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Molecular differential expression of voltage-gated sodium channel α and β subunit mRNAs in five different mammalian cell lines

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Abstract Voltage-gated sodium channels are composed of one α subunit and one or more auxiliary β subunits. A standard reverse transcription–polymerase chain reaction assay was used to detect the mRNAs encoding for seven α subunits (Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.5, Nav1.6, Nav1.9) and for the two non-covalently linked β1 and β3 auxiliary subunits in five different cell lines from rat, mouse and human origin. A semi-quantitavive RT-PCR analysis allowed to evaluate in each cell line, the relative expression level of each NaCh subunit previously detected. The expression profile of the cell lines was compared with that obtained from rat and mouse neural, skeletal muscle and cardiac tissues. This data provide a standard for the study of the modulation of the sodium channel expression in mammalian excitable tissues.

Keywords Voltage-gated sodium channel · α subunit · β subunits · mRNA expression

Introduction

Voltage-dependent sodium channels (NaChs) play a key role in cellular excitability (Hille 2001). Thus, the balance between activation and inactivation of NaChs is crucial for neuronal signaling, skeletal muscle contraction, and heart rhythm. In fact, slight alterations in NaCh gating lead to pathologies involving cellular excitability and whole animal physiology, such as generalized epilepsy, myotonia, paralysis, long QT syndrome or cardiac conduction defects (Abriel et al. 2001; Bennett et al. 1995; Spampanato et al. 2003; Splawski et al. 2002; Wallace et al. 1998; Wang et al. 1995).

Voltage-gated sodium channels are composed of one α subunit and one or more auxiliary β subunits. The pore-forming α subunits are large proteins and consist of four homologous domains, each with six transmembrane segments, being both the N- and the C-termini intracellular (Catterall 1992; Felipe et al. 1994). Nine different voltage-gated sodium channel α subunits (Nav1.1-Nav1.9) and a related, non-voltage-gated atypical isoform (NavX), all encoded by a different gene, were cloned in mammals (Yu and Catterall 2003; Catterall et al. 2005; Goldin et al. 2000). These genes produce proteins with a high degree of amino acid sequence identity. Six NaCh subunit isoforms (Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.6, Nav1.7) are tetrodoxin (TTX)-sensitive. Nav1.1, Nav1.2, Nav1.3 and Nav1.6 are all expressed at high level in the central nervous system (CNS) and in the peripheral nervous system (PNS), Nav1.1 and Nav1.6 are also expressed in the heart, Nav1.4 is abudant in adult skeletal muscle, and Nav1.7 is present primarily in the peripheral nervous system. The remaining α subunit isoforms (Nav1.5, Nav1.8 and Nav1.9) are TTX-resistant. Nav1.5 is expressed predominantly in heart, brain and denervated skeletal muscles, while the last two isoforms are mainly expressed in the sensory neurons (Black et al. 1991; Felts et al. 1997; Schaller and Caldwell 2000, 2003; Hartshorne and Catterall 1984).

In addition, post-transcriptional processing, alternative splicing and RNA editing also introduce diversity in sodium channel α subunits. Alternative splicing has been reported for six isoforms which are present in the mammalian nervous system, Nav1.1, Nav1.2, Nav1.3 (Schaller et al. 1992; Gustafson et al. 1993; Lu and Brown 1998; Kasai et al. 2001), Nav1.5 (Raymond et al. 2004), Nav1.6 (Plummer et al. 1997), Nav1.7 (Belcher et al. 1995). Various factors, including age, the tissue of origin and the presence of

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modulatory agents, such as dibutyryl cyclic AMP, modulate the proportion of differentially spliced transcripts (Gustafson et al. 1993; Dietrich et al. 1998; Oh and Waxman 1998).

The voltage-gated sodium channel β subunit family includes four members, β1, β2, β3, β4, and a splice variant of β1 designated as β1A/B. (McClatchey et al. 1993; Makita et al. 1994a, b; Eubanks et al. 1997; Morgan et al. 2000; Yu et al. 2003; Kazen-Gillespie et al. 2000; Qin et al. 2003). β subunits are transmembrane proteins with type I topology: they contain an extracellular N-terminus, a single transmembrane segment and an intracellular C-terminus. All four β molecules contain a high conserved extracellular immunoglobulin (Ig) domain which is structurally homologous to the V-set of the Ig superfamily including CAMs (Isom and Catterall 1996). This unique property of the sodium channel auxiliary subunits was first discovered following sequence analysis of β2, revealing that its extracellular domain contained an Ig fold and an extended region with similarity to the CAM contactin (Isom et al. 1995). The β1 and the β3 subunits are non-covalently associated with the α subunits, whereas the β2 and the β4 subunits are linked through a disulfide bond to the α subunit. Structurally, β1 is more closely related (~45% sequence identity) to β3, whereas β2 is more similar to β4 (35% identity) (Morgan et al. 2000). NaCh β-subunits are all detectable in brain tissues, peripheral nerves and heart, β1, β3 and β4 are also constitutive subunits of the skeletal muscle sodium channels (Isom et al. 1992; Candenas et al. 2006; David et al. 2008; Makita et al. 1994a, b; Stevens et al. 2001).

Although NaCh α subunits are sufficient to form functional channels when expressed alone in heterologous expression systems, the presence of regulatory β-subunits fine-tunes the channel activity (Catterall 2000). NaCh β subunits are able to regulate channel cell-surface expression levels, to modulate channel functions and to affect channel kinetics and voltage dependence (Isom 2001; Hanlon and Wallace 2002; Ferrera and Moran 2006; Moran et al. 2003). NaCh β subunits also function in vitro as hemophilic and/or heterophilic cell adhesion molecules that recruit cytoskeletal ankyrin following hemophilic cell adhesion (Malhotra et al. 2000, 2002), and may be responsible for targeting sodium channel complexes to specialized areas, such as the node of Ranvier and the axon initial segment. They may also stabilize the high density of sodium channels in these regions (Srinivasan et al. 1998; Ratcliffe et al. 2001; Brackenbury et al. 2008).

Mutations in β subunit genes result in a variety of human neurological and cardiovascular diseases, such as familial generalized epilepsy, hyperkalemic periodic paralysis, long QT syndrome or the Brugada syndrome (Rojas et al. 1991; Wallace et al. 1998; Escayg et al. 2000; Antzelevitch 2003; Fish and Antzelevitch 2003). Thus β proteins are critical elements not only when studying the physiological role of NaChs in excitable cells and tissues but also as therapeutic targets for the recognition and the treatment of human pathologies related to disfuctions of the NaChs.

In this study, we have selected five different cell lines from rat, mouse and human origin as model systems to assess the tissue-specific expression profile of the mRNAs encoding for the sodium channel α subunits named Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.5, Nav1.6 and Nav1.9 and for the non-covalently bound β1 auxiliary subunit, whose mutations play a pivotal role in the onset of many human neurological and cardiac diseases. In our analysis, we included also the β3 subunit because of its structural and functional similarity to the β1. In order to achieve our goal, we first conducted standard RT-PCR reactions to detect the NaCh subunit mRNAs expressed in the five cell lines and then used a semi-quantitative approach to determine the relative expression level of each NaCh transcript previously found.

Methods

Cell culture

The pituitary rat cell line GH3, the heart-myoblast rat cell line H9C2, the mouse neuroblastoma cell line N1E-115, the mouse skeletal muscle-myoblast cell line C2C12 and the human neuroblastoma cell line SH-SY5Y were grown as a monolayer culture at 37 °C in a CO_2 incubator that provided a humidified environment (95% air and 5% CO_2). The six cell lines were grown in standard culture medium, consisting respectively of Ham's F10 medium for the GH3 cells, of Dulbecco's Modified Eagle Medium (DMEM) for the H9C2, C2C12 and for the N1E-115 cells and of Ham's F10/DMEM (1:1) for the SH-5Y5Y cells. All media were supplemented with 2 mM L-glutamine and 10% FBS. The maintenance cultures, grown in 25 cm^2 polystyrene flasks (Corning Costar, Cambridge, MA, USA), were split once a week by using a mild trypsinization to remove the cells, and by replating at 20% of original density in a new flask. The medium was changed on day 3 after plating, and every 2 days thereafter. To prevent the loss of differentiation potential, cells were not allowed to become confluent. All culture media components were purchased from Sigma-Aldrich (St. Louis, MO, USA).

RNA isolation

Cells were harvested on day 6 after cell plating, detached by a mild trypsinization, and collected by centrifugation. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of total RNA for each sample was determined by optical density measurements at 260 and 280 nm. The integrity of the RNA was assessed by electrophoresis in a 1% agarose

gel. Total RNA was also isolated from the brain, the heart and the skeletal muscle of an adult Wistar rat and from an adult C57BL/6 mouse using TRIZOL Reagent (Invitrogen Ltd, Paisley, UK) according to the manufacturer's instructions. Procedure to remove tissues from anesthetised animals comply with the current local laws. Total RNA samples from these tissues were used as positive controls during the identification of NaCh channel RNA in rat and mouse cell lines, respectively. Total RNA samples were stored at −80 °C until analyzed by RT-PCR.

RT-PCR

First-strand cDNA was synthesized from 2.5 μg of each RNA using RevertAid First Strand cDNA Synthesis Kit and random hexamers according to the manufacturer's instructions (Fermentas, Burlington, Canada). The resulting cDNA samples were amplified by RT-PCR with specific oligonucleotide primer pairs (Bio-Fab Research, Roma, Italy) designed with the analysis software Primer 3 (Rozen and Skaletsky 2000) according to published NaCh subunit mRNA sequences (http://www.ncbi.nlm.nih.gov/nuccore). The sequences of the primers used and the technical details of the PCR reactions are shown in Table 1. Table 1 also shows the sequence of the oligonucleotide primers used to amplify glyceraldehyde-3-phosphate-dehydrogenase (GDH), which was assayed as housekeeping gene (Radonić et al. 2004).

RT-PCR products were resolved by electrophoresis in 1% agarose gels, stained with ethidium bromide and visualized under an UV transiluminator. Amplicon sizes were determined by comparison with a DNA molecular weight marker (Fermentas, SM0333) that was routinely run on the gels. Controls without reverse transcriptase were used to demonstrate the absence of contaminang DNA. Each experiment, including the RNA extraction, RT-PCR and gel electrophoresis was done in triplicate.

Optimization of RT–PCR conditions for amplification of Na channel cDNAs

In order to establish the optimal conditions for semi-quantitative RT-PCR, the exponential phase of the PCR reaction was defined using the GDH mRNA signal, which provided a constitutive marker for determining relative levels of channel transcripts. In order to avoid rapid saturation of signal intensity, reaction mixtures containing 2.5 μg of total RNA of each cell line in a final volume of 50 μl were retro-transcripted and used as template for RT-PCR reactions in these experiments. For instance, Fig. 1a shows the amount of PCR product generated at different cycle numbers using 2.5 μg of the total RNA of the GH3 cell line and the pair of primers for GDH. The amount of GDH mRNA (band intensity) from each cycle was quantified by densitometric analysis. From the results in Fig. 1b, it can be seen that the rate of amplification was exponential between 18 and 30 cycles, and that the four groups of data points within this range were well fitted assuming a 100% amplification efficiency. For further NaCh subunits analysis, cycle 30 was selected, as it was expected to result strong enough for easy quantification (20–30% of the maximal signal), but still within the exponential phase of the amplification before saturation. These determinations were repeated for each cell line. On the basis of these results, subsequent RT-PCR reactions were carried out untill cycle 30 of the RT-PCR reaction, using the optimized conditions summarized in Table 1.

Semi-quantitative RT-PCR

Relative levels of NaCh mRNAs were determined in each cell line by semi-quantitative RT-PCR (Horikoshi and Sakakibara 2000). Channel transcript levels were normalized to GDH mRNA signal, which was amplified in parallel with each NaCh RNA sample and served as an internal control. Equal amounts (4 μl) of each PCR product were run in triplicate on 1% agarose gels containing ethidium bromide and visualized under an UV transiluminator. The intensity of the electrophoretic bands was quantified from digital images using a custom procedure developed under IgorPro (Wavemetrics, Lake Oswego, OR, USA). The intensity of each RT-PCR product was normalized for its amplicon size and for the intensity of the band corresponding to GDH amplicon in order to obtain an estimation of the relative concentration for each transcript. Data from three separate experiments on different RNA preparations for each cell line were expressed as mean±standard error of the mean (sem).

Results

Identification of mRNAs encoding Na channel subunits

Standard RT-PCR was first performed on total RNA extracted from rat pituitary GH3, from embryonic myoblast H9C2, from mouse neuroblastoma N1E-115, from mouse cardiac myoblast C2C12 and from human neuroblastoma SY-SY5Y cell lines using sequence-specific primers for NaCh α subunits and for the two non-covalently linked sodium channel auxiliary β1 and β3 subunits (see Table 1). As shown in Fig. 2a, in the GH3 cell line, PCR products of the predicted sizes were detected when primers specific for the Nav1.1, Nav1.2, Nav1.3, Nav1.6, β1 and β3 mRNA sequences were used. In contrast, no PCR product could be detected in GH3 cells when primers specific for Nav1.4, Nav1.5 and Nav1.9 mRNA sequences were used. In the H9C2 cell line (Fig. 2b), PCR products of the predicted

Table 1 Sequences of forward and reverse primers used to amplify NaCh α and β subunits and size expected for each RT-PCR-amplified product. The sequence to amplify the "housekeeping" gene GDH is also shown. Database entry codes correspond to those of the NCBI (http://www.ncbi.nlm.nih.gov/nuccore)

protein	gene	database entry	Forward primer	Reverse primer	Amplicon size (bp)
Rat Na+channel α subunits					
Nav1.1	Scn1a	NM_030875	5′-CAACCTGGTGTTCATTGTGC-3′	5′-ACATCCCAA AGATGGCGTAG-3′	341
Nav1.2	Scn2a	NM_012647	5′-TCCCGATTTGAAGGAATGAG-3′	5′-GGTGGCTACTTGGAGCAGAG-3′	309
Nav1.3	Scn3a	NM_013119	5′-CCAGACCATGTGCCTTATTG-3′	5′-CCGTGTTATTGGACATGCAG-3′	293
Nav1.4	Scn4a	NM_013178	5′-CTTCATCGGTGTCATCATCG -3′	5′-GGAGACAGATGACCAGAGCC-3′	269
Nav1.5	Scn5a	NM_013125	5′-GTCTTCAAGCTGGCCAAGTC-3′	5′-ATTCAGGACCACAAGGTTGC-3′	360
Nav1.6	Scn8a	NM_019266	5′-CTCCAAGAAGCCACAGAAGC-3′	5′-ATGGAGAGGATGACCACCAC-3′	303
Nav1.9	Scn11a	NM_019265	5′-GCA AACCAAACGACTGTCCC-3′	5′-TTCTTAACGCACAGCCACTG-3′	216
Rat Na+channel β subunits					
β1	Scn1b	NM_017288	5′-TGAGACCGAGGCAGTGTATG -3′	5′-GTCGCCAGAGTGGTTGTAGG-3′	277
β3	Scn3b	NM_139097	5′-ACATCCTCCTGGTCTTCCTC -3′	5′-TATCCAGTCCCTCAGCACTC-3′	204
Mouse Na+channel α subunits					
Nav1.1	Scn1a	NM_018733	5′-CAACCTGGTGTTCATTGTGC-3′	5′-ACATCCCAA AGATGGCGTAG-3′	341
Nav1.2	Scn2a	NM_001099298	5′-GAGCCAGAAGCCTGTTTCAC-3′	5′-TTCGCTGCTCGATGTAAATG-3′	211
Nav1.3	Scn3a	NM_018732	5′-GCCTTCTTATCGCTGTTTCG -3′	5′-TCAACTGCTCCAACATCTGC-3′	244
Nav1.4	Scn4a	NM_133199.2	5′-GGCCATCATCGTCTTCATCT-3′	5′-ACAGGAGAGCCAGGAACAGA-3′	284
Nav1.5	Scn5a	NM_021544	5′-ACCACGGTTACACCAGCTTC -3′	5′-GCCTCGTGTTCCTTCTTGAG -3′	295
Nav1.6	Scn8a	NM_001077499	5′-CTCCAAGAAGCCACAGAAGC-3′	5′-ATGGAGAGGATGACCACCAC-3′	303
Nav1.9	Scn11a	NM_011887	5′-GCA AACCAAACGACTGTCCC-3′	5′-TTC TTAACGCACAGCCAC TG-3′	216
Mouse Na+channel β subunits					
β1	Scn1b	NM_011322	5′-CGAGTGGTGTGGAACGGTAG-3′	5′-AAGAGAGGAGGCCGAAGAGG-3′	966
β3	Scn3b	NM_153522	5′-ATTGGGTTGTCCTTCACTGC-3′	5′-GATGCTTCCTTGGCTTCTTG-3′	283
Human Na+channel α subunits					
Nav1.1	SCN1A	NM_006920	5′-GAAGAACAGCCCGTAGTGGAA-3′	5′-TTCAAATGCCAGAGCACCA -3′	225
Nav1.2	SCN2A	NM_021007	5′-GAAGGCAAAGGGAAACTCTGG-3′	5′-CAGTGAGACATCAACAATCAGGAAG-3′	297
Nav1.3	SCN3A	NM_001081676	5′-AAACCCCAACTATGGCTACACAA-3′	5′-TCCTAACCCACCTATTCCACTGA -3′	367
Nav1.4	SCN4A	NM_000334	5′-CATCAACACCACCACCTCTG-3′	5′-CACGAGGTCATACACCATGC-3′	484
Nav1.5	SCN5A	NM_198056	5′-GCTACACCAGCTTCGATTCC-3′	5′-GGGTGAGGCTGAGATGATTC-3′	486
Nav1.6	SCN8A	NM_014191	5′-TGGAGCACAACTGGTTTGAG-3′	5′-ACCAGGCATTGGTGAAGAAC-3′	210
Nav1.9	SCN11A	NM_014139	5′-GCA AAC CAA ACG ACT GTC CC-3′	5′-TTCTTAACGCACAGC CAC TG-3′	216
Human Na+channel β subunits					
β1	SCN1B	NM_001037	5′-CTTCAGACACGCACTTCTGG-3′	5′-TTCAAGGCTGGTGAGAGAGG-3′	231
β3	SCN3B	NM_018400	5′-GTCCATCACTGTGCTCAACG-3′	5′-AAGTCCTCTCCAGCCTCCTC-3′	150
glyceraldehyde-3-phosphate-dehydrogenase (GDH)					
GDH		NM_017008	5′-CAAGGTCATCCATGACAACTTTG-3′	5′-GTCCACCACCCTGTTGCTGTAG-3′	496

Reaction mixtures were processed in a GeneAmp PCR System 2400 thermocycler (Applied-Biosystems, Carlsbad, California). The PCR reactions mixture contained 1 μM of primers, 1.5 U of Taq DNA Polymerase (Sigma-Aldrich, Saint Louis, MO, USA), the buffer supplied, 2.5 mM $MgCl_2$, 200 μM dNTP and cDNA in 50 μl. After a hot start (3 min at 94 °C), the parameters used for PCR amplification were: 30 s at 94 °C, 30 s at 54/58 °C, 50 s at 72 °C for 30 cycles, and a final elongation step at 72 °C for 5 min

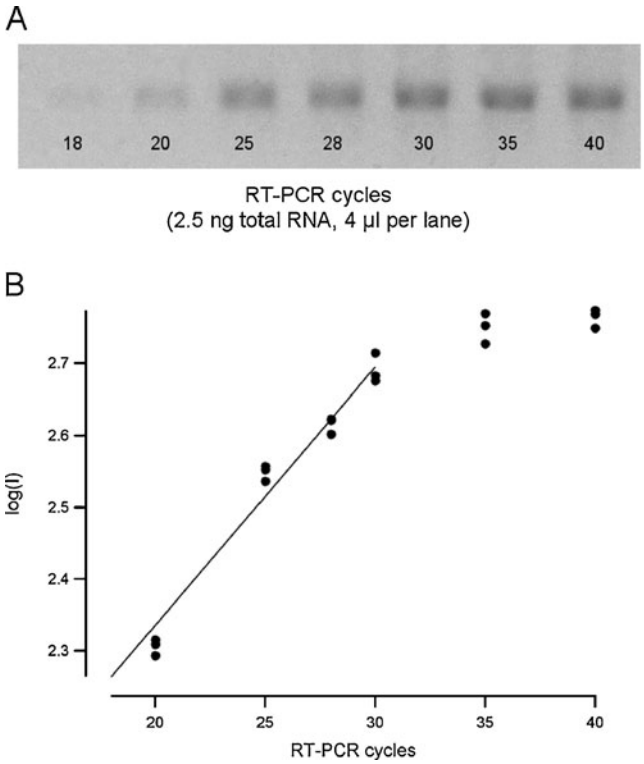

Fig. 1 Amplification of the cDNA for the GDH housekeeping gene as a function of the number of RT-PCR cycles. **a** Representative gel showing the amount of PCR product generated at the indicated cycle numbers when using 2.5 µg of total RNA from GH3 cells in the reaction mixtures (50 µl of final volume) and loading 4 µl of sample per lane. The product ran at the expected size of 496 bp. **b** Logarithmic plot of the protein density versus the number of PCR cycles. Data were derived from the densitometric analysis of three gels per RT–PCR reaction. The continuous line represents the fit of the first four data point groups with a linear function. An efficiency of 100% for the amplification process was assumed in this fitting

sizes were detected when primers specific for the Nav1.5, β1 and β3 mRNA sequences were used. On the contrary, no PCR products could be detected in H9C2 cells when primers designed against the Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.6, and Nav1.9 mRNA sequences were used.

Figure 3 shows the PCR products obtained from the total RNA extracted from rat brain (A), skeletal muscle (C) and heart (E), which served as positive controls. In these cases, the specific rat primers amplified products corresponding to the predicted sizes of Nav1.1, Nav1.2, Nav1.3, Nav1.5, Nav1.6, β1 and β3 mRNAs in brain, corresponding to Nav1.4, β1 and β3 mRNAs in skeletal muscle and to Nav1.1, Nav1.5, Nav1.6, β1 and β3 mRNAs in heart. Overall, these results indicate that in the two analyzed rat cell lines the NaCh isoform mRNA expression reflects a tissue-specific distribution, being the GH3 and the H9C2 cell lines from neuro-endocrine and myoblast origin, respectively.

We futher tested for the expression of mRNAs encoding NaCh α, β1 and β3 subunits in the murine N1E-115 and C2C12 cell lines (see Fig. 2c and d). The two pairs of primers designed to amplify these subunits, recognized PCR products corresponding to the Nav1.2, Nav1.3, Nav1.4, Nav1.6, β1 and β3 mRNAs in the N1E-115 cells and to the Nav1.4, Nav1.5, β1 and β3 mRNAs in the C2C12 cells. For comparison, Fig. 3 shows the PCR products obtained from the total RNA extracted from mouse brain (B), skeletal muscle (D) and heart (F), which served as positive controls. As for the rat cell lines, these results confirm a tissue-specific distribuition of the NaCh α and β subunits in the two analyzed mouse cell lines, from neuronal (N1E-115) and from myoblast (C2C12) origin, respectively.

The last cell line that we analyzed was the human derived neuroblastoma SH-SY5Y cell line. Figure 2e shows the PCR products detected using primers specific for the human NaCh α, β1 and β3 subunits. The recognized PCR products correspond, to the Nav1.2, Nav1.3, Nav1.9, β1 and β3 mRNAs. The results shown in Fig. 2 and in Fig. 3 were confirmed in total RNA preparations from three different batches of the five cell lines.

Evaluation of Na channel PCR product levels in the five cell lines

In order to assess the relative abundance of sodium channel transcripts in each cell line, aliquots of RNA from three different batches of the five cell lines were analyzed by semi-quantitative RT-PCR. For a given total RNA preparation, products resulting from separate amplifications with distinct primer sets were run on the same agarose gel, as illustrated in Fig. 2. The relative concentration of each NaCh subunit mRNA was calculated dividing its signal intensity value by its amplicon size and by the intensity value of the band corresponding to the GDH amplicon (see Table 2). The same approach was used to evaluate the relative concentration of the NaCh subunit mRNA expressed in rat and mouse brain, skeletal muscle and heart (see Table 3).

As shown in Table 2, in the GH3 cell line the normalised level of sodium channel transcripts followed the order of β3>Nav1.6>β1>Nav1.1>Nav1.2≈Nav1.3. Thus, the mRNA for the β3 subunit was 1.2–2.6 times more abundant than any other α subunit mRNAs investigated, and 1.45 times more abundant than the β1 mRNA. The relative concentration of the NaCh subunit mRNA expressed in rat brain are shown in Table 3. In rat whole brain, the normalised level of NaCh transcripts followed the order of β3>Nav1.1>Nav1.3>Nav1.2>Nav1.6>Nav1.5≈β1. Thus, as in rat pituitary cell line GH3, in rat whole brain, the mRNA for the β3 subunit was 1.2–2.3 times more abundant than any other α subunit mRNAs investigated, and 2.3 times more abundant than the β1 mRNA.

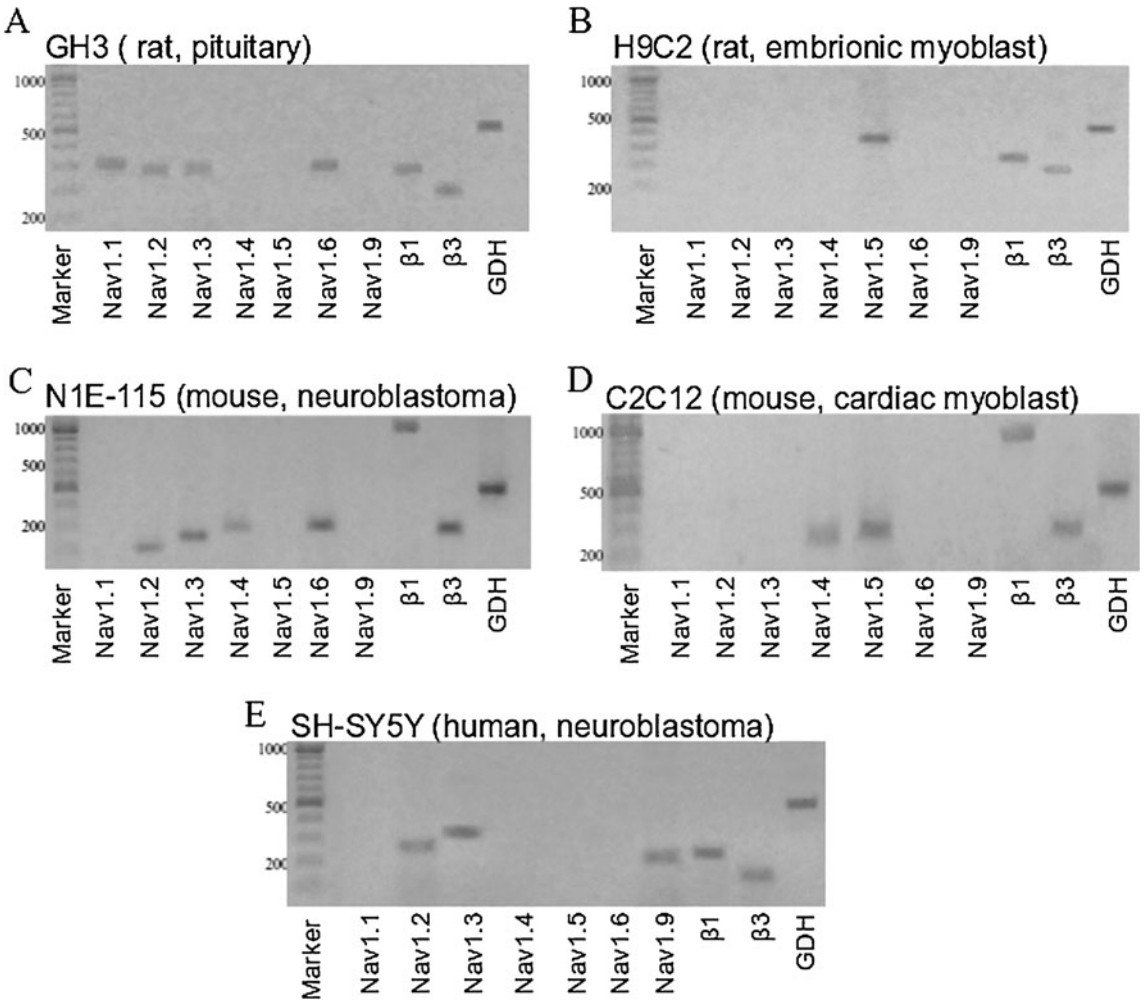

Fig. 2 Detection of transcripts of sodium channel subunits. Total RNA samples isolated from GH3 (**a**), from H9C2 (**b**), from N1E115 (**c**), C2C12 (**d**) and SHSY5Y (**e**) cells analyzed by standard RT–PCR. The primers used are listed in Table 1, along with the predicted sizes of PCR products. Amplified cDNAs were separated by electrophoresis on agarose gels and stained with ethidium bromide. For each cell line, one representative experiment out of three is shown. The relative abundance of each mRNA species is presented in Table 2

Conversely, in the murine neuroblastoma cell line N1E-115 the Nav1.6 and the β3 subunits were the most expressed subunits, being expressed 1.6–3.5 times more than the other NaCh transcripts following the order: Nav1.6≈β3>Nav1.3>-Nav1.2>Nav1.4>β1. In mouse whole brain (Table 3), the Nav1.1 and the β1 mRNA were found to be the most and the less abundant NaCh subunit transcripts, yielding 0.66±0.01 and 0.48±0.01, respectively. The NaCh subunit mRNAs found in this tissue were expressed in the following order: Nav1.1>Nav1.2>Nav1.3>Nav1.6>β3≈Nav1.5>β1 .

In the human derived neuroblastoma SY-SY5Y cell line, the β3 mRNA was found to be the most abundant among the NaCh subunit transcripts, yielding 0.95±0.01. The NaCh subunits mRNAs were expressed in the following order: β3>β1>Nav1.3>Nav1.9>Nav1.2, yielding 0.95±0.01, 0.89±0.02, 0.57±0.02, 0.41±0.06, 0.25±0.08, respectively.

The embryonic cardiac myoblast rat cell line H9C2 expressed exclusively the Nav1.5 as NaCh α-subunit mRNA, which was transcribed about 1.11–1.15 times more than the β1 and β3 subunits, yielding 0.60±0.02, 0.54± 0.04 and 0.52±0.01, respectively. In rat heart, the mRNAs for the Nav1.5 and for the β1 subunits were the most and the less abundant transcripts. The NaCh subunit mRNAs found in this tissue were expressed in the decreasing order: Nav1.5>β3≈Nav1.1>Nav1.6>β1. In mouse heart the NaCh subunit transcript expression of NaCh mRNA was the following: Nav1.5>β1>β3>Nav1.4>Nav1.1>Nav1.6. In particular, the Nav1.5 subunit mRNA is from 2.3 to 1.1 times more abundant than any of the other NaCh subunit mRNAs, confirming its tissue specificity.

The NaCh transcript expression profile of the murine embryonic myoblast cell line C2C12 was: Nav1.5>-Nav1.4≈β3>β1, being the Nav1.5 subunit mRNA 5.6 times more expressed than the β1 subunit. For comparison, we analysed the expression profile in mouse skeletal muscle, where the Nav1.4 subunit was the only NaCh α-subunit

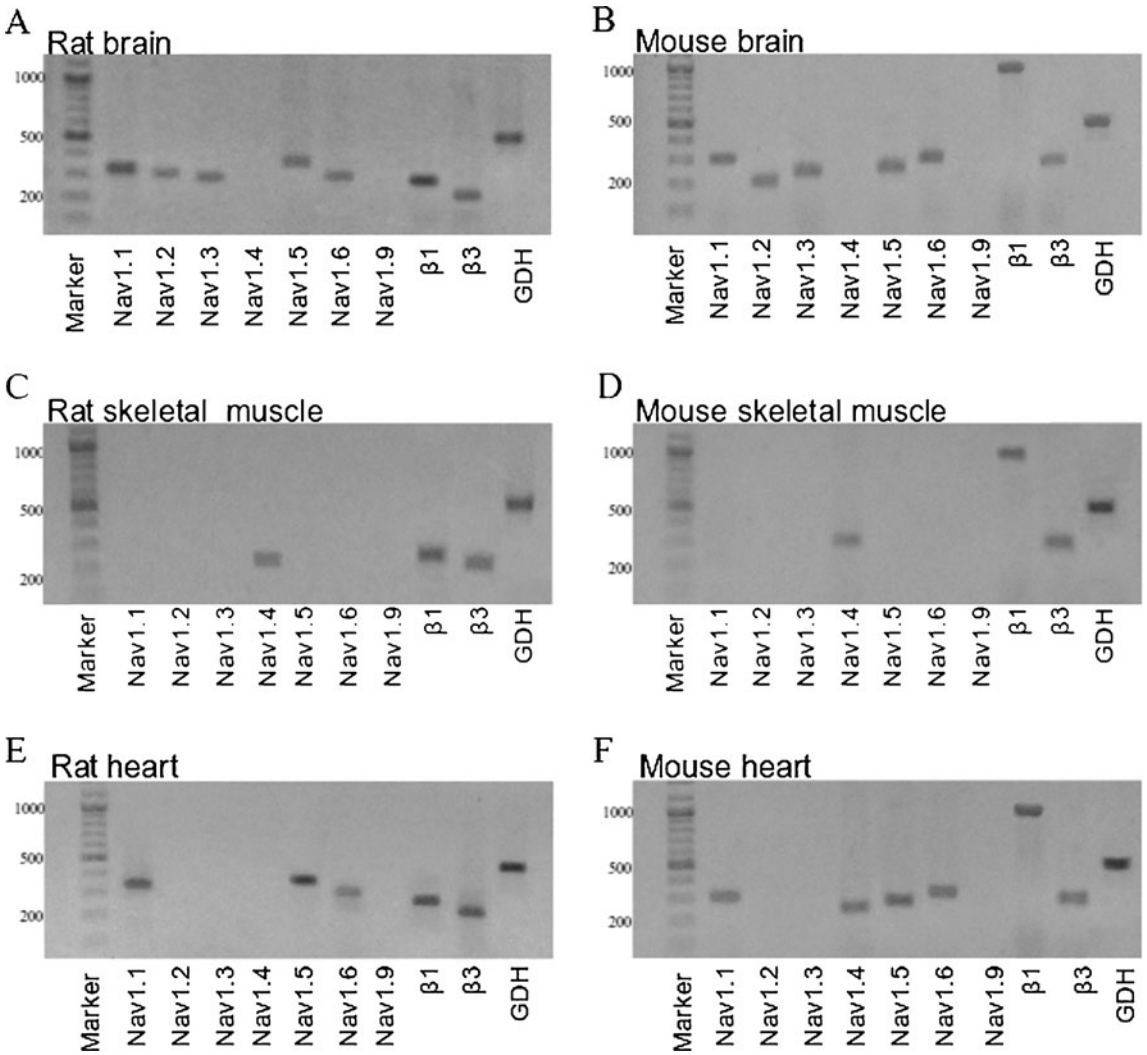

Fig. 3 Detection of transcripts of sodium channel subunits in total RNA samples isolated from adult rat brain (**a**), skeletal muscle (**c**), heart (**e**) and from adult mouse brain (**b**), skeletal muscle (**d**) and heart (**f**) used as a positive control for the identification of NaCh channel subunit RNA in rat and mouse cells. RNA samples were retrotranscribed and PCR-amplified using the primers listed in Table 1. Amplified cDNAs were separated by electrophoresis on agarose gels and stained with ethidium bromide. For each cell line, one representative experiment out of three is shown

expressed, yielding 0.56±0.04. Instead, the two non-covalently linked auxilary β subunits yielded 0.15±0.01 and 0.34±0.03, respectively. Also in rat skeletal muscle Nav1.4 is the only NaCh α-subunit expressed, together with the β1 and β3 subunit mRNAs. The semi-quantitative RT-PCR analysis reveals a yield of the three transcripts of 0.81±0.01, 0.17±0.03 and 0.42±0.05, respectively.

Discussion

We have undertaken a systematic analysis of the expression profile of both the NaCh α-subunit isoforms and the two non-covalently linked auxiliary subunits, β1 and β3. The β1-subunit is an interesting part of the sodium channel that is not essential for the α-subunit functional heterologous expression (Isom 2001; Goldin 1993; Gurnett and Campbell 1996; Makita et al. 1996; McCormick et al. 1999). However, the relevance of this subunit is evident from its correlation with different genetic diseases: generalized epilepsy with febrile seizures plus (GEFS+), the Brugada syndrome or other cardiac conduction defects implying complete left or right bundle branch block and prolonged PR interval on electrocardiogram (Wallace et al. 2002; Tammaro et al. 2002; Audenaert et al. 2003; Watanabe et al. 2008). Since its molecular identification, different, and sometimes opposite, effects on the NaCh function have been ascribed to this ancilary subunit. The effects span from modification of the inactivation, to alteration of the activation, up to α-subunits trafficking localization in the plasma membrane.

The main purpose of this work was to find out a set of cell lines that could be used as a model for the study of the

Table 2 Differential expression of the mRNA coding for each NaCh subunits in the five cell lines. The intensity of each RT-PCR product was normalized for its amplicon size and for the intensity of the band corresponding to GDH amplicon in order to obtain an estimation of the relative concentration of each transcript. Data from three separate experiments on different RNA preparations of each cell line were expressed as mean± standard error of the mean

Subunit	Rat		Mouse		Human
	GH3	H9C2	N1E-115	C2C12	SH-SY5Y
Nav1.1	0.40±0.02	–	–	–	–
Nav 1.2	0.34±0.04	–	0.46±0.02	–	0.25±0.08
Nav 1.3	0.34±0.07	–	0.57±0.01	–	0.57±0.02
Nav 1.4	–	–	0.33±0.05	0.60±0.02	–
Nav 1.5	–	0.60±0.02	–	0.91±0.08	–
Nav 1.6	0.69±0.09	–	0.92±0.01	–	–
Nav 1.9	–	–	–	–	0.41±0.06
β1	0.61±0.02	0.54±0.04	0.26±0.03	0.16±0.03	0.89±0.02
β3	0.89±0.02	0.52±0.01	0.93±0.01	0.60±0.04	0.95±0.01

regulation of the NaCh expression. Hence, we designed a series of experiments to recognize and to semi-quantitatively evaluate the presence of mRNAs encoding for the different NaCh α- subunits as well as for the β1-subunit in five cell lines. We have included also the β3 subunit, as it was described to be analogue to the β1-subunit (Qu et al. 2001; Shah et al. 2001).

So as to obtain the NaCh expression profile, we used RT-PCR to analyse the expression of mRNAs for NaCh α subunits Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.5, Nav1.6 and Nav1.9 and for the two non-covalently linked auxiliary subunits β1 and β3. A successful RT-PCR experiment depends on the quality and specificity of the designed oligonucleotide primers. In our experiments we used specific oligonucleotide primer pairs designed with the analysis software Primer 3 (Rozen and Skaletsky 2000) according to published specie-specific NaCh subunit mRNA sequences (http://www.ncbi.nlm.nih.gov/nuccore). This application allowed us to overcome all the critical steps in designing the RT-PCR oligonucleotide primer pairs. Particular care was observed to design species specific primers, and to consider the alternative splicing forms reported for the NaCh subunits (Schaller et al. 1992; Gustafson et al. 1993; Lu and Brown 1998; Kasai et al. 2001; Raymond et al. 2004; Plummer et al. 1997; Belcher et al. 1995; Gustafson et al. 1993; Dietrich et al. 1998; Oh and Waxman 1998). Under these circumstances, we designed primer pairs that were complementary to the cDNA region common to all the splicing isoforms of the protein, in order to detect at least the presence of one of the different splicing forms expressed in the cell line under analysis.

We detected the mRNAs encoding for the Nav1.1, Nav1.2, Nav1.3 and Nav1.6 α subunits in rat and mouse brain extracts, being in both cases the Nav1.1 transcript the most expressed among the α pore-forming subunits. We also detected the transcripts of the two non-covalently linked auxiliary subunit mRNAs. It is worth noting that the expression of the β3 mRNA was higher in rat than in mouse brain.

Table 3 Differential expression of the mRNA coding for each NaCh subunits in rat and mouse brain, skeletal muscle and heart, respectively. The intensity of each RT-PCR product was normalized for its amplicon size and for the intensity of the band corresponding to GDH amplicon in order to obtain an estimation of the relative concentration of each transcript. Data from three separate experiments on different RNA preparations of the three rat or mouse tissues are expressed as mean±standard error of the mean

Subunit	Rat			Mouse		
	Brain	Skeletal muscle	Heart	Brain	Skeletal muscle	Heart
Nav1.1	0.75±0.01	–	0.72±0.01	0.66±0.01	–	0.49±0.01
Nav 1.2	0.60±0.03	–	–	0.64±0.01	–	–
Nav 1.3	0.68±0.02	–	–	0.62±0.04	–	–
Nav 1.4	–	0.81±0.01	–	–	0.56±0.04	0.69±0.02
Nav 1.5	0.39±0.02	–	0.83±0.03	0.51±0.02	–	0.97±0.01
Nav 1.6	0.41±0.02	–	0.41±0.01	0.58±0.02	–	0.43±0.03
Nav1.9	–	–	–	–	–	–
β1	0.39±0.03	0.17±0.03	0.30±0.03	0.48±0.01	0.15±0.01	0.89±0.01
β3	0.89±0.01	0.42±0.05	0.73±0.02	0.52±0.02	0.34±0.03	0.80±0.03

We compared the NaCh subunits expressed in three different neuroblastoma cell lines with those expressed in rat or mouse brain in order to find out if any of these neuro-ectodermal cell lines could be used as a representative model system to study the functional properties of neuronal NaChs. The neuro-endocrine cell line GH3 expressed the mRNAs for four NaCh channel α subunits (Nav1.1, Nav1.2, Nav1.3 and Nav1.6) and for the two non-covalently linked auxiliary subunits (β1 and β3). Transcripts for the other subunits investigated (Nav1.4, Nav1.5 and Nav1.9) were not detectable in these cells. The RT-PCR analysis of the mouse-derived N1E-115 neuroblastoma cell line detected the expression of the transcripts for the sodium channel Nav1.2, Nav1.3, Nav1.4 and Nav1.6 α subunits together with the expression of the β1 and β3 auxilary subunits. Instead, in this cell line we did not find any expression of the neuronal form of the Nav1.1 transcript that we detected in the GH3 cell line. In the human derived neuroblastoma cell line SH-SY5Y our RT-PCR analysis revealed the presence of the mRNAs for Nav1.2, Nav1.3 subunits which are mainly expressed in the central nervous system, but also of the mRNA encoding for the Nav1.9 α subunit. This is compatible with the origin of this cell line from the peripheral nervous system where the Nav1.9 is highly expressed. In these neuro-ectodermal cell lines as well as in the previously mentioned whole brain extracts, the expression level of the transcript for the β3 subunit was always higher than that of the β1 subunit. Our findings point out that none of the three analysed cell lines exactly reflects the NaCh subunit expression of the nervous system, although our NaCh subunit expression profile data suggest that the GH3 could be assumed as a good model to study the functional properties of the NaChs of the central nervous system, while the SH-SY5Y could be used as a representative cell line of the peripheral nervous system, because of the presence of the Nav 1.9 subunit transcript.

Our RT-PCR analysis on rat and mouse skeletal muscle extracts revealed the presence of the mRNAs encoding for the Nav1.4 α subunit and for the two auxiliary subunits, β1 and β3. Also in mouse skeletal muscle C2C12 cell line, we detected a high expression of the Nav1.4 transcript. This is not surprising, being the Nav1.4 subunit primarily expressed in muscle (George et al. 1992; Gellens et al. 1992). Instead the presence in the C2C12 cell line of the mRNA encoding for the Nav1.5 subunit is probably due to the origin of this cell line. In fact myoblasts are not yet completely differentiated cells. These results, togheter with the finding that in these cells the over-expression of the trascription factor MRF4 is able to dramatically elevate the Nav1.4 gene expression, render the C2C12 cell line a good model system to study the functional properties of the NaCh subunits expressed in skeletal muscle (Thompson et al. 2005).

In rat and mouse heart, the "cardiac" Nav1.5 subunit was strongly expressed (Gellens et al. 1992). Our results also showed the expression of the Nav1.1 and Nav1.6 mRNAs in rat and of the Nav1.1, Nav1.4 and Nav1.6 mRNAs in mouse heart. In both tissues we detected also a high expression of the mRNAs encoding for the two auxiliary β subunits. Instead, in the rat cardiac myoblast cell line H9C2 the only α subunit expressed was the Nav1.5, together with the β1 and β3 subunit mRNAs. Although this cell line retains characteristics typical of undifferentiated cells, it could be chosen as a representative model to study the behaviour of the Nav1.5 in the heart. Furthermore it could be used to study its interaction with the auxiliary β1 subunit in human cardiac diseases.

This systematic study of the NaCh subunit expression profile highlighted that none of the five cell lines under analysis properly reflects the distribution of the NaCh subunits of an adult tissue. This can be due to many factors. First, we have to consider that cell lines are transformed cells that may have diverse differentiation conditions with respect to adult tissues. Moreover, when studying a cell line, we focused our attention on a well-identified homogeneous cell population, while the cell population of a tissue is quite heterogeneous. Nevertheless, our results strongly suggest that these cells have features that are closely related to those of adult cells present in the original tissue, that may be further regulated by appropriate growing factors.

These findings encourage us to use these cell lines to study the functional role of NaChs in cellular excitability, and to evaluate how slight changes in NaCh subunit expression profile could lead to human pathologies related to NaCh disfuctions.

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