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Research report

Sodium/calcium exchanger subtypes NCX1, NCX2 and NCX3 show cell-specific expression in rat hippocampus cultures

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Abstract

Na^+/Ca^{2+} exchange activity is known to be expressed throughout the brain in both glial and neuronal tissue. mRNA of all three major subtypes of the mammalian Na^+/Ca^{2+} exchanger protein (NCX1, NCX2, NCX3) has been detected in most brain areas, albeit at varying densities. [The term 'subtype' is used for exchangers that are products of different genes (NCX1, NCX2, NCX3); 'isoform' is used for splice variants of a single gene product]. However, for lack of subtype specific labels, the cellular expression pattern of this transport protein has remained largely unknown. We have now used three subtype-specific antibodies, two monoclonal and one polyclonal, to identify the cellular distribution of the exchanger subtypes in rat hippocampus cell cultures. Surprisingly, we found little overlap for the expression of this membrane protein in different cell types. NCX1 labeled mainly the membranes of neuronal cells and their associated dendritic network. It was found in nearly all neuronal cells of the population growing in culture. In cultures maintained for more than 3 weeks, NCX1 was increasingly detected in the membrane of glia cells. NCX2 immunoreactivity was predominantly localized in various types of glia cells. It was also detected in the membranes of a few neuronal cell bodies but never in the dendritic network. In addition to labeling membranes, the NCX2 antibody strongly cross-reacted with an unidentified glial fibrillar protein. NCX3 expression appeared very low in hippocampus cultures and was restricted to a small subpopulation of neuronal cells. It was never detected in glia cells. Our results provide novel information on the cell-specific expression of the three Na^+/Ca^{2+} exchanger subtypes (NCX1, NCX2 and NCX3) in mammalian brain. These data may reflect functional differences among the subtypes that are not obvious from studies in recombinant cell lines and hence, may help to understand the functional role of specific glia- or neuron-associated Ca^{2+} transport systems.

Theme: Excitable membranes and synaptic transmission

Topic: Other ion channels

Keywords: Sodium/calcium exchanger; Cellular distribution pattern; Immunocytochemistry; Confocal microscopy; Monoclonal antibodies; Membrane protein expression

1. Introduction

The Na^+/Ca^{2+} exchanger that is expressed in the plasma membrane of a variety of excitable and non-excitable tissues uses a Na^+ gradient to activate the transport of Ca^{2+} in the opposite direction. The exchanger exhibits a coupling ratio of 3:1 Na^+/Ca^{2+} ions and hence, under resting conditions with an inwardly directed Na^+ gradient, will contribute significantly to maintaining a low intracellular Ca^{2+} concentration (for review see Ref. [1]). A mammalian cardiac Na^+/Ca^{2+} exchanger (NCX1) was first cloned by Nicoll et al. [16]. Further subtypes that are products of different genes (NCX2 and NCX3) were later identified in rat brain [13,17].[1] In addition, alternative

Abbreviations: NCX, sodium/calcium exchanger protein; mAb, monoclonal antibody; BHK, baby hamster kidney cells; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; GFAP, glia fibrillar acidic protein

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[1]In the text 'subtype' will be used for exchangers that are products of different genes (NCX1, NCX2, NCX3). The term 'isoform' will be used for splice variants of a single gene product.

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splicing variants of the NCX1 and NCX3 subtypes have been detected [12,21]. The specific tissue distribution of the three subtypes, as inferred from the mRNA levels, shows a characteristic pattern. While NCX1 is ubiquitously expressed, the distribution of NCX2 and NCX3 seems to be restricted to brain and skeletal muscle. In the central nervous system the exchanger protein is widely present both in neuronal cells [11,27] and glia [5,26]. Nevertheless, the mRNA of each of the three subtypes shows a distinct distribution pattern in different brain regions [28]. Overall, the expression of NCX2 appears to exceed by several fold the expression of the two other subtypes [24,28]. Studies with the NCX1-selective monoclonal antibody (mAb) R3F1 have shown that this exchanger subtype is present in neuronal cell membranes. It is particularly enriched in synaptic regions of dendrites where Ca^{2+} plays an important role in neurotransmitter release [22]. On the other hand, it has remained unclear whether individual cells may contain more than one exchanger subtype.

Functionally, the three exchanger subtypes and their splice variants seem to have remarkably similar properties though a few distinctions can be made [9,14]. The highly restricted expression of individual exchanger proteins suggests that this distribution might be associated with specific physiological functions. However, no such link has been established so far.

In the present study, we have used antibodies specific for individual NCX subtypes to assess their distribution at the protein level in different cell populations of primary rat hippocampus cell cultures. Our results suggest that there is little co-expression of exchanger subtypes in individual cells. Moreover, the NCX2 protein appears to occur almost exclusively in glia while NCX1 and NCX3 are mainly restricted to neuronal cells. These results provide a basis for a better understanding of the physiological significance of subtype expression patterns.

2. Materials and methods

2.1. Tissue culture

2.1.1. Culture of hybridoma cells

Hybridomas producing monoclonal antibodies were generally maintained in a medium prepared by mixing equal parts of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml amphotericin B and 1% Nutridoma NS supplement (Roche Molecular Biochemicals). The cells were grown in a H_2O-saturated atmosphere containing 5% CO_2 and 95% air while supernatant was harvested every 24 h.

2.1.2. Culture of baby hamster kidney (BHK) cells

Recombinant BHK cells expressing either canine cardiac NCX1 or rat brain NCX2 or rat brain NCX3 exchanger subtypes were kindly provided by Dr B. Linck, University of Münster, Germany. The cells were grown in DMEM supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1 μg/ml fungizone. To eliminate possible revertants positively transfected cells were transiently grown in the original selection medium containing 2.5 mM amethopterin.

2.1.3. Culture of primary rat hippocampus cells

Isolation and cultivation of hippocampal cells followed established methods [15,22,23]. Briefly, cells from the CA1–CA3 regions were isolated by digesting small tissue sections with trypsin type XI (Sigma) and DNase type IV (Sigma). The cells were plated on poly-L-ornithine coated cover slips at a density of 6000–20 000 cells (depending on whether glia or neuronal cells were studied, respectively) within an area confined by cloning cylinders (28 mm^2) tightly attached to the cover slips using silicon grease. The culture medium was prepared from Eagle's minimal essential medium supplemented with 29.2 mg/l glutamax I (Gibco), 6 g/l glucose, 25 mg/l insulin, 100 mg/l transferrin, 5 mg/l gentamycin, 5% fetal calf serum and 2% B-27 neuronal growth supplement (Gibco). To inhibit glia cell growth, cytosine-β-D-arabinofuranoside (Ara-C, 3 μM) was added if a high proportion of neuronal cells was desired. The cultures were maintained at 37 °C in a water-saturated 8% CO_2/92% air atmosphere for 14–21 days prior to immunohistochemistry. During this period, the medium was changed every 2–3 days.

2.2. Antibody preparation

2.2.1. Preparation of fusion proteins

Fusion proteins composed of the long cytoplasmic loops of NCX2 and NCX3 with six His tags at the amino termini were synthesized for antigens. The fusion proteins were constructed by PCR amplification. The PCR primers included restriction enzyme sites for *Bam*HI and *Hin*dIII (forward NCX2 primer: ATCATCGGATCCGAGGGAGACCCGCCC and reverse NCX2 primer: GGATGGAGGCTTCTCCTCACGAGACCC; forward NCX3 primer: ATCATTGGATCCGAGGGTGAACACCCTA, and reverse NCX3 primer: TGATGGAAGCTTCTCCTCTCCAGATTCAT). The PCR products were subcloned into the pSK^+ vector (Stratagene), sequenced, then digested with *Bam*HI and *Hin*dIII, and subcloned into the pQE-30 vector (Qiagen) to generate p30NCX2L and p30NCX3L.

Fusion proteins containing residues E272–E716 of NCX2 [13] or E276–722 of NCX3 [17] were generated after inducing cells containing p30NCX2L or p30NCX3L with IPTG. Fusion protein was isolated under denaturing conditions in the presence of urea with Ni-NTA resin (Qiagen) according to the manufacturer's instruction. Urea

was removed from the purified fusion protein and replaced with PBS by step-wise dialysis.

2.2.2. Preparation of monoclonal antibody R3F1

This antibody was generated as described previously using as antigen recombinant canine cardiac exchanger (NCX1) expressed in baculovirus-infected Sf9 insect cells [20].

2.2.3. Preparation of monoclonal antibody W1C3

This antibody was raised against the 30NCX2L fusion protein. The overall procedure for antibody generation followed the one described in Porzig et al. [20]. From immunizations with this antigen, the only subtype-specific antibodies that could be recovered were of the IgM class. Purification of these mAbs by methods requiring adsorption to column materials (ion-exchange, hydroxyl apatite) resulted in severe loss of immunoreactivity. Therefore, immunoglobulins were first precipitated from the culture supernatant in 50% ammonium sulfate, centrifuged at 10 000×*g* and re-solubilized in 1/10th of the original volume with Na–Tris solution (100 mM Tris–Cl, 150 mM NaCl, pH 8.0) [6]. The protein solution was further concentrated by ultrafiltration using a 10 000-MW cutoff membrane (Centriprep-10 tubes, Amicon), followed by size exclusion chromatography with FPLC (Sephacryl S-300 High Resolution column, Amersham Pharmacia Biotech). The column was eluted with Na–Tris solution and the eluate collected in 5-ml fractions. The IgM mAbs, appearing in the first protein peak after the void volume peak, were further concentrated by ultrafiltration. The final stock solution containing 4–7 mg/ml protein was mixed 1:1 with glycerol and stored at −20 °C. A single freez–thaw cycle resulted in almost complete loss of immune reactivity. Analysis of the partially purified preparation by SDS–PAGE and immunoblotting with anti-mouse IgM suggested that the specific mAb probably accounted for less than 10% of the total protein concentration.

2.2.4. Preparation of the polyclonal NCX3 antibody

Polyclonal antibodies against the purified 30NCX3L fusion protein coupled to KLH were generated at the UCLA Antibody and RadioImmunoassay Core Facility by Helen Chu Wong and John H. Walsh.

2.2.5. Immunoblotting with NCX antibodies

1. BHK cell homogenate. BHK cells were scraped from culture dishes and immediately transferred into ice-cold (4 °C) phosphate-buffered saline (PBS) containing a proteinase inhibitor cocktail (Complete mini®, Roche Diagnostics). The cells were homogenized with a Potter-type glass-Teflon® homogenizer. The final preparation contained 1.8–4.4 mg protein/ml and was stored at −20 °C.
2. Hippocampus homogenate. Hippocampus cell cultures were washed in PBS, scraped from the growth surface in PBS supplemented with protease inhibitors as above and either stored at −20 °C or immediately dissolved in SDS sample buffer for PAGE.
3. Cardiac membrane vesicles. Myocardial membranes were purified from equine hearts according to standard methods as described in Ref. [19].

Polyacrylamide gel electrophoresis (PAGE), Western blotting and immune reactions were performed according to standard procedures with or without prior membrane protein extraction. For plasma membrane protein extracts, the cell homogenate was first sedimented at 14 000×*g*, and then resuspended in lysis buffer (NaCl 137 mM, Tris–Cl 20 mM, DTT 0.5 mM, Triton X-100 1% and including the CompleteMini® protease inhibitor cocktail). The mixture was incubated on ice for 30 min and again sedimented. After separation of supernatant and sediment, both fractions were separately subjected to PAGE and blotting procedures. Peroxidase-labeled second antibodies were detected with the enhanced chemoluminescent (ECL) assay of Amersham or with the Lumilight® preparation of Roche Diagnostics.

2.2.6. Protein determination

The protein content of antibody solutions and tissue samples was measured with the colorimetric bicinchoninic acid (BCA) method using the Micro BCA assay kit from Pierce (Rockford, IL).

2.2.7. Immunohistochemistry

Rat hippocampus cultures confined by cloning cylinders were first rinsed three times in PBS and then fixed and permeabilized in pre-cooled absolute ethanol at minus 20 °C for 6 min. Following rehydration in PBS, the cultures were washed 2×5 min in blocking solution (BS, 10% FCS in PBS) and incubated overnight at 4 °C in BS in the presence of primary antibodies (W1C3 20–30 μg/ml, R3F1 5–10 μg/ml), rabbit anti-NCX3 antiserum as well as commercial antibodies directed against vimentin, glia acidic fibrillar protein (Transduction Laboratories, Lexington, KY) and tubulin (Sigma, Buchs, Switzerland) were used in 1:50 or 1:100 dilutions). At the end of the incubation period, the cultures were washed four times in BS and incubated in the dark for an additional 2 h at room temperature in the presence of 1:50 dilutions of fluorescein- or rhodamine-conjugated second antibodies (Affini-Pure goat anti-mouse IgG (H+L), goat anti-mouse IgG Fcγ fragment specific, donkey anti-mouse IgM, μ-chain specific, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The cover-slip cultures were subsequently washed for about 50 min in four changes of BS and finally mounted on glass slides using 80% v/v glycerol and 0.2% paraphenyldiamine in 0.1 M phosphate, pH 8.6 as a bleach-inhibiting medium. The cover-slips

were sealed to the slide with nail-polish to avoid evaporation.

2.2.8. Laser scanning microscopy

Stained cultures were examined using a Zeiss LSM 510 laser scanning confocal microscope equipped with argon and HeNe lasers and 100×, 63× and 40× oil objectives that was driven by the Windows NT 4.0 based software of the manufacturer. The fluorescein isothiocyanate (FITC) fluorophore was excited with the 488-nm line of the argon laser and its emission band collected between 503 and 530 nm. The tetramethyl rhodamine isothiocyanate (TRITC) fluorophore was excited by the 543 nm line of the HeNe laser and its emission band collected between 560 and 615 nm. In dual labeling experiments great care was taken to balance brightness and contrast such that neither green nor red fluorescence dominated the emission. The pinhole was usually set to values allowing 0.75–1.5 μm depth of focus. Images were scanned with a resolution of 1024×1024 pixels per frame using 0.75–1.5-μm sections reaching from the basal glia layer to neuronal cell bodies positioned on top of the glia cells. Differential interference contrast (DIC) images were scanned in transmission mode using the 488 or 543 nm lines of the argon or HeNe laser, respectively. For all conditions and instrument settings, background fluorescence was checked using unspecific mouse IgG or IgM (rather than the specific antibodies) at the same concentration together with the FITC- or rhodamin-labeled second antibodies. Background fluorescence was extremely faint, such that no structures were visible on the corresponding printouts. Therefore, no such controls have been included in Fig. 3. Printouts were generated by a Sony UP-D2500 digital color printer or a Tektronix Phaser 750 Plus laser color printer.

3. Results

3.1. Characterization of subtype-specific NCX antibodies

Possible antigenic cross reactivity among the three antibodies directed against NCX1, NCX2 or NCX3 was analyzed using three recombinant baby hamster kidney (BHK) cell lines expressing exclusively each one of the three exchanger subtypes [14] and horse cardiac sarcolemma vesicles, expressing exclusively NCX1. The result of this analysis is shown in Fig. 1A–D.

The mAb R3F1 has been characterized earlier and was known to react preferentially with the NCX1 exchanger subtype. In cardiac sarcolemma this antibody labeled a prominent band close to 120 kDa. Often a minor band at 70 kDa was also visible. In NCX1-expressing BHK cells, a single strong antibody-labeled band always appeared close to 205 kDa. Most likely this band resulted from a partially irreversible aggregation of the protein in NCX1-overexpressing cells. In Triton X-100 extracts of these cells the aggregate band could be partially resolved thus revealing the 120 kDa and the 70 kDa bands known from cardiac preparations (Fig. 1 B). No major bands were labeled in BHK cells expressing NCX3 or in control BHK cells. However, a weak cross-reaction was sometimes visible with a 60-kDa band in homogenates from NCX2 expressing BHK cells (not shown).

The mAb W1C3 reacted prominently only with NCX2 expressing BHK cells while all other BHK cell lines (including wild type) shared some weakly labeled unspecific bands. In NCX2 cells, the number of bands labeled by the antibody was strongly dependent on the concentration of protease inhibitors during sample preparation. With the most efficient cocktail (Complete mini® from Roche Diagnostics), we observed a major band close to 60 kDa. Labeling in the high molecular weight range between 120 and 206 kDa varied in strength (compare Figs. 1C and 2C). Neither the high molecular weight bands nor the strong band at 60 kDa was shared by NCX1 or NCX3 expressing cells, nor was there a specific reaction with cardiac sarcolemma (not shown). This observation is compatible with the assumption that the NCX2 protein is particularly prone to proteolytic cleavage by endogenous proteases [14]. During sample preparation, a major fraction of the large intracellular loop of NCX2 containing the epitope for mAb W1C3 appears to be cleaved very efficiently by proteolytic activity. Unlike the NCX1 protein, NCX2 could not be extracted from BHK cell homogenates with the Triton X-100 method. The immune reactivity remained quantitatively in the triton-insoluble sediment (Fig. 2C).

The polyclonal rabbit antibody reacted prominently with NCX3 expressing BHK cells labeling a single major band close to 120 kDa (Fig. 1D).

3.2. Labeling of hippocampus cell homogenates with subtype-specific NCX1–3 antibodies

To test whether the three NCX subtypes are all present in hippocampus tissue cultures, we prepared cell homogenates and Triton extracts from cells cultivated for 14–23 days on glass cover slips as well as from freshly dissected hippocampus preparations. A rough estimate of the relative abundance of the three exchanger subtypes in neuronal or glia cells was attempted by using either ‘neuron-rich’ or ‘glia-rich’ cultures for the preparation of PAGE samples. ‘Glia rich’ cultures contained few isolated neurons. ‘Neuron rich’ cultures, were maintained in the presence of Ara-C to inhibit glia cell growth. Nevertheless, these cultures still contained a thin glia cell layer covering a major fraction of the growth surface. Neuronal cells grew in densely interconnected groups on top of the glia cells. Further suppression of glia cell growth resulted invariably in protracted neuronal cell death. Immunoblots from neuron rich cultures are presented in Fig. 2A–C.

In culture homogenates all of which contained a large

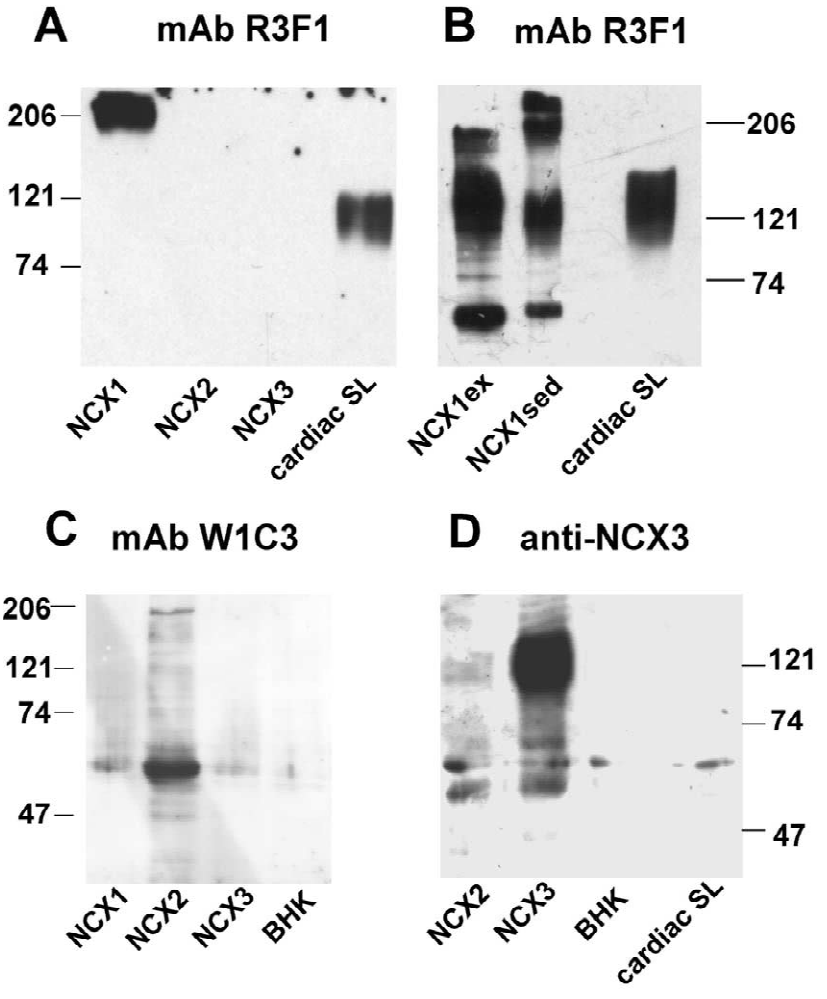


Fig. 1. Immunoblots for three exchanger subtype-specific antibodies used in the immunohistochemical studies. (A) Homogenates (5 μg protein/lane, each) of recombinant BHK cells expressing NCX1, NCX2 or NCX3 and equine sarcolemma vesicles (cardiac SL, 5 μg protein) were separated by PAGE, blotted onto nitrocellulose and exposed to mAb R3F1 (3 μg/ml). mAb binding was detected using peroxidase-conjugated rabbit anti-mouse IgG (whole molecule, 1:3000) and the Lumilight chemoluminescence kit. (B) BHK cell homogenates from NCX1-expressing cells were extracted with Triton X-100 prior to gel electrophoresis. Five μg protein/lane were applied to the separating gel, both of the Triton soluble (NCX1ex) and insoluble (NCX1sed) fractions. Procedure otherwise as in (A). (C) The Triton X-100-insoluble fractions from NCX1-, NCX2-, and NCX3-expressing BHK cell homogenates (3.5 μg/lane) or from wild type BHK cells (BHK) were treated as described in A and the blots reacted with mAb W1C3. A peroxidase-conjugated μ-chain-specific goat-anti mouse IgM (1:3000) was used as second antibody to detect mAb binding. No immunolabeling was detected in the Triton X-100 soluble fraction of the homogenates (not shown here but compare Fig. 2C). (D) Homogenates from NCX2, NCX3, wild type BHK cells or cardiac sarcolemma vesicles (5 μg protein/lane) were treated as described in A. Blots were reacted with a polyclonal anti-NCX3 antibody. A peroxidase-conjugated goat anti-rabbit IgG (1:3000) was used as second antibody. The film generated from the chemoluminescence reaction was scanned and the resulting .tiff file imported into Adobe Photoshop. The figures were trimmed. Contrast and brightness were adjusted such as to reach a faithful rendering of the original film.

fraction of glia cells, NCX1 labeling with mAb R3F1 was generally weak although a 120-kDa band could be detected throughout (Fig. 2A). A strong double band close to 120 kDa was labeled after Triton extraction of freshly dissected hippocampus (Fig. 2A). Significantly, the 70-kDa band that appeared prominent in the NCX1 expressing BHK cells was generally lacking.

The most prominent feature in immunoblots with the NCX2-specific mAb W1C3 was a strong band at 60 kDa (corresponding to the main immunoreactive band in NCX2 expressing BHK cells) that was accompanied by a similarly strong band at 54 kDa. The strength of this double band was directly proportional to the glia cell content of the cultures (Fig. 2C). Since the band at 54 kDa had no clear equivalent in NCX2 over-expressing cells, it could not be identified as a proteolytic fragment of NCX2. Since glia cells are very rich in a 50 kDa fibrillar protein (glia fibrillar acidic protein, GFAP), we suspected a cross-reaction of mAb W1C3 with GFAP. However, a specific antibody directed against GFAP detected a band that was clearly separable from the 54-kDa band labeled by W1C3 (not shown). Immunohistochemical results reported below confirmed the conclusion that the 54 kDa band and GFAP represent separate proteins. In control experiments, we tested whether both bands resulted from an immune reaction due to a shared epitope. In the experiment shown

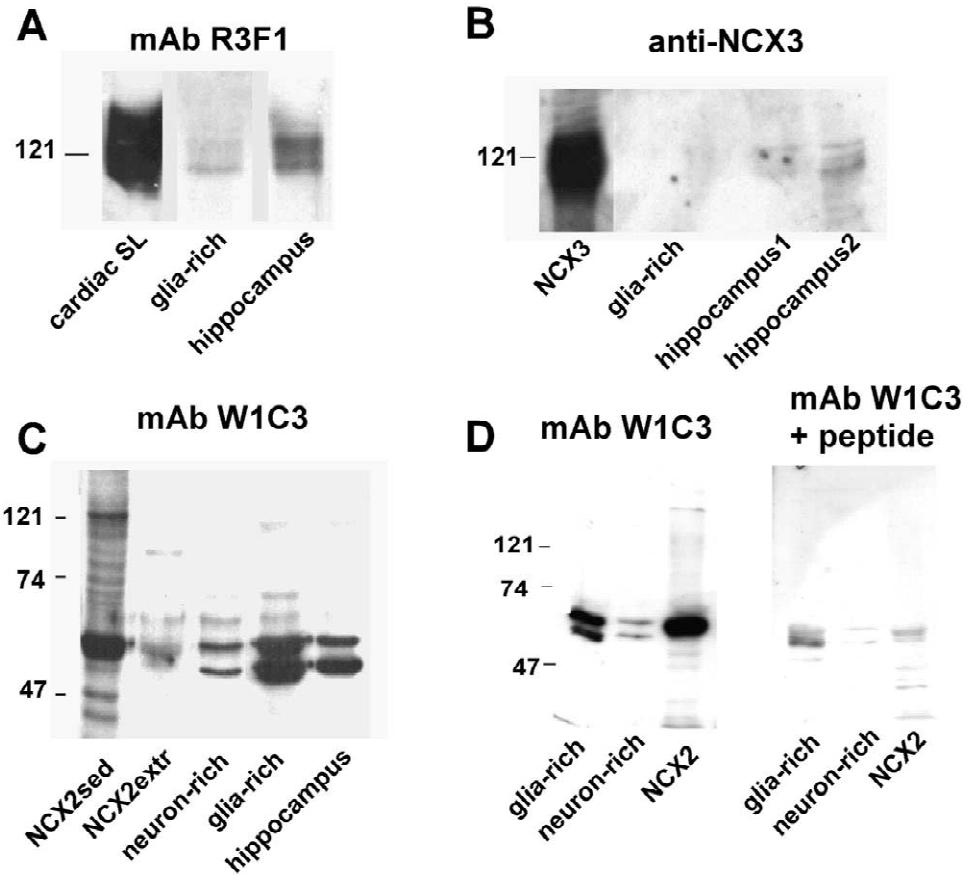


Fig. 2. Detection of Na/Ca exchanger subtypes in immunoblots of hippocampus cultures using subtype-specific antibodies. (A) Western blots of PAGE-separated cardiac sarcolemma vesicle protein (5 μg/lane), of hippocampus culture homogenate (glia-rich, 4.8 μg protein) and of Triton X-100-extracted whole hippocampus homogenate (4.8 mg protein) were labeled with mAb R3F1 (3 μg/ml) and detected with peroxidase-conjugated goat anti-mouse IgG (1:3000). Lanes from the same gel were cut and trimmed to show the relevant samples side by side. (B) Blots of NCX3 expressing BHK cell homogenate (5 mg protein), of homogenate from a glia-rich hippocampus culture (7 μg protein) and of native hippocampus homogenate (hippocampus 1: 2.5 μg, hippocampus 2: 5 μg protein) were labeled with polyclonal NCX3 antibody (anti-NCX3, 1:1000) and detected with a goat anti-rabbit peroxidase-conjugated second antibody (1:3000). The positive control (anti-NCX3 reacting with NCX3 expressing BHK cell homogenate) was cut from the same gel and placed side by side to the corresponding tissue culture samples. (C) Reaction of protein samples from NCX2 expressing BHK cells and from hippocampus cultures with mAb W1C3 (1:500 of partially purified stock). The following samples were applied left to right: NCX2sed: Triton X-100-insoluble sediment from NCX2 expressing BHK cell homogenate (3.4 μg protein); NCX2ex: Triton X-100-soluble extract from the same cells (3.7 μg protein); neuron-rich: neuron-rich hippocampus culture homogenate (5 μg protein); glia-rich: glia-rich hippocampal culture with few neurons (5 μg protein); hippocampus: Triton X-100 insoluble extract of native hippocampus homogenate (4.4 μg protein). (D) Inhibition of specific mAb W1C3 binding by the fusion peptide that was used as antigen to rise mAb W1C3. Identical sediment samples from Triton X-100 extracts of cell homogenates were applied to lanes 1–3 and 5–7 of a separating gel (glia-rich: 3.1 μg protein from glia-rich hippocampus culture; neuron-rich: 2.9 μg of neuron-rich hippocampus culture; NCX2: 3.4 μg of NCX2-expressing BHK cells). mAb W1C3 was used as primary antibody in both cases. The left hand panel was exposed to mAb W1C3 in the simultaneous presence of 22 μg/ml of the antigenic peptide. A peroxidase-conjugated goat anti-mouse IgM (μ-chain-specific, 1:3000) was used as second antibody throughout.

in Fig. 2D, immunoblots from the same PAGE separation were incubated either with mAb W1C3 alone or in the additional presence of an excess (22 μg protein/ml) of the NCX2 fusion protein. As described in Section 2, this protein had been used as the immunization antigen for the generation of mAb W1C3. Hence, it is expected to compete for specific antibody binding sites. As is obvious from Fig. 2D, mAb binding to both immunoreactive bands is largely suppressed. Possibly, the reduction is slightly less marked in the lower band. Based on this finding, we assume that a true cross reaction due to shared epitopes causes the immunolabeling of the second band by mAb W1C3.

Unlike NCX1- and NCX2-specific antibodies, the polyclonal NCX3 antibody reacted very weakly, if at all, with proteins from hippocampus tissue culture homogenates (Fig. 2B).

Together these results suggest that NCX1 and NCX2 are both expressed in hippocampus tissue cultures but seem to be unevenly distributed between neurons and glia cells. On

the other hand, the expression of NCX3 appears to be exceedingly low and seems to be restricted to neuronal cells.

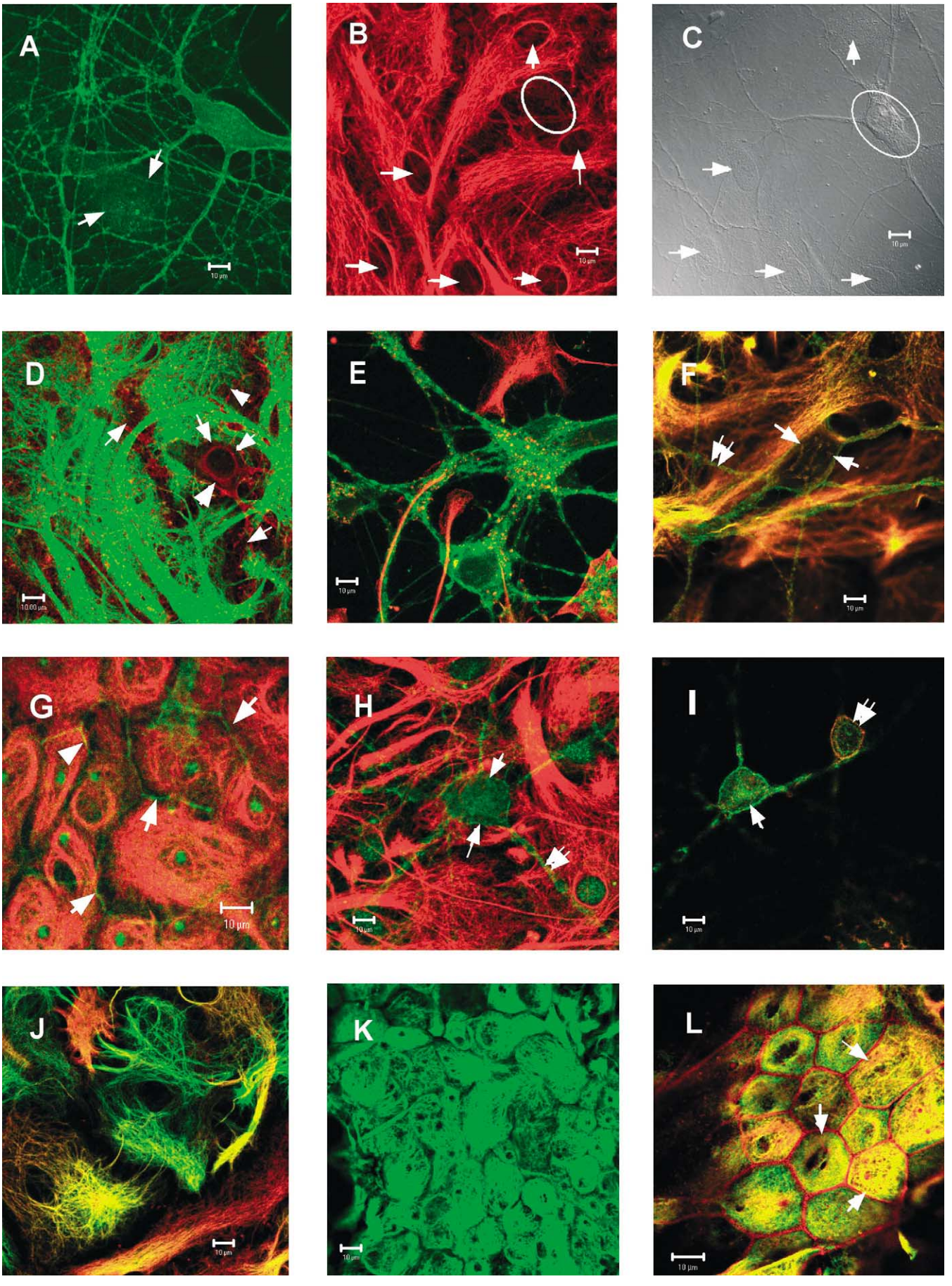
3.3. Immunohistochemical studies with NCX subtype-specific antibodies

In a first set of experiments, we assessed the distribution of NCX1 and NCX2 in alcohol-fixed hippocampus cultures containing neurons and different types of glia cells. Co-localization studies were facilitated by the fact that the two primary subtype-specific mAbs belonged to different immunoglobulin classes (IgG, IgM). Hence, they could be detected simultaneously with class-specific secondary antibodies. Single staining of NCX1 with R3F1 using FITC-labeled anti-mouse IgG resulted in an almost exclusive labeling of neuronal cell membranes and of the whole dendritic network between neighboring cells. Glia cells, if at all visible, showed only a rather dim, diffuse staining. Glia cell membranes could not be discriminated (Fig. 3A). By contrast, W1C3 labeled with a rhodamine-conjugated anti-mouse IgM, stained glia cells very brightly while failing to detect most neuronal cells (Fig. 3B,C). Surprisingly, the antibody strongly labeled an intracellular fibrillar network in all subtypes of glia cells, that was present in cell bodies and extended into the glia fibers that surrounded the neuronal cell bodies. In these morphologically highly complex neuron/glia co-cultures it was impossible to determine if NCX2 immunoreactivity was also present in glia cell membranes. Since exchanger transport activity can be measured in glia cells [5,26], failure of the antibody to label an exchanger protein in glia cell membranes would cast serious doubts on its specificity. Therefore, we prepared glia-rich cultures containing only very few neurons. Under these conditions glia cells tend to grow in a cobblestone pattern that facilitates the detection of cell borders. Fig. 3L shows that mAb W1C3 clearly detected a membrane protein in addition to its labeling of cellular fibrils.

As a further control, we stained the cell membranes in mixed glia/neuron cultures with CM-Dil (Molecular Probes) a strongly red fluorescent membrane tracker dye with a high photon yield, in an attempt to localize glia cell membranes within the glia fiber network. The typical result of a CM-Dil/W1C3 co-staining is shown in Fig. 3D. With the focal plain set to the glia cell level (as done here), no clear staining of glia fiber membranes could be observed, while shifting the focal plain towards the neuronal cell level revealed bright red labeling of the cell membranes as well as of the dendritic network (not visible in Fig. 3D). The glia cells could only be traced by the W1C3-labeled cellular fibrils (green) that were detected by a fluorescein-conjugated IgM antibody. We conclude from these findings that the fluorescence signal emanating from the highly extended glia cell membrane was simply too weak to be picked up by our detection system. Most likely, the failure to detect membrane-associated mAb W1C3 in glia/neuron cultures did not result from a lack of antibody specificity. In pure glia cell cultures (comparable to the one shown in Fig. 3 L), CM-DIL stained glia cell membranes brightly red (not shown).

How exclusive is the association of NCX1 and of NCX2 with neurons and glia, respectively?

This question was tackled by using two different techniques. First, mixed neuron/glia cultures were simultaneously stained with R3F1 and W1C3. The two mAbs were separately detected with a FITC-conjugated γ-chain-specific anti-mouse IgG and a rhodamine-conjugated μ-chain-specific anti-mouse IgM. With these fluorescent labels, areas of apparent co-localization would stain yellow. Visual fields containing both neuronal and glia cells were systematically scanned for structural elements stained by both mAbs by moving the focal plain in steps of about 1 μm from the glia layer on the bottom of the growth surface to the neuronal cell bodies located in the topmost cell layer. In a first approximation, this approach allows to discriminate mere overlay of two differently stained structures from more intimate co-localization in the same structure, e.g. the cell membrane. With this method, virtually no co-localization could be observed (Fig. 3E). The yellow spots on the neuronal cells probably represent points of contact between glia cell extensions and neuronal cell body membranes. As a positive control for true co-localization, and also as a more stringent exclusion of neuronal labeling with the NCX2-specific mAb W1C3, we used, together with the μ-chain-specific anti-IgM described above, a FITC-labeled anti-mouse IgG (whole molecule) that strongly cross-reacted with mouse IgM. Under this condition, all structures carrying binding sites for W1C3 should stain yellow because binding of W1C3 would made secondary sites available for an interaction with the cross-reacting IgG. By contrast, all structures that were exclusively labeled by R3F1 would appear green because they would be recognized only by the FITC-labeled anti-IgG. When hippocampus cultures labeled with W1C3 and R3F1 were stained with this latter combination of secondary antibodies, nearly all glia cell structures appeared yellow or yellow/red, while most neuronal membranes still showed exclusively green fluorescence (Fig. 3F). Only in a few visual fields did we observe single neuronal cell bodies labeled with W1C3 (not shown). Finally, we also doubly stained pure glia cell cultures with mAbs R3F1 and W1C3 using IgG- and IgM-specific secondary antibodies. In most cases only mAb W1C3 immunoreactivity could be detected in glia cell membranes. To test whether the developmental stage of the culture would affect the expression of NCX subtypes, we decided to complement these experiments by studying aged glia cell cultures more than 28 days after seeding. Under these conditions, isolated patches of glia cells were detected that clearly expressed NCX1 immunoreactivity within their membranes (Fig. 3G). A few cells contained both, mAb R3F1 and mAb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10 µm

W1C3 reactive material. Together these results confirmed that NCX1 was mainly confined to neuronal membranes while NCX2 was the dominant exchanger subtype in glia cells. Yet, aging of the cells might be associated with a shift towards NCX1 expression in glia cell membranes. The very low signal level with R3F1 in glia cells confirmed that this mAb, in spite of its weak cross reaction with NCX2 over-expressing BHK cells, can be considered as NCX1-specific in normal brain tissue. The spots of green fluorescence associated with glia cell nuclei in Fig. 3G seem to result from unspecific staining of some nuclear material by the FITC-conjugated secondary antibody and do not indicate nuclear localization of mAb R3F1.

In further experiments, we tried to detect NCX3-specific immunolabeling in hippocampus cultures. As predicted by the weak signals in immunoblotting experiments (compare Fig. 2B), very few cells could be labeled with the NCX3-specific antibody. All NCX3-positive cells were neurons that expressed this exchanger subtype in the membranes of their cell bodies and of the dendritic network (Fig. 3H). No glia cells could be detected that stained with anti-NCX3. Fig. 3I shows the exceedingly rare event of two neighboring neurons one of which expressed predominantly NCX3 (in this case stained green) while the other expressed NCX1 (in this case stained red). Probably less than 5% of the neuronal cells in a given culture could be labeled with anti-NCX3, some of which seemed to express both NCX1 and NCX3.

In a final set of experiments we tried to identify the nature of the fibrillar structures that appeared to cross-react with the NCX2-specific antibody W1C3. The most obvious approach seemed to be a search for co-localization of W1C3 with antibodies specific for proteins in different types of fibrils. We first used an IgG mAb directed against glial fibrillary acidic protein (GFAP), the most abundant intermediary filament protein in glia cells. With this mAb, the staining was restricted to glia cells and revealed a pattern that resembled superficially the one obtained with mAb W1C3. However, co-staining of the same cultures with anti-GFAP and mAb W1C3 showed a very heterogeneous picture (Fig. 3J). The green areas in this single focal plain out of a stack of frames indicate structures exclusively labeled with the GFAP mAb. Areas of overlap between GFAP and the W1C3-stained fibers appear yellow. In addition some reddish cells are visible, indicating dominant staining with W1C3. Scanning through different focal plains revealed that the W1C3-labeled fiber network was usually located close to the lowest focal plain adjacent to the growth surface, while GFAP dominated in the more superficial layers of the culture. This resulted in a stratigraphic distribution of the different stains even within single cells, such that each focal plain showed characteristic differences in fluorophore distribution. By contrast, a projection of all focal plains into one picture fails to show this heterogeneity. Immunohistochemistry using a γ-chain-specific IgG antibody (lacking cross-reaction with mouse IgM) was fully consistent with these conclusions (not shown). Together our results suggest that GFAP and W1C3 fail to co-localize.

Next, we studied whether mAb W1C3 co-localized with vimentin, another prominent intermediary fiber protein located in developing glia cells. On its own, an IgG-mAb

Fig. 3. Immunocytochemical staining of ethanol-fixed hippocampus cultures with exchanger subtype-specific antibodies as viewed by confocal microscopy. Size bars in the lower left or right corner give always 10 μm. (A) Labeling with mAb R3F1 (anti NCX1, 10 μg/ml) of a mixed glia/neuron culture. Antibody binding detected with FITC-labeled second antibody (1:50). Note that only neuronal cells and their dendritic network are clearly stained. Two arrows point to a shadowy glia cell overlaying some dendrites. (B) Labeling with mAb W1C3 (anti-NCX2, 1:200 dilution of stock). Detection of the antibody with a rhodamine-conjugated anti-mouse IgM second antibody. The visual field contains several glia cells together with one neuronal cell (panel C corresponds to a transmission (DIC optics) photomicrograph of the same area). The arrows in B and C point to the position of glia cell nuclei. The circle gives the position of the neuronal cell. Note that the neuronal cell remains entirely unstained while the antibody labels a rich fibrillar network within the glia cells. (D) Double labeling with mAb W1C3 and CM-DIL, a red fluorescent membrane tracker dye. mAb W1C3 was detected with a FITC-conjugated second antibody. The arrows point to a brightly red stained neuronal cell and its dendrites. In this co-culture with neuronal cells, the plasma membrane structure of glia cells can not be resolved. (E) Double labeling with mAb R3F1 (detected with FITC-conjugated second antibody) and mAb W1C3 (detected with rhodamine-conjugated second antibody). Note the lack of co-localization within neuronal (green) and glial (red) structures. The punctuated yellow staining on the neuronal cell bodies probably corresponds to glial contact points. (F) Double labeling with mAb R3F1 (detected with a FITC-conjugated second antibody (goat anti-mouse IgG (H+L)) and mAb W1C3 (detected with goat anti-mouse IgM (μ-chain-specific)). Note the orange-yellow staining of glial structures due to the detection of mAb W1C3 by both second antibodies. Single arrows point to the cell membrane of a neuronal cell overlaying the glia structure. Double arrows points to a dendrite of the same cell. Note that no IgM (mAb W1C3) is detected in the neuronal cell under these conditions. (G) Double labeling of a 4-week-old glia-rich culture with mAb W1C3 (detected with rhodamine-conjugated donkey anti-mouse IgM) and mAb R3F1 (detected with FITC-labeled goat anti-rabbit IgG). Note that glia membrane labeling by mAb R3F1 (arrows) can be detected in this aged culture as fine green lines between the red stained fibrillar network within glia cells. Unlike in younger cultures, the secondary antibody showed an unspecific interaction with some nuclear material in glia cells (green spots associated with glia nuclei). (H) Double labeling with mAb W1C3 (detected with rhodamine-conjugated donkey anti-mouse IgM) and anti-NCX3 (detected with FITC-labeled goat anti-rabbit IgG). Note the complete lack of co-localization between W1C3 (red) and anti-NCX3 (green). The arrows point to the cell body of a NCX3-positive neuron. Double arrows point to a dendritic fiber of the same neuron. (I) Double labeling with mAb R3F1 and anti-NCX3. The panel represents a single focal plain showing two neurons one of which is NCX1 positive (red, double arrow), the other NCX3 positive (green, single arrow). (J) Double labeling of glia cells with mAb W1C3 (second antibody conjugated with rhodamine) and anti-GFAP (second antibody conjugated with FITC). Note the lack of co-localization. Yellow staining results from overlay. (K) Labeling of a glia-rich culture with an anti-vimentin mAb. Detection with a FITC-conjugated anti-mouse IgG. The cells contain densely packed fibrils, cell membranes remain unstained. (L) Same type of culture as in K, doubly labeled with mAb W1C3 and anti-Vimentin. Second antibodies were conjugated to rhodamin or FITC, respectively. The arrows point to the brightly red stained cell membranes. The cell interior is heterogeneously stained due to the overlay of red and green-stained fibrillar structures.

directed against vimentin stained a dense fibril network that also showed some similarity with the W1C3-labeled structure (Fig. 3K). However, co-staining with both antibodies did not reveal any significant co-localization. As mentioned above, in Fig. 3L a focal plain has been selected from a glia cell culture where the FITC-labeled intracellular vimentin contrasts with the purely rhodamine (W1C3)-labeled cell membranes. Yellow staining indicates areas where W1C3-labeled fibers overlay the vimentin network.

Additional experiments tested the co-localization of W1C3 with actin and tubulin fibers. Even though specific antibodies clearly detected such fibers in glia cell layers, albeit in low density, W1C3 did not co-stain any of the two proteins.

4. Discussion

The most surprising result of the present investigation is the rather strict cell-specific expression pattern of the three NCX subtypes in rat hippocampus tissue cultures. Earlier studies, based mainly on mRNA expression patterns, detected by Northern blotting or RT-PCR amplification, suggested co-localization of NCX1, NCX2 and NCX3 in most brain areas as well as in neurons and glia cells [18,21,26,28]. The appreciable regional variability of mRNA levels suggested a subtype protein expression pattern that might be unique for each brain area. Nevertheless, it is usually difficult or impossible to derive estimates of membrane protein concentrations from measured mRNA levels. Moreover, rather discrepant estimates for mRNA expression of rat brain exchanger subtypes have been published. Yu and Calvin [28] reported that NCX2 transcript levels generally exceeded the ones for NCX1 and NCX3 by one order of magnitude. On the other hand, He et al. [7] observed that the concentration of NCX1 message both in neurons and glia, was significantly higher than the one of the NCX2 message.

The presence of the Na^+/Ca^{2+} exchanger protein in neuron and glia cells has also been confirmed by functional studies and by immunohistochemistry in brain tissue and glia cell cultures [5,8,10,26]. However, up to now, all immunohistochemical studies suffered from the fact that antibodies of unknown subtype specificity had been used. Therefore, exchanger subtype localization on the protein level has not been possible previously. From the three antibodies used in the present study, only the NCX1 antibody R3F1 showed a slight cross-reaction with NCX2 while the two others seemed to label exclusively one subtype. In the following discussion, we shall consider separately the results for each individual antibody and shall try to reconcile them with the data in the literature. To what extent our data on membrane transport protein expression in neuronal tissue culture faithfully reflect the situation in intact tissue will have to be analyzed in future studies.

4.1. Data obtained with mAb R3F1

Results with this antibody, though superficially rather clear-cut, seem to disagree with earlier observations. In our study, immunostaining was mainly restricted to cell membranes of neuronal cell bodies and the dendritic network (see also Ref. [22]). However, several studies have confirmed the expression of NCX1 mRNA splicing variants in primary brain astrocytes [7,21]. Yet, the NCX1 selective mAb detected very little immunoreactivity in hippocampus glia cells. Even in the absence of neuronal cells, when glia cells form relatively homogeneous cell layers, labeling of the characteristic plasma membrane pattern (compare Fig. 3L) was scarce and could be detected only in aged cultures. There are several possible explanations. (1) The actual protein concentration in hippocampus glia cells might be much lower than in cortical astrocytes used by Quednau et al. [21] or He et al. [7]. (2) Both groups of authors point out that astrocytes predominantly express the ‘B’-type splicing variants of NCX1 while the ‘A’ variants are found in neurons. ‘A’ and ‘B’ denote two mutually exclusive exons that define two families of NCX1 splice variants. The sequence coded for by these two exons forms part of one of the two neighboring epitopes identified for mAb R3F1 [20]. Since the original antigen used to raise mAb R3F1 (cardiac NCX1) contained only A variants, its affinity to proteins with the Exon B sequence might be considerably lower. These two explanations may be complementary since the loss of affinity alone does not abolish immunolabeling by R3F1. The NCX1 splicing isoforms expressed in kidney belong to the ‘B’ family of variants but still are well recognized by this antibody [3,25]. Finally, and this is also supported by our results, it is possible that exchanger subtype expression is developmentally regulated, such that the relative abundance in different brain tissues may differ in newborn and adult animals. An analogous developmentally regulated expression of NCX1 and NCX3 subtypes in skeletal muscle has been recently described [4].

4.2. Data obtained with mAb W1C3

This antibody is the first to react specifically with NCX2 but has the distinct disadvantage of cross-reacting with a glial fibrillar protein. The abundance of this fibrillar protein appeared to be similar to the one of the exchanger protein (Fig. 2C). Nevertheless, glia cell membranes are also clearly labeled. This is consistent with the expected localization of the exchanger generating the transport activity which has been measured repeatedly in glia cells [5,8,26]. Since NCX1 and NCX2 seem to share very similar transport kinetics [14], the functional studies in astrocytes mentioned above would not have discriminated between the two exchanger subtypes.

The preference of NCX2 for glia cells was not absolute. We detected a few neurons that were stained with W1C3.

However, this staining was restricted to neuronal cell bodies and a few dendritic membranes close to the cell body. The extended dendritic network in hippocampus cultures was never labeled. Even after the strong enhancement of the W1C3 fluorescent signal by double labeling with two different secondary antibodies, little neuronal co-localization with NCX1 could be detected. This observation suggests that the enrichment of immunoreactive exchanger in synaptic boutons observed by Reuter and Porzig [22] is entirely confined to the NCX1 subtype. Consequently, rate and duration of synaptic storage vesicle exocytosis and neurotransmitter release that are sensitive to Ca^{2+} movements via the Na^+/Ca^{2+} exchanger [2] rely on NCX1 rather than NCX2 activity.

4.3. Data obtained with NCX3

Staining by the NCX3-specific polyclonal antibody was confined to an exceedingly small neuronal cell population in the rat hippocampus. This was somewhat surprising as the abundance of the hippocampus NCX3 message in the study of Yu an Colvin [28], reached about half the value for NCX1. It might be speculated that the NCX3 expressing cell population does not survive the initial period of tissue culture, leaving predominantly NCX1-expressing cells in well developed mature cultures. It is also possible that culture conditions affect the expression of NCX3. If intercellular contacts were involved in modulating the expression of individual exchanger subtypes, the in vivo distribution pattern would be disturbed by random mixing of cells in culture. Whatever the reason for this discrepancy, it seems clear that NCX3, like NCX1, is not significantly expressed in glia cells. Like NCX1, NCX3 was expressed in neuronal cell bodies as well as in the associated dendritic network. Therefore, it is possible that NCX3 contributes to the regulation of neurotransmitter release in dendritic synaptic boutons of the respective neurons.

In conclusion, our results show a marked cell-specific distribution of the three Na^+/Ca^{2+} exchanger subtypes that would not have been predicted from expression studies on the mRNA level. NCX1 and NCX2 appear to dominate high capacity Ca^{2+} extrusion systems for neurons and glia cells, respectively, in rat hippocampus. This intriguing distribution pattern may have developed on the basis of still unknown different functional requirements for cellular Ca^{2+} homoeostasis in the two cell types.

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