Cloning and functional expression of a new epithelial sodium channel δ subunit isoform differentially expressed in neurons of the human and monkey telencephalon

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Abstract

Epithelial sodium channel (ENaC) is a member of the ENaC/degenerin family of amiloride-sensitive, non-voltage gated sodium ion channels. ENaC α, β and γ subunits are abundantly expressed in epithelial tissues, where they have been well characterized. An ENaC δ subunit has also been described in the human nervous system, although its histological distribution pattern remains unexplored. We have now isolated a novel ENaC δ isoform (δ2) from human brain and studied the expression pattern of both the known (δ1) and the new (δ2) isoforms in the human and monkey telencephalon. ENaC δ2 is produced by a combination of alternative transcription start sites, a frame shift in exon 3 and alternative splicing of exon 4. It forms functional amiloride-sensitive sodium channels when co-expressed with ENaC β and γ accessory subunits. Comparison with the classical ENaC channel (αβγ) indicates that the interaction between δ2, β and γ is functionally inefficient. Both ENaC δ isoforms are widely expressed in pyramidal cells of the human and monkey cerebral cortex and in different neuronal populations of telencephalic subcortical nuclei, but double-labelling experiments demonstrated a low level of co-localization between isoforms (5–8%), suggesting specific functional roles for each of them.

Keywords: alternative splicing, degenerin/epithelial sodium channel family, epithelial sodium channel, human cerebral cortex, pyramidal neurons, Xenopus oocytes.


Members of the degenerin/epithelial Na⁺ Channel (DEG/ENaC) family of non-voltage gated ion channels are abundantly expressed in different tissues including the nervous system (Alvarez de la Rosa et al. 2000; Kellenberger and Schind 2002). The DEG/ENaC family includes acid sensing ion channels (ASICs), which are proton-gated channels expressed in neurons of the CNS (Price et al. 1996; Garcia-Añoveros et al. 1997; Lingueglia et al. 1997; Waldmann et al. 1997; Alvarez de la Rosa et al. 2003) and PNS (Price et al. 1996; Lingueglia et al. 1997; Waldmann et al. 1997; Chen et al. 1998; Alvarez de la Rosa et al. 2002). ASICs have been involved in mechanoreception, nociception and retinal transduction in the PNS and in pH sensing, modulation of learning and memory (Kristtal 2003).

Another branch of this ion channel family includes the ENaC, which was cloned by functional screening in Xenopus oocytes and is composed of three different subunits: the pore-forming α subunit and the accessory β and γ subunits (Canessa et al. 1994b). Subsequently, a fourth subunit, named δ, was identified and characterized as a pore-forming subunit that associates with β and γ to produce amiloride-sensitive Na⁺ currents (Waldmann et al. 1995). ENaC δ mRNA is abundant in human brain as reported by northern blot analysis (Waldmann et al. 1995; Yamamura et al. 2004),
although its histological expression pattern has not been investigated. Recent data suggest that there might be more than one form of the human ENaC δ subunit. A random sequencing project of human testis cDNAs (Ota et al. 2004) reported a clone ‘similar to amiloride-sensitive sodium channel delta subunit’ (GeneBank AK093239). Subsequently, a fragment of this putative isoform was detected by PCR in human cultured epithelial cells (Ji et al. 2006). We now demonstrate that the new ENaC δ isoform, which we name δ2, is expressed in several human tissues alongside with the previously described cDNA (Waldmann et al. 1995), herein referred to as δ1. Cloning of the full-length δ2 cDNA from human brain and expression in Xenopus oocytes show that δ2 forms functional amiloride-sensitive, voltage-insensitive Na$^+$ channels when co-expressed with ENaC β and γ accessory subunits. Furthermore, we describe for the first time the distribution of both δ2 and δ1 isoforms in human and monkey telencephalon. Our results reveal that both δ isoforms are expressed in pyramidal cells of the cerebral cortex and in different neuronal populations of telencephalic subcortical centres with the exception of striatum. Double-labelling experiments demonstrate that most neurons express a single isoform, suggesting specific functional roles for each of them.

Material and methods

Sequence analysis
GenBank database searches were performed with the basic local alignment search tool at the National Center for Biotechnology Information web server (http://www.ncbi.nlm.nih.gov/BLAST/), using the published δ ENaC (Waldmann et al. 1995) as query sequence. Protein and cDNA sequence alignments were performed with Clustal W (Thompson et al. 1994), available at the web site of the European Bioinformatics Institute (Cambridge, UK). A ENaC δ subunit gene model was generated automatically by the Ensembl database (Hubbard et al. 2005) version 39, June 2006 (available online at http://www.ensembl.org, Gene ID ENSG00000162572). The search for specific functional motifs in the N-terminus sequence of the ENaC δ isoforms was performed with the Eukaryotic Linear Motif resource at http://elm.eu.org/ (Puntervoll et al. 2003). Putative kinase target detection was performed using NetPhosK 1.0 (http://www.cbs.dtu.dk/services/NetPhosK/) (Blom et al. 2004).

RT-PCR, DNA cloning and cRNA synthesis
Reverse transcription of total human RNA obtained from different tissues (Clontech Laboratories, Mountain View, CA, USA) was performed with the First Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) using oligo-dT as primer. Aliquots of the RT products were used to amplify specific cDNA fragments by PCR using Taq polymerase and oligonucleotides described in Table S1. An I.M.A.G.E. Consortium cDNA Clone (Lennon et al. 1996) containing a partial sequence of human ENaC δ1 cDNA (clone ID 5163898) was obtained from Geneservice Ltd. (Cambridge, England). Full-length δ2, α and γ ENaC cDNAs were obtained by RT-PCR from human brain, kidney or lung using specific oligonucleotides designed after available sequences (GeneBank AK093239; McDonald et al. 1994; Voille et al. 1995) and a proof-reading DNA polymerase cocktail (High Fidelity PCR kit; Roche Diagnostics, Mannheim, Germany). PCR products were cloned by topoisomerase I-mediated ligation in pSc-C (StrataClone PCR cloning kit; Stratagene, Cedar Creek, TX, USA) or by restriction enzyme cutting and ligation in pcDNA3.1(+) (Invitrogen). C-terminal epitope tagging of δ2 and α cDNAs was performed by PCR using oligonucleotides that incorporate a tail of FLAG- or hemagglutinin (HA)-coding sequences (Alvarez de la Rosa et al. 2002; Coric et al. 2004). Fluorescently labelled δ2, α and β subunits were generated by attaching the cDNA of enhanced yellow fluorescent protein (eYFP) to the 3′-end of ENaC subunits and subcloning in pcDNA3.1(+) (Invitrogen). Plasmids were purified using a commercially available kit (Qiagen, Valencia, CA, USA) and insert identity was confirmed by dideoxy-nucleotide sequencing. After linearization with the appropriate restriction enzymes, constructs were used as templates for in vitro cRNA synthesis using a commercial system (mMessage mMachine; Ambion, Austin, TX, USA). cRNAs were purified by phenol-chloroform extraction and isopropanol precipitation.

ENaC heterologous expression and electrophysiology
Oocytes were harvested from Xenopus laevis adult females under benzocaine anesthesia by partial ovariectomy and collagenase IA dispersion. Stage V to VI oocytes were selected and microinjected with 0.1 ng of full-length ENaC subunits cRNAs alone or in combination as specified for each experiment. Cells were then incubated for 2 days (for electrophysiology) or 2–5 days (for imaging experiments and western blots) at 18°C in oocyte Ringer’s (in mmol/L: 82.5 NaCl, 2 KCl, 2 CaCl$_2$, 2 MgCl$_2$, 1 Na$_2$HPO$_4$ and 10 HEPES, at pH 7.5) supplemented with 100 μmol/L amiloride (Sigma Aldrich, Stenheim, Germany). Oocytes whole-cell currents were recorded using a two-electrode voltage clamp system as described previously (McNicholas and Canessa 1997; Völlter et al. 2000). The bath solution contained (in mmol/L): 1.8 CaCl$_2$, 2 MgCl$_2$, 4 KCl, 5 BaCl$_2$, 5 HEPES, pH 7.2 and 150 mmol/L Na$^+$ or K$^+$ gluconate. ENaC-specific currents were calculated as the difference before and after the addition of 100 μmol/L amiloride to the bath. Current-voltage curves were generated by increasing voltage from −180 to +60 mV in sequential 20 mV steps of 200 ms duration each. Currents were recorded at 1 kHz. Stimulation and data acquisition were controlled using the Pulse + PulseFit software (HEKA Elektronik, Lambrecht, Germany) running on a Macintosh computer. Data analysis was performed with the programs PulseFit (HEKA Elektronik) and Igor-Pro (WaveMetrics, Lake Oswego, OR, USA). ENaC FLAG-tagged δ2 protein expression was detected by western blot analysis of Xenopus oocytes extracts using an anti-FLAG monoclonal antibody (Sigma Aldrich) as previously described (Giraldez et al. 2005). ENaC HA-tagged α protein was detected with a HA-Y11 monoclonal antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Expression of β and γ ENaC subunits was detected using previously characterized rabbit polyclonal antibodies (Coric et al. 2004). Cell surface expression of fluorescently labelled channel subunits was detected by collecting emission spectra from whole oocytes using a laser scanning confocal microscope (Olympus).
In situ hybridization

The expression of both ENaC δ isoforms was studied by in situ hybridization (ISH) histochemistry in human cerebral cortex and monkey telencephalon. Human brains came from patients (two men and one woman; average age 55.7 ± 7.2 years) who died without history of drug abuse or neurological or psychiatric illness. The absence of degenerative or vascular disease was confirmed by pathological examination in each case. Brains were removed after a post-mortem period of 12.2 ± 4.4 h. Blocks containing frontal and temporal cortices were washed in 0.1 mol/L phosphate-buffered saline pH 7.4 (PBS) and immediately immersed in 4% p-formaldehyde in PBS for 72 h at 4°C. Four adult male rhesus monkeys (Macaca fascicularis; 5 to 8-years old, 3.5–4.8 kg) were administered an overdose of sodium pentobarbital and transcardially perfused with heparinized ice-cold 0.9% saline followed by 3–4 L of 4% p-formaldehyde in PBS. Brains were removed, cut into blocks and immersed in fixation solution overnight. Human and monkey samples were cryoprotected by consecutive immersion in 10%, 20% and 30% sucrose in PBS (24 h each), frozen and cut into 30-μm thick sections perpendicular to the long axis of the cortical gyri in human cortex and in the coronal axis in monkey forebrain, with a freezing microtome. ISH probes consisted on sense and anti-sense 40-mer oligonucleotide probes. ENaC δ1 isoform probe corresponds to the 3’-end of exon 1 (P61; Table S1). ENaC δ2 isoform was detected with two 40-mer oligonucleotide probes (PA82 and PB82; Table S1) designed to cover exon 4, which is specific to δ2. Sense oligonucleotides were used as control for non-specific binding. Digoxigenin (DIG)- or biotin-labelled probes were generated using a commercial kit (DIG oligonucleotide tailing kit, 2nd Generation; Roche Diagnostics). This system uses terminal transferase to generate tail oligonucleotides that incorporate a mixture of up to 40 dATP and DIG- or biotin-dUTP residues at the 3’-end in a template-independent reaction. Labelling efficiency was determined by dot-blot comparison with standards provided by the manufacturer. Single ISH labelling was performed using DIG-probes essentially as described (Gonzalez et al. 2004). Briefly, sections were pre-hybridized at 45°C for 2 h in hybridization solution (50% formamide, 5x SSC and 40 μg/mL denatured salmon DNA). Probes were added to the hybridization mix at 400 ng/mL and sections were incubated at 45°C for 16 h. Post-hybridization washes included: 2x SSC at 22°C for 10 min, 2x SSC at 55°C for 15 min and 0.1x SSC at 55°C for 15 min. The slides were then equilibrated for 5 min in TN buffer (100 mmol/L Tris–HCl and 150 mmol/L NaCl, pH 7.5) and incubated for 2 h at 22°C with alkaline-phosphatase conjugated anti-DIG monoclonal antibody (1 : 5000 final dilution in TN with 0.5% blocking reagent; Roche Diagnostics). After washes, the slides were equilibrated for 5 min in TNM buffer (100 mmol/L Tris–HCl, 100 mmol/L NaCl and 50 mmol/L MgCl2, pH 9.5) and incubated in substrate solution (Nitro-Blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt in TNM buffer; Roche Diagnostics). Staining was stopped in TE (10 mmol/L Tris–HCl and 1 mmol/L EDTA, pH 8.0), and the slides were dehydrated and mounted in Entellan® (Merek, Darsstadt, Germany). For double ISH labelling biotin- and DIG-labelled probes were simultaneously added to the hybridization mix. The combination of probes that gave optimal results was biotin-61 and DIG-62. After hybridization and post-hybridization washes, the biotin-labelled probe was detected using the tyramide signal amplification procedure (NEF, Boston, MA, USA) as described by the supplier. Briefly, sections were immersed for 30 min in 3% H2O2 to inactivate endogenous peroxidase, washed several times in TNT buffer (100 mmol/L Tris–HCl pH 7.5, 150 mmol/L NaCl and 0.05% Tween), equilibrated in TNB (100 mmol/L Tris–HCl pH 7.5, 150 mmol/L NaCl and 0.5% blocking reagent) and incubated for 30 min in 1 : 100 streptavidin-conjugated horseradish peroxidase (NEF) in TNB. Sections were then washed several times in TNT buffer, incubated for 10 min in biotinyl-tyramide diluted 1 : 50 in amplification buffer, washed in TNT buffer and incubated again in 1 : 100 streptavidin-conjugated horseradish peroxidase. The colourimetric reaction of the biotin-labelled probe was visualized after incubation for 5 min in 0.005% 3‘-3‘-diaminobenzidine tetrahydrochloride (Sigma Aldrich) and 0.001% H2O2 in 50 mmol/L caccodylate buffer, pH 7.6. After several rinses in TN, sections were processed for the DIG-labelled probe as described above. Cells containing the biotin-labelled probe showed a brown reaction, those containing the DIG-labelled probe showed a blue reaction, and those with both probes showed a blue-brown reaction.

Selected sections of human cerebral cortex processed for single ISH labelling were also immunostained for glial fibrilar acidic protein (GFAP), a marker of astrocytes, and calbindin (CB) and parvalbumin (PV), two markers of cortical non-pyramidal cells. After endogenous peroxidase inactivation, sections were incubated for 60 min at 22°C in 4% normal goat serum (Jackson ImmunoResearch, West Grove, PA, USA) in PBS and overnight in one of the following primary antibodies: mouse monoclonal anti-PV (Sigma Aldrich) 1 : 3000, mouse monoclonal anti-CB (Sigma Aldrich) 1 : 2000 and rabbit polyclonal anti-GFAP (Dako, Glostrup, Denmark) 1 : 10 000 in PBS. After several rinses, sections were incubated for 2 h in either a biotinylated goat anti-mouse (for PV and CB) or anti-rabbit (for GFAP) antiserum (1 : 1200; Jackson ImmunoResearch) and 0.5% normal goat serum in PBS. Immunoreactions were visible after incubation for 1 h at 22°C in ExtrAvidin-peroxidase (1 : 5000; Sigma Aldrich) in PBS and after 10 min in 3‘-3‘-diaminobenzidine tetrahydrochloride solution. Control experiments omitting the probe or the primary antibody or using a sense probe were included in each ISH or immunohistochemistry experiment. In addition, adjacent sections were stained with the Nissl method to provide the basic cytoarchitecture of human and monkey samples. Images were obtained under a Leica DMR® microscope (Leica Microsystems GmbH, Wetzlar, Germany) and compiled using Adobe Illustrator® (Adobe Systems, San Jose, CA, USA).

Results

Characterization, molecular cloning and tissue-specific expression of a new ENaC δ subunit isoform

A search of the nucleotide databank at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD, USA) using the published ENaC δ subunit
cDNA as query sequence (herein referred to as δ1, GeneBank accession number U38254) revealed a clone containing a N-terminal variant of ENaC δ subunit (GeneBank AK093239). This cDNA, which we name δ2, was identified in a random sequencing project of human testis cDNAs (Ota et al. 2004) and annotated as ‘highly similar to amiloride-sensitive sodium channel delta-subunit’. Translation of the cDNA predicts a 704 amino acid protein that differs from the originally described ENaC δ1 638 amino acid sequence (Waldmann et al. 1995) by having a longer N-terminus (Fig. 1a). It has been determined experimentally that DEG/ENaC proteins have two transmembrane (TM) domains with both the N- and the C-termini located intracellularly and a long extracellular loop (Canessa et al. 1994a; Saugstad et al. 2004). Therefore, the divergent N-terminus sequence in ENaC δ isoforms is predicted to be located in the intracellular side of the protein (Fig. 1b). Close inspection of the predicted gene structure of human ENaC δ subunit (Ensembl database, v.39, June 2006) suggests that the divergent N-terminus sequence is generated by different transcription start sites (located in exon 3 for isoform δ1 and in exon 2 for isoform δ2, Fig. 1c), alternative splicing of exon 4, which is

Fig. 1 Primary sequence and structure of epithelial Na⁺ channel (ENaC) δ isoforms. (a) Protein sequence comparison of ENaC δ1 and δ2 N-termini. The location of the first transmembrane domain (TM1) is indicated with a solid line above the sequence. ‘.’, identical residues; ‘:’, conserved substitutions; ‘.’, semi-conserved substitutions. (b) Schematic representation of ENaC δ1 and δ2 topology with the localization of the divergent sequences indicated in blue (δ1) and red (δ2). Extracellular (Ext) and intracellular (Int) sides of the membrane are indicated. (c) The predicted human ENaC δ subunit gene structure and putative mechanisms of isoform generation. Exons are represented as boxes with the exon number underneath. For simplicity, exons 6–14 have been omitted from the figure. Exons common to both isoforms are represented by black boxes. White boxes indicate untranslated regions. Bent arrows indicate alternative transcription start sites and ATG1 and ATG2 represent initiation codons for isoforms δ1 and δ2, respectively. In each case, exons not present in one of the isoforms mRNA are drawn as white boxes with grey outline. Solid lines represent putative mRNA splice events (blue for δ1 and red for δ2). Note that the 5’ boundary of exon 2 differs for the two isoforms. (d) Partial alignment of ENaC δ1 and δ2 cDNAs showing the alternative reading frames used in exon 3, which gives rise to δ1 (blue) or δ2 (red) protein sequences. Putative exon boundaries are shown with grey shading.
absent in isoform δ1 (Fig. 1c), and a frame shift in exon 3 (Fig. 1d).

In order to investigate the expression of the novel ENaC δ2 isoform, we performed RT-PCR using as template cDNAs obtained from different human tissues. To that end, we used primers that should amplify both ENaC δ isoforms, giving PCR products of different sizes due to the alternative splicing of exon 4 (primers exon3-F and exon5-R; Table S1). We obtained specific bands of the predicted size (84 bp for PCR products of different sizes due to the alternative splicing (Fig. 1d).

Fig. 2 Expression of epithelial Na⁺ channel (ENaC) δ1 and δ2 in human tissues. (a) Agarose-gel electrophoresis of RT-PCR products obtained from human tissues using primers common to a N-terminal region of both isoforms. The size of RT-PCR products is indicated in base pairs. An I.M.A.G.E. clone (clone ID 5163898) containing a partial sequence of human ENaC δ1 was included as a control. Panel (a) also shows RT-PCR products from human tissues using primers specific for human β-actin. (b) RT-PCR amplification of the full-length coding sequence (open reading frame; ORF) of human ENaCδ2 from brain cDNA.

and sequencing confirmed that the PCR product was the full-length ENaC δ2 coding sequence.

Functional expression of ENaC δ2 in Xenopus oocytes
We used heterologous expression in X. laevis oocytes to determine if the newly described ENaC δ2 isoform is able to form functional ion channels and to investigate their properties. Currents from cRNA-injected oocytes were recorded using a two-electrode voltage clamp system. Expression of δ2 alone did not result in detectable amiloride-sensitive current (Fig. 3b). It has been previously demonstrated that ENaC δ1 forms channels when co-expressed with β and γ ENaC accessory subunits (Waldmann et al. 1995). Co-expression of δ2, β and γ ENaC subunits gave rise to an amiloride-sensitive Na⁺ current that is voltage-independent (Figs 3a and b), while co-expression of β and γ subunits alone did not result in any detectable amiloride-sensitive current (Fig. 3b). As control, oocytes were co-injected with ENaC α, β and γ subunits. This combination gave rise to amiloride-inhibitable, voltage-independent Na⁺ currents (Fig. 3c), as expected (Canessa et al. 1994b). For the external Na⁺ concentrations used in this study the expected reversal potential (E_r) for ENaC currents was around +60 mV. As ENaC channels are constitutively active and despite keeping oocytes in 100 μmol/L amiloride, they were overloaded with Na⁺ and thus E_r was shifted to more negative values (Fig. 3b). We observed a larger shift of E_r for the δ2βγ subunit combination (Fig. 3b). This is probably due to the lower sensitivity of ENaC δ subunits to amiloride (Waldmann et al. 1995), a property which is conferred by the extracellular loop and therefore conserved between δ isoforms.

Comparison between current magnitudes elicited by αβγ and δ2βγ (Fig. 3d) revealed that the former produces 10-fold more current than the latter. We first tested whether the lower current magnitude was due to poor expression of δ2. To that end, we performed western blot analysis of protein extracts from oocytes injected with epitope-tagged δ2-FLAG or α-HA subunits, along with β and γ subunits. The protein products were detected at comparable levels in both groups (Fig. 3e). Therefore, the lower δ2βγ currents could be due either to poor channel expression in the plasma membrane or alternatively to the presence of non-active channels or channels with very low open probability. To distinguish between these two possibilities, we investigated cell-surface expression of fluorescently labelled δ2 or α channel subunits. Non-injected oocytes yielded very low endogenous fluorescence (Fig. 4a). Oocytes co-expressing α-eYFP/β/γ subunits showed a strong cell-surface labelling when compared with δ2-eYFP/βγ- or β-eYFP/γ-injected oocytes (Fig. 4a). To verify that the fluorescence signal observed was specific from the eYFP-labelled ENaC subunits, we obtained the emission spectra of oocytes excited at 515 nm (Fig. 4b). Only α-eYFP/β/γ produced an emission spectrum consistent with
suggests that the smaller whole-cell currents detected with eYFP, whereas signals from the other subunit combinations were not above background fluorescence. This result strongly suggests that ENaC α, β and γ subunits are absent or expressed in very low amounts in the human cerebral cortex, and therefore are not likely to be physiological interacting partners for the δ subunit.

**ENaC δ1 and ENaC δ2 expression in the human cerebral cortex and monkey telencephalon**

Northern and dot-blot experiments have shown ENaC δ1 subunit mRNA expression in different regions of the human brain (Yamamura et al. 2004), but no data is available regarding the specific areas and cell types expressing this subunit or the novel δ2 isoform. Based on the small nucleotide sequence divergence between δ1 and δ2 mRNAs (Fig. 1), we generated DIG- or biotin-labelled oligonucleotide probes specific for each of the ENaC δ isoforms (Table S1) and used them in ISH experiments in sections obtained from human cerebral cortex and monkey telencephalon.

In situ hybridization using single DIG-labelled probes showed a similar distribution pattern for both ENaC δ isoforms in human cerebral cortex. Many neurons displaying a genuine pyramidal morphology throughout layers II to VI and the underlying white matter of the frontal and temporal cortices were stained for ENaC δ1 as well as for ENaC δ2 (Figs 5a and c), suggesting that ENaC δ subunits are preferentially expressed in pyramidal neurons of the cerebral cortex. The combination of ISH for each ENaC δ isoform with immunohistochemistry for GFAP (a marker of astroglial cells) and the calcium binding proteins CB and PV (two markers of non-pyramidal cells) did not detected double-labelled cells (Figs 5e–h). This result supports the idea that ENaC δ isoforms are exclusively found in pyramidal cells. Double ISH labelling for ENaC δ1 and δ2 probes revealed that most pyramidal neurons express a single isoform (Figs 5i–k) and that their distribution pattern varies from one cortical region to another, without following a precise cytoarchitectural pattern. In some regions, they form separate groups, with δ1-expressing neurons arranged in clusters separated by δ2-expressing neurons, or vice versa (Fig. 5i). These clusters differ in shape and size and occupy a variable number of cortical layers. In other cortical regions, neurons expressing a single ENaC δ isoform are intermingled with those expressing the other, and those co-expressing both forms (Fig. 5j), giving rise to a ‘mosaic-like’ organization (Fig. 5k). The percentage of neurons co-expressing both isoforms in the human cortex is very low (around 5–8% of the total number of stained cells), but the percentage of those expressing one of the two forms varies from one region to another.

In the monkey cerebral cortex (cingular, frontal, orbital, insular, temporal, piriform and entorhinal cortices), the expression pattern was in general similar to that found in...
humans, with ENaC δ isoform expression restricted to pyramidal neurons. Double-labelling experiments show clusters or large regions where only one isoform is preferentially expressed, and regions where ENaC δ1-, δ2- and double-labelled neurons are intermingled (Figs 6a-d). In subcortical telencephalic structures, ENaC δ isoforms were also extensively expressed. Single δ1 and δ2 DIG-probes revealed numerous stained neurons in the claustrum (Fig. 6e), amygdaloid nuclei (Fig. 6f–h), medial and lateral septal nuclei, vertical and horizontal limbs of the diagonal band, olfactory tubercle, substantia innominata, basal nucleus of Meynert, bed nucleus of the stria terminalis and globus pallidus (Figs 6i and k). Only a few weakly stained cells were found in the accumbens nucleus and the ventral pallidum (data not shown), and interestingly, no labelled cells were detected in putamen and caudate nucleus (Figs 6i and j). Double ISH-stained sections showed that in subcortical nuclei δ1-, δ2- and δ1/δ2-expressing neurons are intermingled in variable proportions (Figs 6g, h and l), as described above for cortical regions.

Discussion

In this study, we have characterized a new transcript of human ENaC δ subunit gene that differs from the originally described δ subunit (Waldmann et al. 1995) by having a longer intracellular N-terminus, while the rest of the protein, including both TM domains and the extracellular loop, is conserved between isoforms. This new transcript, which we have named ENaC δ2, is expressed in several human tissues, including the CNS. Molecular cloning of the full-length δ2 cDNA followed by heterologous expression in Xenopus oocytes showed that the new isoform is able to form functional amiloride-sensitive, voltage-insensitive sodium channels when co-expressed with accessory ENaC subunits. Importantly, comparison between δ2βγ channels and classical ENaC αβγ channels indicates that the interaction between δ2, β and γ is functionally inefficient. Moreover, β and γ ENaC subunits are absent or expressed at very low levels in the human brain cortex, making it...
unlikely that these subunits are interacting partners for the δ subunit. The use of oligonucleotide probes and ISH allowed us to specifically detect both ENaC δ1 and δ2, providing for the first time a detailed description of ENaC δ subunit isoforms distribution in human and monkey telencephalon.

Fig. 5  Epithelial Na⁺ channel (ENaC) δ1 and δ2 expression in the human cerebral cortex. (a–d) Single labelling for ENaCδ1 (a and b) and ENaCδ2 (c and d) using digoxigenin-labelled antisense (a and c) and sense (b and d) probes. Insets correspond to boxed areas in (a and c). (e–h) Double labelling by digoxigenin-labelled probes and immunohistochemistry for glial fibrilar acidic protein, calbindin (CB) and parvalbumin (PV). (i–k) Double labelling for ENaCδ1 (brown) and ENaCδ2 (blue) in the temporal cortex. (k) Corresponds to boxed area in (j). As shown in (a and c), both isoforms display a similar labelling pattern, localized in many neurons with a pyramidal morphology (insets) in different cortical layers. The combination of in situ hybridization for each ENaC δ isoforms and immunohistochemistry for glial fibrilar acidic protein (e and f), CB (g) and PV (h) revealed no double-labelled cells. Sections hybridized with both isoforms showed that most neurons only express one of them, forming different clusters (i) or being intermingled in the same region (j), where a few of them co-express both forms (k arrows). as, antisense probe; s, sense probe. Bar in (a–d) 300 μm; in the insets 80 μm; in (e–h) 20 μm; in (i and j) 250 μm; in (k), 40 μm.
Identification and functional characterization of a new ENaC δ subunit isoform

The existence of a novel human ENaC δ subunit isoform was initially detected in a random sequencing project of human testis cDNAs (Ota et al. 2004), which is available in the GeneBank database. Recently, Ji et al. (2006) studied ENaC δ subunit mRNA expression in human epithelial cell lines of lung, pancreas and colon origin. RT-PCR experiments using primers covering the region between the beginning of the open reading frame and the first TM domain gave two bands in the majority of the cell lines tested. The authors identified one of the bands as corresponding to a novel ENaC δ subunit cDNA, but no further characterization was provided (Ji et al. 2006). In this study, we have confirmed the expression of the new δ2 isoform in several human tissues, including the CNS and have cloned the full-length cDNA from human brain, allowing for functional studies in an heterologous expression system1.

The most important biophysical and pharmacological features that characterize channels formed by ENaC δ subunit, such as high selectivity for Na+, lower amiloride

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1While this paper was under review, an article describing the full-length cloning and functional expression of a sequence identical to the one reported here as ENaC δ2 has been published (Yamamura et al. 2006).
sensitivity than α channels (Waldmann et al. 1995; Ji et al. 2004) and current potentiation by protons (Ji and Benos 2004; Yamamura et al. 2004), are determined by the two TM domains and the extracellular loop, which are conserved in both δ1 and δ2 isoforms. Furthermore, a His-Gly motif present in the N-terminal intracellular domain and involved in ENaC gating (Kellenberger and Schild 2002) is also conserved. Therefore, we predicted that all the properties mentioned above would be conserved between δ isoforms. This has been experimentally confirmed by Yamamura et al. (Yamamura et al. 2006). The functional consequence of the N-terminal variation between isoforms remains to be elucidated. It could possibly be involved in differential regulation of the channel activity and/or traffic by intracellular signals. The search for functionally important amino acid patterns in the divergent sequences between δ isoforms revealed several putative kinase consensus sequences and a proline-rich domain that could interact with SH3 motif-containing proteins in δ2, but not in δ1. The functional relevance of these motifs remains to be tested.

**Differential expression of ENaC subunits in the CNS**

Our present work and studies published by others show that both δ isoforms (Waldmann et al. 1995; Ji and Benos 2004; Yamamura et al. 2006) produce amiloride-sensitive Na⁺ channels when co-expressed with ENaC β and γ accessory subunits in heterologous systems. Furthermore, a recent study describes how ENaC δ physically interacts and functionally modifies αβγ channels (Ji et al. 2006), reinforcing the idea that different combinations of ENaC subunits could form channels with heterogeneous properties (McNicholas and Canessa 1997; Kellenberger and Schild 2002). Interestingly, we have found that the currents obtained by co-expression of δ2, β and γ subunits are smaller than those obtained by co-expression of α, β and γ subunits. The smaller current levels of δ2βγ channels correlate with lower cell-surface expression levels, indicating that channel assembly and/or traffic to the membrane occur with lower efficiency than for αβγ channels. Furthermore, our results show that the β and γ subunits are absent or expressed at very low levels in the human brain, in contrast with previous reports describing the detection of α subunit mRNA by RT-PCR in the mouse cerebral cortex (Dyka et al. 2005) or studies in the rat showing widespread expression of ENaC α and β subunits in different brain areas (Amin et al. 2005). Our results suggest that other members of the DEG/ENaC family expressed in the human CNS could be channel-forming partners of the ENaC δ isoforms. ASIC1 and two isoforms of ASIC2 (ASIC2a, also known as BNaC1α, and ASIC2b) are also found in pyramidal neurons of rat (Linguegilia et al. 1997; Alvarez de la Rosa et al. 2002; Duggan et al. 2002) and human (Giraldez and Alvarez de la Rosa, unpublished observations) brain cortex, making them potential partners of ENaC δ subunit isoforms, although detailed information regarding the distribution patterns of these subunits in the human or monkey CNS is lacking.

Double-labelling experiments showed little co-localization of ENaC δ1 and δ2 isoforms in cortical and subcortical neurons of the telencephalon (Figs 5 and 6). Instead, a complex distribution pattern that includes clusters of δ1- or δ2-expressing neurons as well as areas where both types of neurons are intermingled suggests that ENaC δ isoform expression is carefully regulated. Other genes encoding proteins from the DEG/ENaC family, including ASIC1–3 genes, give rise to different transcripts (Kellenberger and Schild 2002), but there is limited information regarding the relative distribution of the different isoforms in the CNS. ISH experiments using ³²P-labelled probes in rat brain sections showed that ASIC2 isoforms (ASIC2a and ASIC2b) localize to the same areas, suggesting a high level of co-expression (Linguegilia et al. 1997). Further information about the relative expression of other DEG/ENaC protein isoforms is clearly needed to establish whether ENaC δ1 and δ2 differential distribution in CNS neurons is a unique feature of this gene.

**The physiological function of ENaC δ subunit**

As discussed above, the physiological partners of ENaC δ isoforms in CNS neurons and the functional consequence of ENaC δ isoform interaction with other members of the DEG/ENaC family remain to be established. The precise distribution of ENaC δ1 and δ2 in neurons of cortical and subcortical areas described in this study suggests specific roles for each isoform. On the other hand, ISH experiments do not provide information about the subcellular distribution of ENaC δ isoforms in neurons. It has been recently described that ENaC δ channel activity is potentiated by protons (Ji and Benos 2004; Yamamura et al. 2004), suggesting a role for this subunit as an extracellular pH sensor in neurons. This has been proposed as one of the putative roles of ASIC channels in the CNS (Krishtal 2003), which are thought to be involved in signal transduction following ischemic episodes. ENaC δ channels have very slow activation and desensitization kinetics in response to a decrease in extracellular pH (Ji and Benos 2004), in contrast with the fast activation and desensitization properties of most ASIC channels (Alvarez de la Rosa et al. 2002; Zhang and Canessa 2002; Hesselager et al. 2004). The slow proton response would make ENaC δ channels good sensors of slow extracellular pH changes as may be found during ischemia.

Unlike ASICs, which require a decrease in extracellular pH to become active, ENaC δ channels have a constitutive, pH-independent activity. A persistent Na⁺ conductance in the membrane would have a depolarizing effect on the resting membrane potential. If such persistent sodium conductance was also present in the post-synaptic membrane, it could have an amplification effect on synaptic potentials, making it...
easier for a graded potential change to reach the threshold of the neuron, triggering an action potential. It has been suggested that ASIC channels could have a similar role modulating action potentials in hippocampal neurons in response to small changes in extracellular pH (Vukicevic and Kellenberger 2004). Recently published data suggests that sperm capacitation-associated hyperpolarization depends on a cAMP-dependent inhibition of an amiloride-sensitive depolarizing Na⁺ current, which is likely to be due to ENaC channels formed either by α or δ subunits (Hernandez-Gonzalez et al. 2006). This study supports the idea that ENaC δ channels could be involved in the control of membrane potential and cell excitability. Information about the subcellular localization of ENaC δ isoforms in neurons is clearly needed in order to further advance our understanding of the roles of these proteins in the CNS.

In summary, we have characterized a new isoform of human ENaC δ subunit (δ2). This new isoform forms functional amiloride-sensitive Na⁺ channels when co-expressed with ENaC β and γ accessory subunits, even though this interaction is inefficient, pointing towards other ENaC/DEG proteins as physiological channel-forming partners of δ2. Furthermore, we provide for the first time detailed localization of ENaC δ1 and δ2 isoforms in human and monkey telencephalon. Our data show a striking differential distribution pattern of both isoforms in cortical pyramidal neurons and subcortical neurons, pointing towards a strict regulation of expression and specific and distinct roles of different δ subunits in the human CNS.

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Supplementary material

The following supplementary material is available for this article online:

Table S1 Oligonucleotides used in this study.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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