. F–J: ventral third ventricle (V3V) and adjacent areas. No significant staining is seen in the periventricular nucleus (PeVN) and ependyma when the primary antibody is omitted (F). With inclusion of the primary antibody, cells in the PeVN show staining for α- (G; brown-black) and β- (H; brown-black) ENaC subunits and MR (J; red) but undetectable staining of γ-ENaC (I; red). The ependyma of the ventral third ventricle is shown in the insets. Scale bar = 37 μm. K: double staining for β-ENaC and GFAP, most β-ENaC-positive cells (brown) are glial fibrillary acid protein (GFAP) (red) negative. L–O: paraventricular nucleus (PVN) also shows staining in adjacent sections for α- (L; brown-black), β- (M; brown-black), and γ- (N; brown-black) ENaC and MR (O; red). Note the lack of significant staining for γ-ENaC and MR in the parvocellular parts. Inset in M shows a magnified magnocellular neuron expressing β-ENaC. Scale bar = 37 μm.
Cardiovascular Regulatory Centers of Hypothalamus and Brain Stem

Hypothalamic nuclei such as the MnPO, SON, and magnocellular PVN showed prominent MR and ENaC immunoreactivity. MR expression was previously documented in the SON and PVN (1, 21, 38). Pietranera et al. (34), however, did not find immunoreactivity in the SON and PVN of Sprague-Dawley rats with the MCR N-17 antibody. An explanation of the different findings is not readily apparent, but variations in experimental conditions and fixatives could be contributing. The immunosignal in the SON and PVN for MR and all ENaC subunits was as strong as the signal in the hippocampus. Lack of GFAP costaining and morphological criteria suggests expression in the magnocellular neurons, which are vital in osmoregulation and salt-sensitive hypertension via synthesis of a range of hormones and neurotransmitters, including vasopressin and possibly ouabain-like compounds (43). ENaC and MR were further detected in regions of the anteroventral third ventricle, including the SFO and OVLT. AV3V lesions inhibit salt sensitivity and mineralocorticoid hypertension (17). The actual pathways activated by ENaC in these brain areas remain to be determined.

Hippocampus, Amygdala, and Other Brain Areas

MR and ENaC α- and β-subunits were abundantly expressed in the amygdala, hippocampus, and different cortical areas. The γ-ENaC signal was weak or undetectable in several of these areas. In the amygdala, aldosterone via MR modulates salt appetite (37). In the hippocampus and cortical areas MR modulate the hypothalamo-hypophysial-adrenal axis and higher brain functions (7, 16, 29, 45). One may speculate that ENaC may be the actual mediator of these actions.

ENaC and MR in Regulation of CSF Na⁺ Concentration

Prominent expression of all ENaC subunits and MR in the choroidal epithelia and ependyma is consistent with a role of ENaC in regulation of CSF Na⁺ concentration. In most tissues, Na⁺ transport by ENaC is dependent on the electrochemical gradient generated by Na⁺-K⁺-ATPase. In renal tubules, Na⁺-K⁺-ATPase is localized on the basolateral membrane and ENaC on the apical membrane, favoring unilateral transport of Na⁺ from the tubular lumen toward the interstitium. In the choroid plexus and blood-brain barrier, Na⁺-K⁺-ATPase activity is primarily located on the abluminal membrane, but 25%
the luminal (mainly associated with each membrane show different ouabain sensitivities, consistent with the presence of different isoenzymes at the luminal (mainly α2/α3) and abluminal (mainly α1) membranes (39). Blockade of Na\(^+\) channels by amiloride decreases the rate of Na\(^+\) entry into the CSF and choroid plexus both in vivo and in vitro (32). Our finding that ENaC is indeed expressed in the choroidal epithelia supports a functional role for ENaC in regulation of CSF Na\(^+\) concentration.

**Brain Blood Vessels**

All three ENaC subunits and MR were expressed in the brain vasculature in the endothelia as well as smooth muscle cells (Fig. 8, M–P). In contrast, Drummond et al. (9) detected mRNA of all three ENaC subunits by PCR from isolated cerebral vessels but could not detect α-ENaC expression by immunobassay or RT-PCR of freshly isolated smooth muscle cells or vessels. It was postulated that ENaC as stretch receptors in blood vessels may be involved in autoregulation of cerebral blood flow. Increased incidence of ischemic cerebrovascular events appears to occur in individuals with ENaC polymorphisms (22), possibly a consequence of dysregulation of the activity is of luminal origin. Na\(^+\)-K\(^+\)-ATPase activities and increased incidence of ischemic cerebrovascular events appear to occur in individuals with ENaC polymorphisms (22), possibly a consequence of dysregulation of the activity is of luminal origin. Na\(^+\)-K\(^+\)-ATPase activities associated with each membrane show different ouabain sensitivities, consistent with the presence of different isoenzymes at the luminal (mainly α2/α3) and abluminal (mainly α1) membranes (39). Blockade of Na\(^+\) channels by amiloride decreases the rate of Na\(^+\) entry into the CSF and choroid plexus both in vivo and in vitro (32). Our finding that ENaC is indeed expressed in the choroidal epithelia supports a functional role for ENaC in regulation of CSF Na\(^+\) concentration.

**Perspectives**

In salt-sensitive rats (Dahl S and spontaneously hypertensive), increases in CSF Na\(^+\) concentration on high-salt intake and enhanced neuronal response to CSF Na\(^+\) contribute to the development of sympathetic hyperactivity and hypertension (24, 41, 43). In rats, following myocardial infarction, sympathetic activity increases contributing to progressive cardiac dysfunction and heart failure (23). In both scenarios, sympathetic hyperactivity and disease progression can be prevented by central infusions of spironolactone or benzamil (23, 24, 41–43). These functional studies suggest a pivotal role for MR in regulation of cardiovascular function and CSF Na\(^+\). Decreased Na\(^+\) concentration as well as development, memory, and synaptic plasticity related to proteins involved in neurodegeneration. Nature 361: 463–467, 1994.

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