Electron microscopy localization of NCX1, 2, 3 isoform protein exchangers in neuronal astrocytes

S. Salucci, A. Minelli, P. Gobbi

Dipartimento di Scienze dell'Uomo, dell'Ambiente e Della Natura, Università degli Studi di Urbino "Carlo Bo"

Corresponding author: Sara Salucci
Dipartimento di Scienze dell'Uomo, dell'Ambiente e Della Natura, Università degli Studi di Urbino, Campus Scientifico
Località Crocicchia 61029 Urbino (PU)
Tel. +39.0722.304244 - Fax +39.0722.304244
E-mail: sara.salucci@uniurb.it

Summary

Na^+/Ca^{2+} exchangers (NCX1, 2 and 3) play relevant role in neural cells, where variations of cytosolic Ca^{2+} concentration represent a pivotal event in many physiological and pathological processes. Astrocytes display a type of excitability based on changes in intracellular Ca^{2+} concentration.

In the present study, electron microscopic immunohistochemistry was applied to investigate the expression of the three NCX1-3 protein isoforms, in astrocytes of cerebral cortex and hippocampus. Results showed that a conspicuous population of astrocytic cells expressed NCX1–3 in both brain areas. Immunolabeling for NCX1-3 was observed in many glial profiles of various size, notably in distal astrocytic processes in contiguity of synaptic structures, suggesting the involvement of NCX in shaping astrocytic $[Ca]_i$ transients evoked by adjacent synaptic activity. NCX1-3 immunoreactivities (irs) were expressed in astrocytic mitochondria, indicating an important contribution to mitochondrial Ca^{2+} regulation in this cell type *in situ*. In addition, all NCX isoforms were consistently expressed in perivascular astrocytic endfeet, suggesting an important role in regulating the barrier function of blood-brain barrier (BBB). Present immunomorphological work showed that in both brain regions all NCX isoforms were expressed in astrocytes, thus pointing to a widespread role of the three exchangers in maintaining Ca^{2+} homeostasis in glial cells and suggesting that distinct NCX isoforms may share analogous physiological roles in the brain *in vivo*.

Keywords: Na^+–Ca^{2+} exchangers, synapses, glial cells, blood-brain barrier, immunohistochemistry.

Introduction

Since the initial microscopic studies of the nervous system, glial cells were considered to play a simple supportive role for neurons (Perea and Araque, 2005a). Glial cells and, in particular, astrocytes play an active role in many functions of the nervous system (Araque, 1999; Volterra and Meldolesi, 2005) such as differentiation, proliferation and trophic support and survival of neurons.

Glial cells display a form of excitability that is based on variations of the Ca^{2+} concentration in the cytosol rather than electrical changes in the membrane. This excitability may serve as a cellular information element, which suggest the ability of glia to play more active roles in the nervous system (Perea and Araque, 2005a).

New findings have recently proposed the existence of bidirectional communication between astrocytes and neurons, where the astrocyte Ca^{2+} signal plays a crucial role (Simpson and Russell, 1998; Wallace, 1999; Pivovarova *et al.*, 2002; Gunter *et al.*, 2004; Minelli *et al.*, 2007).

Na^+ -Ca^{2+} exchanger (NCX) is a plasma membrane antiporter mainly involved in maintaining cytosolic Ca^{2+} homeostasis. NCX couples uphill Ca^{2+} extrusion to downhill Na^+ influx (forward mode); alternatively, it can operate as Na^+ efflux-Ca^{2+} influx pathway (reverse mode), depending on membrane potential and intracellular ions con-

centration (Phlipson and Nicoll, 2000; Annunziato *et al.*, 2004).

The Na^+/Ca^{2+} exchanger (NCX) participates in controlling $[Ca]_i$ and $[Na]_i$ homeostasis in neural cells. In the central nervous system (CNS), variations of $[Ca]_i$ represent a pivotal event in a variety of physiological processes, such as neural development and synaptic transmission and plasticity, while deregulation of $[Ca]_i$ and $[Na]_i$ homeostasis is involved in neuronal and glial injury occurring in hypoxia-anoxia conditions and in several neurodegenerative diseases (Lipton, 1999; Annunziato *et al.*, 2004). Therefore, NCX function might have relevant impacts in all these conditions. In fact, contribution of NCX to buffering $[Ca]_i$ following different stimulation paradigms has been demonstrated in neurons and astrocytes from various brain structures both in cultures (White and Reynolds, 1995; Takima *et al.*, 1996; Hoyt *et al.*, 1998; Ranciat-McComb *et al.*, 2000; Smith *et al.*, 2000; Korkation *et al.*, 2004) and in vivo (Fierro *et al.*, 1998; Goldberg *et al.*, 2003; Kim *et al.*, 2005). Three genes have been cloned encoding for distinct NCX isoforms, NCX1-3, all expressed in adult central nervous system (CNS) (Lee *et al.*, 1994; Li *et al.*, 1994; Nicoll *et al.*, 1996; Yu and Colvin., 1997; Minelli *et al.*, 2007).

Here, electron microscopic immunohistochemistry has been used to investigate the cellular and subcellular localization of NCX1-3 isoforms in cerebral cortex and hippocampus astrocytes of adult rat *in situ*.

Materials and Methods

Rat tissue preparation

Sprague-Dawley adult albino rats weighing 200-300 grams were used in these studies. Care and handling of animals were done in compliance with the regulations of the Ethical Commettee of the University of Urbino. Rats were deeply anesthetized with 12% chloral hydrate and perfused through the ascending aorta with physiological saline solution followed by 3,5% paraformaldehyde (PFA) and 1% glutaraldehyde in phosphate buffer (PB, 0.1M; pH 7.4). Brains were removed and postfixed in 4% PFA for 2-12 h. 30 µm sections were then cut on a Vibratome in either coronal or parasagittal plane and collected in phosphate buffered-saline (PBS).

Antibodies

NCX1 protein was detected by using a commercially-available mouse monoclonal IgG antibody (R3F1; Swant, Bellinzona, Switzerland) (Philipson and Nicoll, 2000). For NCX2 protein detection, a monoclonal IgM antibody (W1C3) (Thurneysen *et al.*, 2002), obtained by hybridoma cells, has been used. Finally, NCX3 protein was detected by a rabbit polyclonal IgG antibody generously provided by Dr KD Philipson (Thurneysen *et al.*, 2002).

Immunoperoxidase procedure

Free-floating sections were pretreated for 30 min in PBS containing 1% H_2O_2 for quenching endogenous peroxidase and rinsed for 1 hr in 1% sodium borhydride in PBS to minimize aspecific binding due to aldehydic residues. Sections were then preincubated for 1 hour in 10% non-immune goat serum (NGS) in PBS, and then incubated overnight at 4°C in primary antibodies against NCX1-3 diluted in PBS plus 1% NGS. NCX1 antibody was used at concentrations of 1:750, NCX2 at 1:500 and NCX3 at 1:500. The next day, after several washes in PBS, sections were incubated first in NGS (10% in PBS for 15 min) and then for 1 hour in the appropriate biotinylated secondary antibodies: goat anti-mouse IgG, goat anti-mouse IgM, goat anti-rabbit IgG (for NCX1, NCX2 and NCX3, respectively; Vector Lab, Burlingame, CA) diluted 1:200 in PBS plus 1% NGS. After further rinsing in PBS, sections were processed according to the avidin-biotin peroxidase complex procedure (Vector; PK-6100; 30 min). Finally, the reaction product was demonstrated by 3',3-diaminobenzidine tetrahydrochloryde (DAB; 40 mg/50 mL) with 0.03% hydrogen peroxide.

Preembedding silver-enhanced immunogold procedure (SEI)

Sections were incubated with 1% bovine serum albumin (BSA) in PBS (PBS-BSA), then overnight in primary antibodies (concentrated as above) in PBS-BSA. After several washes in PBS-BSA, sections were incubated for 1hour in the appropriate secondary antibodies conjugated to 1.4nm colloidal gold particles: goat anti-mouse IgG and goat anti-rabbit IgG for NCX1 and NCX3 detection, respectively (Nanoprobes, Yaphank, NY), diluted 1:200 in PBS-BSA with 1% NGS; for NCX2 detection, sections were first incubated for 1 hour with biotinylated goat anti-mouse IgM secondary antibody and then for 2 hours with a gold-conju-

gated goat anti-biotin IgG (Nanoprobes, Yaphank, NY), diluted 1:200 in PBS-BSA with 1% NGS. Sections were rinsed in PBS-BSA, then in PBS and post-fixed in 1% glutaraldehyde alone in PBS (10 min). After a brief washing in deionized water, colloidal gold labelling was intensified using a silver enhancement kit (HQ silver, Nanoprobes, Yaphank, NY) for 3-5 minutes at room temperature in dark room. Finally, sections were washed and collected in PB.

For both immunoperoxidase and immunogold ultrastructural studies, controls were performed by omitting primary antisera from immunocytochemical procedure and/or by substituting it with NGS 10% in PBS-BSA; in these cases, signal was virtually absent and showed no preferential association with plasma membranes or specific subcellular structures.

Electron microscopy

After completion of the immunohistochemical procedure, sections for both immunoperoxidase and immunogold studies were postfixed for 30 min in 2,5% glutaraldehyde in PB, and then for 1h in OsO_4 (1% and 0.2% in PB for immunoperoxidase and immunogold material, respectively). After dehydration in graded series of ethanol, sections were cleared in propylene oxide, flat-embedded in Epon-Spurr between acetate sheets (Aclar; Ted Pella, Redding, CA), and polymerized at 60°C for 72 hours. After polymerization, embedded sections were examined under a dissecting microscope. Identified areas of interest, corresponding to cortical gray matter and hippocampal CA1 and CA3 subfields, were excised with a razor blade and mounted on resin pyramidal blocks utilizing a cyanoacrylic glue. 1µm semithin sections were cut with a Reichert ultramicrotome and collected on glass slides without counterstaining for light microscopic inspection. 70-100 nm-thick ultrathin sections were cut either from the surface or from the edge (i.e. perpendicular to the plane section), counterstained with uranyl acetate and lead citrate, and examined with a Philips CM 10 transmission electron microscope.

Data analysis

All data were collected from a region of the parietal cortex corresponding to the first somatic sensory cortex and from hippocampal CA1 subfield. Identification of immunolabeled and unlabeled profiles was based on established morphological and morphometrical criteria (Minelli *et al.*, 2007).

Results

Both neocortex and hippocampus astrocytes expressed high levels of NCX1-3 ir as previously reported (Minelli *et al.*, 2007). Immunoperoxidase reaction and silver intensification were particularly intense in thin, distal astrocytic processes; astrocytes appeared with an irregular contour adapting to the profile of adjacent neuropilar elements and labelling was distributed trough the cytoplasm and usually the internal side of plasma membrane was intensely stained (Figures 1A-H; 2A-F). Distal labelled astrocytes were often in contiguity of synaptic structure; they were often found adjacent to unstained axon terminals making asymmetric contacts with proximal or distal dendrites and with dendritic spines, some of which were also labelled (Figure 1A-G). SEI confirmed DAB ultrastructural localization: in fact gold particles for NCX1-3 were detected on astrocytic processes plasma membrane directly in contact with synaptic structures (Figure 2A-C, 2F).

Ultrastructural analisys revealed that NCX1-3 irs were present also in many astrocytic cell bodies and thick processes; here, granular patches of reaction product were scattered in the cytoplasm and small clumps of ir were associated to organelles, plasma membrane and mitochondria (Figure 2D, 2F). Both DAB (Figure 1E-H) and Silver (Figure 2D-F) immunolocalization showed that astocytic mitochondria were sometimes completely surrounded by a rim of intense NCX1-3 ir, especially those located just beneath the plasmalemma .

NCX1-3 irs were also intense in perivascular astrocytic end-feet (Figure 3A-C) in direct apposition to the basal lamina around the endothelial wall of blood vessels.

Discussion

Ultrastructural observations reveal that all isoforms are consistently expressed in neuronal astrocytes, suggesting that NCX1-3 activity in the two brain regions in situ may be diffusely involved in regulating Ca^{2+} homeostasis in glial cells. The modulation of $(Ca)_i$ has been implicated in the control of a variety of astrocytic functions, including reciprocal astrocyte-neuron signalling and glial toxicity (Verkhrasky *et al.*, 1998; Perea and Araque, 2005a). A contribution of NCX activi-

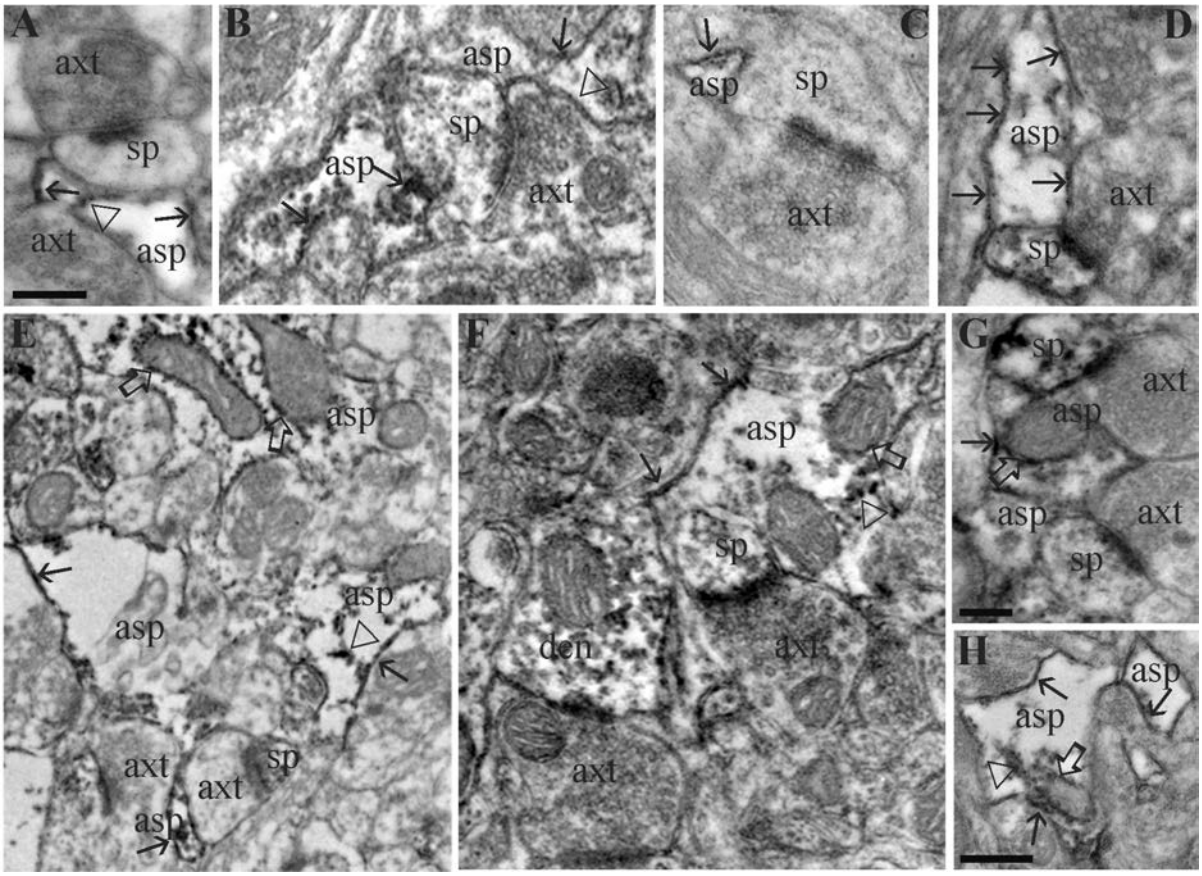

Figure 1. Ultrastructural localization of NCX1-3 with immunoperoxidase reaction. NCX1 (A, cerebral cortex), NCX2 (B, cerebral cortex) (C, hippocampus), NCX3 (D hippocampus): distal astocytic processes (asp) labelled in plasma membrane (black arrows) and in the cytoplasm (open arrowheads); all astrocytes are in apposition to dendritic spines (sp, some of which labelled) those that received unlabelled axon terminals (axt). (E, hippocampus) NCX1 ir in several labelled astrocytic processes: labelling appears scattered in the cytoplasm (open arrowheads) or associated to plasma membrane (black arrows) or mitochondria (open arrows). (F) A perisynaptic hippocampal astrocytic processes labelled in the plasma membrane (black arrows), cytoplasma (open arrowhead) and mitochondria (open arrow). (G-H) Cerebral cortex. Intense NCX3 ir is visible on plasma membrane (black arrows), cytoplasma (open arrowhead) and mitochondria(open arrows) of two distal astrocytic processes, one of which is close to synaptic structure. Bars: in A 0.25 µm for A-D, F; 0.25 µm for G; 0.5 µm for H.

ty in the regulation of $(Ca^{2+})i$ transients has been documented in cultured astrocytes following glutamate receptor stimulation and strain-induced traumatic injury (Floyd *et al.*, 2005; Goldman *et al.*, 1994; Golovina *et al.*, 1996; Kim *et al.*, 1994, 2005; Smith *et al.*, 2000) but, so far, direct evidence in vivo proving an active role of NCX in modulating astrocytic Ca^{2+} signalling are scanty. Present anatomical results show that, in rat neocortex and hippocampus, NCX isoforms are all remarkably expressed in distal astrocytic processes surrounding asymmetric synapses (i.e. presumably glutamatergic) (De Felipe *et al.*, 1988; Dori *et al.*, 1989), thus indicating the presence in situ of NCX1-3 exchangers on astrocytic membrane regions in proximity of synaptic sites of transmitter release. Since spilled-out glutamate can diffuse from synapses and evoke calcium responses in surrounding astrocytes in vivo (Grosche *et al.*, 1999; Aguado *et al.*, 2002; Peters *et al.*, 2003; Perea and Araque, 2005b), our findings strongly suggest that glial NCX1-3 are well sited to play a role in shaping $(Ca^{2+})i$ transients evoked in astrocytes by adjacent synaptic activity.

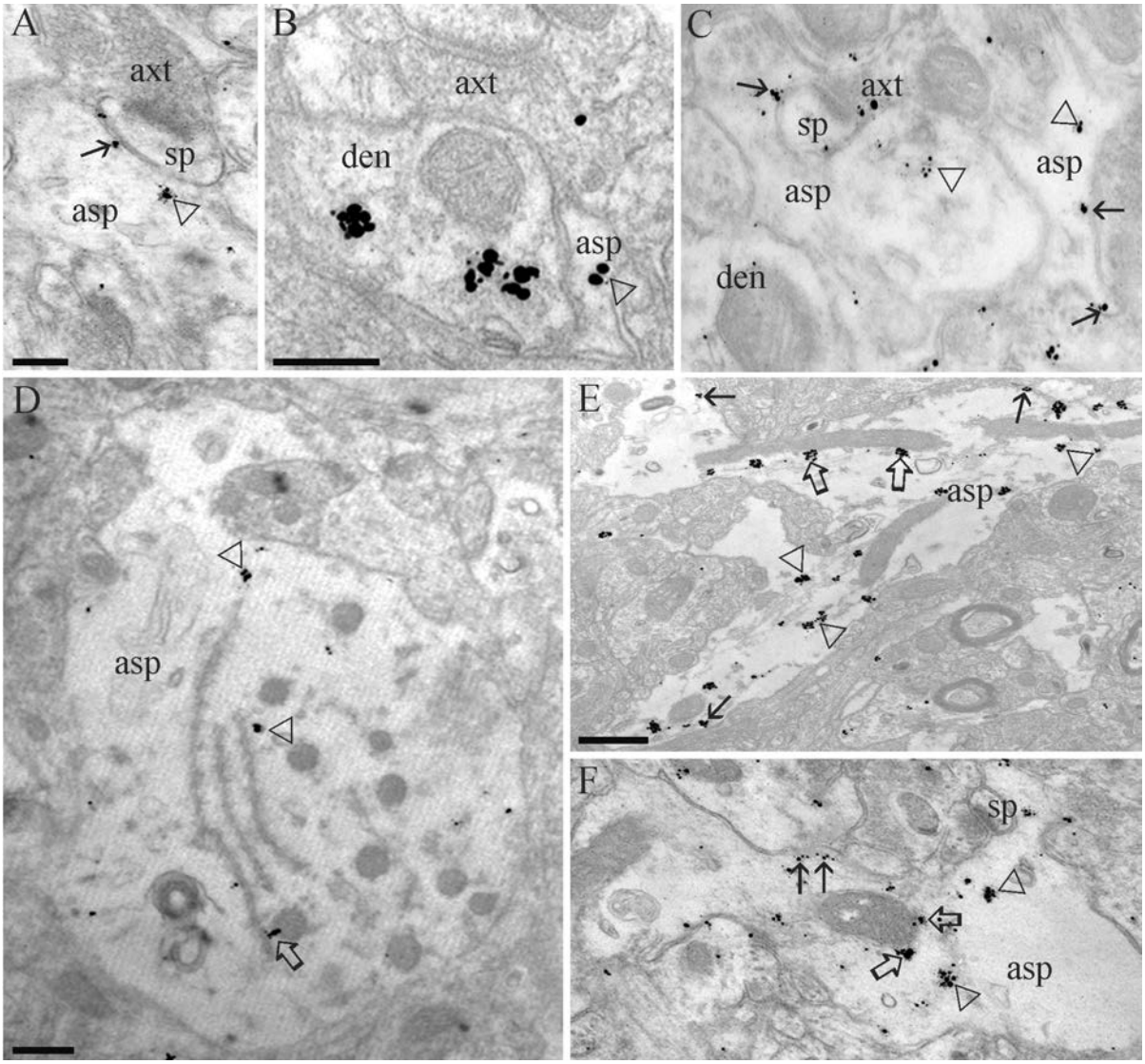

Figure 2. Ultrastructural localization of NCX1-3 with silver enhancement. NCX1 (A, hippocampus) NCX2 (B, cerebral cortex), NCX3 (C, hippocampus). Exemple of astrocytic processes (asp) sheating synaptic structure: immunogold labelling is associated to plasma membrane (black arrows) and cytoplasm (openarrowheads). (D) NCX1 ir in a proximal astrocytic process where mitochondria (open arrow) and cytoplasm (openarrowheads) are labelled. (E; hippocampus) (F; cerebral cortex) Two astrocytic processes show NCX2 and NCX3-immunoparticles associated to plasma membrane (black arrows), cytoplasma (open arrowheads) and mitochondria (open arrows). Bars: in A 0.25 µm for A, C, F; 0.25 µm for B; 0.5 µm for D; 1 µm for E.

On the other hand, not all distal astrocytic processes sheathing synapses display the same level of NCX1-3 expression, and some of them are totally devoid of staining, thus pointing to a functional heterogeneity of NCX activity in regulating Ca^{2+} responses in different subpopulations of perisynaptic glial processes. Interestingly, recent studies showing that astrocytes selectively respond to different synapses suggest that glial cells are capable to discriminate between the activity of specific synapses (Perea and Araque, 2005a). Our results seems in line with these find-

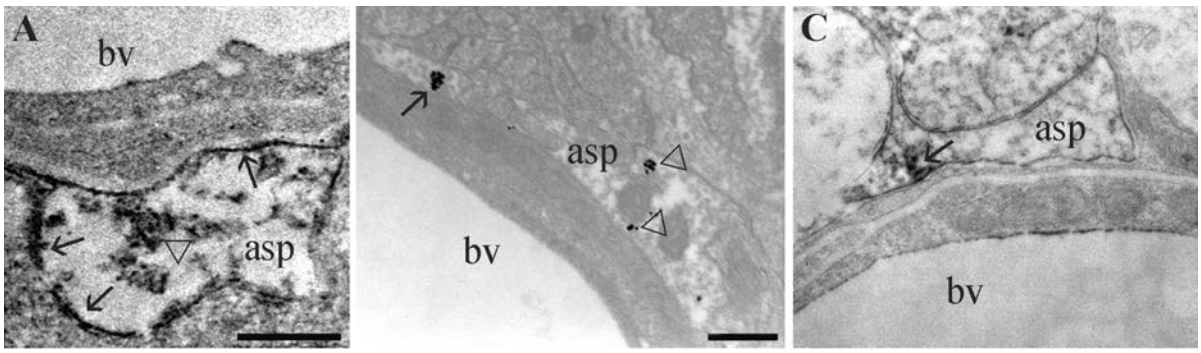

Figure 3. NCX1-3 in astrocytc and-feet processes. (A) NCX1 in a cortical astrocyte and-feet, immunoperoxidase reaction is detected on plasma membrane (black arrows) in apposition to endothelial cell membrane. (B) Cerebral cortex: a perivascular astrocytic profile (asp) bearing NCX2 immunoparticles on plasma membrane (black arrow) and in the cytoplasm (open arrowheads). (C) Hippocampus: NCX3 ir (black arrow) in perivascular astrocytic process (asp) apposed to capillary basal lamina. (arrow). bv: blood vessel. Bars: 0.25 µm for A; in B, 0.5 µm for B, C.

ings, and emphasize a possible contribution of NCX in determining synapse-specific Ca^{2+} responses in astrocytes (Perea and Araque, 2005a, b; Minelli *et al.*, 2007).

Present ultrastructural findings showing a widespread expression of NCX1-3 throughout the astrocytic surface suggest that glial exchangers can operate in diverse cellular regions, thus pointing to a spatially distributed role of NCX function in controlling $(Ca^{2+})_i$ *in vivo* and in regulating the extent of intracellular propagation of astrocytic Ca^{2+} signals.

In addition present data show that, in both neocortex and hippocampus, a conspicuous population of astrocytic mitochondria express high levels of NCX1-3 isoforms, in according with a recent study (Gobbi *et al.*, 2007), thus indicating that all three exchangers are likely to give an important contribution to $(Ca)_{mit}$ regulation in astrocytic cell type *in situ*. In fact previous studies conducted in isolated brain mitochondria have documented that a continuous recycling of Ca^{2+} across the mitochondrial membrane causes reciprocal changes in the Ca^{2+} levels in extra- and intramitochondrial compartments (Nichols, 2004, 2005). We often observed the co-presence of NCX1-3 protein expression on cellular and mitochondrial membranes closely facing each other, thus suggesting the possibility that plasmalemmal and mitochondrial NCX-mediated Ca^{2+} transport could operate in a coordinated manner in buffering $(Ca^{2+})_i$ variations within restricted functional microdomains. This concept was expressed in a recent study showing that in glutamate-stimulated astrocytes mitochondria become trapped near the plasma membrane (Kolokova *et al.*, 2006).

In both neocortex and hippocampus, all NCX exchanger isoforms are consistently expressed in perivascular astrocytic endfeet. This structure and endothelial cell form the BBB, a permeability barrier present in capillaries that selectively limits the influx and efflux of a variety of solutes and substances between blood and brain. Recent studies showing that calcium transients in astrocyte endfeet can cause cerebrovascular vasoconstriction (Mulligan and MacVicar, 2004) suggest that NCX activity in perivascular astrocytes could play an important role in the glial control of brain microcirculation (Zonta *et al.*, 2003).

In conclusion, these anatomical findings attribute a fundamental role to NCX in maintaining astrocytic homeostasis in brain in situ, and in controlling reciprocal communication between astrocytes and neurons in processing synaptic information.

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