Glutamate-Induced ATP Synthesis: Relationship between Plasma Membrane Na\(^+\)/Ca\(^{2+}\) Exchanger and Excitatory Amino Acid Transporters in Brain and Heart Cell Models

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ABSTRACT

It is known that glutamate (Glu), the major excitatory amino acid in the central nervous system, can be an essential source for cell energy metabolism. Here we investigated the role of the plasma membrane Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) and the excitatory amino acid transporters (EAATs) in Glu uptake and recycling mechanisms leading to ATP synthesis. We used different cell lines, such as SH-SY5Y neuroblastoma, C6 glioma and H9c2 as neuronal, glial, and cardiac models, respectively. We first observed that Glu increased ATP production in SH-SY5Y and C6 cells. Pharmacological inhibition of either EAAT or NCX counteracted the Glu-induced ATP synthesis. Furthermore, Glu induced a plasma membrane depolarization and an intracellular Ca\(^{2+}\) increase, and both responses were again abolished by EAAT and NCX blockers. In line with the hypothesis of a mutual interplay between the activities of EAAT and NCX, coimmunoprecipitation studies showed a physical interaction between them. We expanded our studies on EAAT/NCX interplay in the H9c2 cells. H9c2 expresses EAATs but lacks endogenous NCX1 expression. Glu failed to elicit any significant response in terms of ATP synthesis, cell depolarization, and Ca\(^{2+}\) increase unless a functional NCX1 was introduced in H9c2 cells by stable transfection. Moreover, these responses were counteracted by EAAT and NCX blockers, as observed in SH-SY5Y and C6 cells. Collectively, these data suggest that plasma membrane EAAT and NCX are both involved in Glu-induced ATP synthesis, with NCX playing a pivotal role.

Introduction

Glutamate (Glu), the major excitatory amino acid in the central nervous system of mammals, is involved in important brain functions, such as memory and learning (Meldrum, 2000). Glu is also involved in brain energy metabolism, which is of fundamental importance to neuronal functions and survival (Hertz and Dienel, 2002). It has already been established that Glu per se is able to activate neuronal and glial energy metabolism (Hertz and Hertz, 2003; Panov et al., 2009; Magi et al., 2012). After being picked up by astrocytes or neurons, Glu can be converted to \(\alpha\)-keto-glutarate, which, as an intermediate of the Krebs cycle, can increase energy metabolism (Olstad et al., 2007; Amaral et al., 2011). The metabolic fate of Glu in the cells is influenced by its extracellular concentration as observed in astrocytes, in which Glu is preferentially metabolized via the Krebs cycle when its extracellular levels rise up to the low millimolar range (McKenna et al., 1996). Indeed, during synaptic stimulation, Glu can reach millimolar concentrations at the synaptic cleft (Clements et al., 1992; Danbolt, 2001; Nyitrai et al., 2006). The extracellular concentration of Glu is spatially and temporally defined by a very efficient reuptake system located in both neuronal and glial cells and composed of highly specialized proteins, the excitatory amino acid transporters (EAATs) (Danbolt, 2001). EAATs contribute to neurotransmitter recycling and prevent the extracellular Glu concentration from rising to neurotoxic levels (Maragakis and Rothstein, 2004). Recent studies indicate that EAATs may not be a mere glutamate “sink” that terminates glutamatergic synaptic transmission, but they can play a more important role in the control of cell energy metabolism than previously recognized. In this regard, an association of glutamate-aspartate transporter (GLAST) and glutamate transporter 1 (GLT1), two
members of the EAATs family, with glycolytic enzymes and mitochondria has been reported (Genda et al., 2011; Bauer et al., 2012). Moreover, the mitochondrial immunoreactivity of EAATs was reported for three EAATs, namely, GLAST (Ralph et al., 2004; Magi et al., 2012), GLT1, and excitatory amino acid carrier 1 (EAAC1) (Magi et al., 2012), the last of which is specifically involved in the Glu-dependent stimulation of ATP production in mitochondria isolated both from neuronal and glial cells (Magi et al., 2012).

Since EAATs transport Glu using the favorable Na+ gradient (Tzingounis and Wadiche, 2007), a mechanism able to restore the transmembrane Na+ gradient after Glu entry is required. Recent studies suggest that the Na+/K+-ATPase, the antiporter enzyme that maintains the Na+ and K+ ion gradients across the membrane, may regulate Glu uptake via the Glu-dependent Glu transport activity is resistant to ouabain at concentrations (i.e., 0.1–5 mM) known to inhibit the rat Na+/K+-ATPase (Johansen et al., 1987; Volterra et al., 1994; Rose et al., 2009; Genda et al., 2011).

Another transporter that could support Glu entry via EAAT is the Na+/Ca2+-exchanger (NCX). NCX catalyzes the bidirectional and electrogenic exchange of 3 Na+ and 1 Ca2+ across the plasma membrane, operating in either Ca2+-efflux/Na+-influx mode (forward mode) or Ca2+-influx/Na+-efflux mode (reverse mode) (Blaustein and Lederer, 1999; Torok, 2007). It has been already proposed that in astrocytes Glu and Na+ entry via EAAT induces a Ca2+ response as a result of the reverse mode of plasma membrane NCX (Kirischuk et al., 2007; Verkhovsky, 2010). In a recent study, we found that EAAT and NCX also localize within brain mitochondria and that EAAC1 and NCX1 exist as a macromolecular complex that allows Glu entry into the matrix, enhancing ATP production (Magi et al., 2012). In this article, we report findings suggesting that plasma membrane EAAT and NCX are involved in Glu-induced ATP synthesis in neuronal, glial, and cardiac cells and that NCX plays a pivotal role in this phenomenon.

Materials and Methods

Cell Cultures

Cell lines (purchased from the American Type Culture Collection, Manassas, VA) were cultured as a monolayer in polystyrene dishes (100-mm diameter) and grown in RPMI 1640 medium (SH-SY5Y cells) or Dulbecco’s modified Eagle’s medium (C6 and H9c2 cells) (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 1% L-glutamine (200 mM) (Invitrogen), 1% sodium pyruvate (100 mM) (Invitrogen), 100 IU/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). Cells were grown in a humidified incubator at 37°C in a 5% CO2 atmosphere.

Generation and Characterization of H9c2 Cells Stable Expressing NCX1

H9c2 wild-type (WT) cells were plated at a density of 1.5 × 10^5 cells/cm², followed by culture in Dulbecco’s modified Eagle’s medium with 10% FBS. After 24 hours, myoblasts were transfected with pcDNA3.1(+)NCX1 (kindly provided by Dr. K. D. Philipson) carrying the Geneticin (G418) resistance gene by using Lipofectamine reagent according to manufacturer’s instructions. After an additional 24 hours, the cells were cultured with selection medium containing G418 at the concentration of 500 μg/ml until control (untransfected) cells died completely. Individual foci from the transfected cells were then selected, subcultured, and transferred to other plates for further propagation. Clonal cells were cultured under the G418 selection medium until a stable cell line (named H9c2-NCX1) was obtained, as revealed by real-time polymerase chain reaction, immunofluorescence analysis (data not shown), and Western blot. For real-time, the following primers were used: forward 5'-GCTCTGGTCTCTGAGTT-GATT-3' and reverse 5'-TTCTCCCGGATGCTTCTGCTT-3'. NCX1 activity was evaluated in Fluor-4–loaded cells using a superfusion protocol designed to evoke Ca2+ uptake through the reverse mode. Briefly, H9c2-WT or H9c2-NCX1 cells were initially superfused with an extracellular solution containing 140 mM NaCl, 5 mM KCl, 20 mM HEPES, 2 mM CaCl2, 1 mM MgCl2, and 10 mM glucose. Then, NCX1 reverse mode was evoked by exposing the cells to a Na+-free solution containing 140 mM LiCl, 5 mM KCl, 20 mM HEPES, 2 mM CaCl2, 1 mM MgCl2, and 10 mM glucose. The pH was adjusted to 7.1 in the Na+-based and Li+-based solutions with NaOH or LiOH, respectively. None of the H9c2-WT cells analyzed showed a Ca2+ response, as observed in H9c2-NCX1 cells.

Antibodies

NCX1 protein was detected by using a commercially available mouse monoclonal IgG antibody (dilution 1:500, R3F1; Swant, Bellinzona, Switzerland). The following primary antibodies were used to detect EAATs: mouse anti-EAAC1 (dilution 1:1000; Chemicon International, Temecula, CA) (Levenson et al., 2002; Castaldo et al., 2007) and rabbit anti-GLAST and rabbit anti-GLT1 (Castaldo et al., 2007), both purchased from Alpha Diagnostic International, Inc. (San Antonio, TX) and used at 1:1000 dilution.

Western Blot and Coimmunoprecipitation Studies

Experiments were performed on whole lysates and plasma membrane crude fractions from both isolated brain tissue and continuous cell lines (i.e., SH-SY5Y human neuroblastoma, C6 rat glioma, H9c2 cardiac myoblast, and H9c2 cells stably expressing NCX1). Whole lysates were obtained using standard techniques and a cell lysis solution containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), SDS 1%, and a protease inhibitor cocktail mixture (Roche Diagnostics, Milan, Italy); plasma membrane crude fractions were obtained as previously described (Castaldo et al., 2007).

Briefly, cells and brain were homogenized in 6 volumes of ice-cold homogenizing buffer: 4 mM Tris-HCl, pH 7.4, 1 mM EDTA (pH 8.0), SDS 1%, and a protease inhibitor cocktail mixture (Roche Diagnostics, Milan, Italy); plasma membrane crude fractions were obtained as previously described (Castaldo et al., 2007). Briefly, cells and brain were homogenized in 6 volumes of ice-cold homogenizing buffer: 4 mM Tris-HCl, pH 7.4, 0.52 mM sucrose, 1 mM EDTA; 0.25 mM dithiothreitol; and protease inhibitor cocktail mixture (Roche Diagnostics). Homogenates were centrifuged 1000g at 4°C for 15 minutes, and supernatants were then centrifuged at 100,000g at 4°C for 1 hour to obtain the crude membrane fraction. The pellet was resuspended in homogenizing buffer and stored at −70°C for immunoblotting.

Cell membrane proteins were immunoprecipitated by using commercially available mouse monoclonal IgG antibodies directed against EAAC1 (1:50; Chemicon International) (Proper et al., 2002; Yu et al., 2006) and NCX1 (R3F1, 1:50; Swant) (Minelli et al., 2007). To recover the immunocomplexes, samples were incubated with A-Sepharose beads (GE Healthcare, Milan, Italy).

Proteins were separated by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane and then incubated with the appropriate primary antibody. Immunoreactions were revealed by incubation with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (dilution 1:1000) for 1 hour at room temperature. An enhanced chemiluminescence detection system (ECLPlus; Amersham Biosciences, Uppsala, Sweden) was used to detect bound antibodies. Images were captured and stored on a ChemiDoc station (Bio-Rad, Milan, Italy) and analyzed with the Quantity One (Bio-Rad) analysis software (Castaldo et al., 2007).
Real-Time Confocal Imaging

Measurement of Membrane Potential. SH-SY5Y, C6, and H9c2 cells grown for 18 hours on poly-L-lysine–coated glass coverslips were loaded for 1 hour at 37°C with 1 μM fluorescent anionic dye bis(1,3-dibutylthiobarbituric acid)-trimethine oxonol (bis-oxonol) (Molecular Probes, Eugene, OR) before each experiment (Ward et al., 2007). The dye is lipophilic and increases or decreases in fluorescence on depolarization or hyperpolarization, respectively. Importantly, the negative charge on bis-oxonol prevents accumulation in mitochondria; therefore, the dye distributes across cell membranes according to the membrane potential ($V_m$), giving a reliable measurement of relative changes in $V_m$ (Mohr and Fewtrell, 1987). After bis-oxonol loading, cells were washed and transferred to a microscopy chamber in standard buffer solution in the presence of 1 μM bis-oxonol. The dye was allowed to equilibrate for an average of 5 minutes, after which a stable baseline was obtained. Cells were then treated with the indicated compounds. Fluorescence was monitored with excitation at 530 nm and emission at 560–585 nm. Confocal images were obtained using the LSM 510 microscope (Carl Zeiss, Milan, Italy) equipped with a META detection system and a 20× objective. Illumination intensity was kept to a minimum (0.1–0.2% of laser output) to avoid phototoxicity; the pinhole was set to give an optical slice of ~1 μm. For data analysis, fluorescence was expressed as ratios ($F/F_0$) of fluorescence counts ($F$) relative to baseline values before stimulation ($F_0$). Data are presented as change in fluorescence relative to initial fluorescent value for each individual cell. Images were captured once every 30 seconds to avoid excessive bleaching of the dye. Cells on coverslips were then perfused with medium containing bis-oxonol (1 μM) at a constant rate, and fluorescence imaging was started. When DL-threo-β-benzylxoyaspartic acid (DL-TBOA) (300 μM), 2-[4-[(4-nitrophenyl)methoxy]phenyl]methyl]-4-thiazolidinecarboxylic acid ethyl ester (SN-6) (3 μM), or 2-[2-[4-phenyl(ethyl)isothiourea mesylate (KB-R7943) (3 μM) was used, they were added starting from preincubation throughout the end of the experiments. Glu or added drugs were diluted in the perfusion medium and applied by switching the reservoirs of the perfusion system. Cell depolarization by perfusion with increased K+–containing extracellular solution was used as positive control (Supplemental Fig. 1). Na+ in the high K+ solution was reduced equivalently to maintain isosmolality.

Analysis of Cytosolic Ca2+. Stock solutions of 5 mM Fluo-4 AM (Molecular Probes) were prepared and stored in aliquots at −20°C. SH-SY5Y, C6, and H9c2 cells were incubated with Fluo-4 AM (concentration 5 μM) for 50 minutes in the incubator at 37°C (Roychowdhury et al., 2006). Cells were superfused with standard buffer solution and were allowed to equilibrate for 15 minutes. For imaging of the Fluo-4 AM fluorescence, excitation light was provided by an argon laser at 488 nm, and the emission was filtered with a 515-nm–long pass filter. Images were acquired using the photomultiplier of the Zeiss LSM 510.

Analysis of fluorescence intensity was performed offline after the image acquisition by averaging the fluorescence intensity values within boxes overlying the cell somata using the imaging software of Zeiss LSM. Data were normalized and the averages of the intensities were calculated.

**Fig. 1.** Involvement of NCX and EAAT in Glu-stimulated ATP synthesis in SH-SY5Y and C6 cells. ATP production in SH-SY5Y (A) and C6 (B) cells after 1-hour incubation with Glu (black bars) or vehicle (gray bars). DL-TBOA was able to counteract Glu-stimulated ATP synthesis in both cell lines. (C and D) Effect of KB-R7943 and SN-6 on Glu-induced ATP synthesis in SH-SY5Y and C6 cells, respectively. Both NCX inhibitors were able to counteract Glu-induced ATP synthesis. Cells were preincubated for 15 minutes with the inhibitors and then exposed to Glu (black bars) or vehicle (gray bars) for 1 hour. The drugs did not affect the energy level at the steady state. Each bar in A–D represents the mean of almost six different experiments. *P < 0.01 versus any other group (A); *P < 0.001 versus any other group (B–D). Ctrl, control.
Analysis of ATP Production

ATP production was evaluated by using a commercially available luciferase-luciferin system (ATPlite; PerkinElmer, Waltham, MA). Twenty-four hours after plating in 96 multiwell plates (60,000/well), cells were first washed with standard buffer solution containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM HEPES, and 5.5 mM glucose (pH 7.4), adjusted with Tris, and then exposed to Glu (0.5–1 mM) in standard buffer solution for 1 hour at 37°C. When ATP levels were evaluated in the absence of extracellular Na⁺, NaCl was substituted with LiCl on an equimolar basis and pH was adjusted with LiOH. On the other hand, when experiments were conducted in Ca²⁺-free conditions, we substituted MgCl₂ for CaCl₂ in the extracellular solution. ATP levels were analyzed after incubation. All ATP data were normalized to the protein content.

Statistical Analysis

Data were expressed as mean ± S.E.M. P < 0.05 was considered significant. Differences among means were assessed by one-way analysis of variance followed by Dunnet’s post-hoc test.

Drugs and Chemicals

DL-TBOA, SN-6, and KB-R7943 were obtained from Tocris (Bristol, UK). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

Results

DL-TBOA Inhibited Glu-Induced ATP Synthesis. As brain cell models, we initially used two cell lines, SH-SY5Y (neuroblastoma cell line) and C6 (glioma cell line), to test the effect of increasing concentrations of Glu, and we found a dose-dependent increase in ATP production with an EC₅₀ of 101.4 and 303 μM, respectively. These values are closely related to the Kₘ value for the Glu uptake, estimated to be around 100 μM (Danbolt, 2001). Interestingly, we found that the Glu-stimulated ATP synthesis in SH-SY5Y and C6 cell lines was counteracted by the nontransportable EAAT blocker DL-TBOA (Shimamoto et al., 1998; Shigeri et al., 2004) (Fig. 1, A and B). Furthermore, DL-TBOA per se had no effect on ATP levels (Fig. 1, A and B).

Glu-Induced Plasma Membrane Depolarization. Considering that substrate uptake by EAATs is electrogenic (Kanai et al., 1993; Danbolt, 2001), the exposure of cells to increased Glu concentrations is expected to elicit a significant plasma membrane depolarization as a consequence of Na⁺ accumulation. This hypothesis was tested by real-time confocal video-imaging studies in cells loaded with the selective indicator of plasma membrane potential bis-oxonol (Parks et al., 2007). Exposure to Glu resulted in a significant

Fig. 2. Real-time membrane potential analysis in intact cells (SH-SY5Y and C6). Experiments performed in SH-SY5Y (A, C, and E) and C6 (B, D, and F) cells using the plasma membrane potential indicator bis-oxonol (1 μM). Glu perfusion induced plasma membrane depolarization (blue line). DL-TBOA (300 μM) (pink line), SN-6 (3 μM) (green line), and KB-R7943 (yellow line) (3 μM), all perfused from 20 minutes before through the end of recordings, prevented Glu-stimulated plasma membrane depolarization. For each group, more than 50 cells recorded in three different experimental sessions were analyzed, and the maximal depolarization induced after Glu stimulation was used for the statistical analysis. *P < 0.001 versus any other group. (E and F) Inhibitors did not have any significant effect on plasma membrane potential. For each group, more than 30 cells recorded in three different experimental sessions were analyzed. Ctrl, control.
depolarization in both SH-SY5Y and C6 cells (Fig. 2, A–D). In SH-SY5Y cells, the increase in the intensity of bis-oxonol fluorescence was greater than that observed with high K+-evoked depolarization (Supplemental Fig. 1). At present, we can speculate that this effect may depend on the cell type used. In line with previous results, in both cell lines, the depolarization was completely counteracted by DL-TBOA, confirming the key role of Na+/Glu cotransport in this response (Fig. 2, A–D).

**Involvement of NCX in Glu-Induced ATP Synthesis.** Since plasma membrane EAATs cotransport Na+/Glu, maintenance of the Na+ gradient is fundamental to their activities. We previously showed that in brain mitochondria, Glu entry via EAAT requires a functional mitochondrial NCX (specifically, NCX1) (Magi et al., 2012). Considering that NCX transporters are also expressed on cellular surface (Minelli et al., 2007; Torok, 2007), we tested the idea that an equivalent functional interaction between EAAT and NCX also exists for Glu entry into the cells. As shown in Fig. 2, A–D, blockade of NCX conductance with KB-R7943 and SN-6 (Watanabe et al., 2006; Niu et al., 2007) completely prevented the Glu-induced depolarization in SH-SY5Y and C6 cells. In addition, we found that the two NCX inhibitors completely prevented Glu-induced ATP synthesis (Fig. 1, C and D). Collectively, these data suggest that NCX activity is critical for Glu-induced ATP synthesis. Membrane depolarization and intracellular Na+ accumulation observed during Glu exposure may favor the reverse mode of operation of NCX, which in turn will tend to bring Ca2+ into the cytoplasm (Rojas et al., 2013). Therefore, we measured intracellular Ca2+ concentration [Ca2+]cyt in SH-SY5Y and C6 cells before and after Glu exposure. Glu significantly increased [Ca2+]cyt in both SH-SY5Y and C6 cells (Fig. 3, A–D). This effect was completely abolished by DL-TBOA and by the two NCX inhibitors, KB-R7943 and SN-6 (Fig. 3, A–D), confirming a close functional relationship between NCX and EAAT.

![Fig. 3. Real-time intracellular Ca2+ analysis in intact cells (SH-SY5Y and C6). Experiments performed in SH-SY5Y (A, C, and E) and C6 (B, D, and F) cells using the intracellular Ca2+ indicator Fluo-4 AM (5 μM). Glu perfusion induced an increase in intracellular Ca2+ levels (blue line). DL-TBOA (300 μM) (pink line), SN-6 (3 μM) (green line), and KB-R7943 (3 μM) (yellow line), all perfused from 20 minutes before through the end of recordings, prevented Glu-stimulated [Ca2+]cyt increase. For each group, more than 50 cells recorded in three different experimental sessions were analyzed, and the maximal [Ca2+]cyt induced after Glu stimulation was used for the statistical analysis. *P < 0.001 versus any other group. (E and F) Inhibitors did not have any significant effect on [Ca2+]cyt. For each group, more than 30 cells recorded in three different experimental sessions were analyzed. Ctrl, control.](molpharm.aspetjournals.org/doi/abs/10.1124/mol.117.111050)
Finally, we found that all the pharmacological inhibitors (DL-TBOA, SN-6, and KB-R7943) had no effect on plasma membrane potential (Fig. 2, E and F) and \([\text{Ca}^{2+}]_{\text{cyt}}\) (Fig. 3, E and F).

**Plasma Membrane EAAT and NCX: Physical Interaction.** We hypothesized that the functional interaction between NCX and EAAT could be consistent with their physical interaction. In a previous study (Magi et al., 2012), we demonstrated a physical link between NCX1 and EAAC1 within mitochondria, where the two transporters cooperate in the Glu-stimulated ATP synthesis. Since EAAC1 is the EAAT isoform predominately expressed in SH-SY5Y and C6 cells (Fig. 4A) (Magi et al., 2012), we speculated that a similar relationship could exist on the plasma membrane. Coimmunoprecipitation studies performed on membrane protein fractions revealed a strong NCX1 immunoreactivity in the EAAC1 immunoprecipitates and, in line with this result, EAAC1 was pulled down by NCX1 antibody on reverse immunoprecipitation (Fig. 4, B and C).

**Plasma Membrane EAAT and NCX Functional Interaction in Cardiac Cells.** Glu is an important substrate for the intermediary metabolism not only in the brain but also in other organs, such as the heart (Dinkelborg et al., 1996). Several studies have proposed that Glu plays an important role in the recovery of cardiac oxidative metabolism after ischemia (Svedjeholm et al., 1996; Kugler, 2004; Vanky et al., 2006). Similar to what was observed in neuronal and glial cells, such metabolic response to extracellular Glu can only take place if cardiac cells express on their sarcolemma an efficient uptake system. Indeed, functional EAATs are expressed in cardiomyocytes (King et al., 2001; Kugler, 2004). We therefore hypothesized that also in the heart, the observed metabolic response elicited by Glu influx via EAAT could be regulated on the cell surface by the cardiac isoform NCX1 (Shigekawa and Iwamoto, 2001; Menick et al., 2007). To test this possibility, we decided to use as cardiac model the rat heart–derived H9c2 myoblasts (Menard et al., 1999).

In preliminary experiments, we found that our H9c2 clone expresses three EAAT isoforms (GLAST, GLT1, and EAAC1) but lacks endogenous expression of NCX1 (Fig. 5A), even after 7 days of differentiation in 1% FBS or 10 nM retinoic acid (Menard et al., 1999) (data not shown). In addition, in this cell line, we failed to detect any significant response to Glu stimulation in terms of ATP production, plasma membrane depolarization, and \([\text{Ca}^{2+}]_{\text{cyt}}\) increase (Figs. 5B, 6A, and 7A).

We speculated that such unresponsiveness of H9c2 cells to Glu stimulation was due to the absence of NCX1. For confirmation, we decided to use our H9c2 cells (that we named H9c2-WT) and to generate an H9c2 clone (that we named H9c2-NCX1) by stably expressing a functional NCX1 (Fig. 5A; see Materials and Methods). NCX1 expression enabled H9c2 cells to respond to Glu, being ATP production–stimulated, plasma membrane depolarization–induced, and \([\text{Ca}^{2+}]_{\text{cyt}}\)–increased (Figs. 5B, 6A, and 7A).

Concerning membrane depolarization in H9c2-NCX1, the increase in bis-oxonol fluorescence intensity was lower than that observed in SH-SY5Y and C6 cells and lower than that observed in the same cell line with high K\(^+\) (Supplemental Fig. 4).
Fig. 1). We hypothesized that this effect may be related to the specific cell type. Notably, both DL-TBOA and SN-6 counteracted all the Glu-induced responses analyzed (Figs. 5, C and D, 6C, and 7C) but were without effects in unstimulated H9c2-NCX1 cells (Figs. 6B and 7B). Moreover, as observed in SH-SY5Y and C6 cells, coimmunoprecipitation studies performed on H9c2-NCX1 membrane protein extracts revealed a physical interaction between EAAC1 and NCX1 (Fig. 5A). In this regard, H9c2-WT cells, lacking endogenous NCX1 expression, provided a good negative control to confirm the specificity of such interaction. Hence, we carried out coimmunoprecipitation studies on this cell line. When membrane protein extracts from H9c2-WT were pulled down with NCX1 antibody, no immunoreactivity for EAAC1 was detected. Consistent with this result, no signal for NCX1 protein was detected when H9c2-WT protein membrane extracts were pulled down with EAAC1 (Fig. 5A).

Glu-Induced ATP Synthesis: Role of Ca²⁺ and Glu. Our results showed that exposure to Glu was able to increase ATP synthesis in both neuronal and non-neuronal cells through the interplay between plasma membrane EAAT and NCX. We hypothesized a crucial role for NCX1 since, by working on the reverse mode, it probably contributed to maintenance of the Na⁺ gradient required for the EAAT activity. At the same time, as a consequence of NCX reverse mode activity, we observed an increase in [Ca²⁺]_{cyt} that per se could represent a stimulus able to increase cellular ATP content through activation of the mitochondrial Ca²⁺-dependent dehydrogenases (Denton, 2009). On the other hand, Glu is an important substrate that can be used by mitochondria to generate ATP. To sort out these issues definitively and clarify the mechanism underlying ATP production after Glu exposure, we tried to study separately the effects of Ca²⁺ and Glu.

To evaluate the role of Ca²⁺ in the absence of Glu, we measured the ATP content of C6, SH-SY5Y, and H9c2-NCX1 cells after 1-hour exposure to a Na⁺-free extracellular solution, known to evoke NCX reverse mode, and, consequently, to induce a rise in [Ca²⁺]_{cyt} (Amoroso et al., 2000) (Fig. 5A). Conversely, to evaluate the role of Glu, we measured the ATP content of the same cell lines after 1-hour exposure to Glu in the absence of extracellular Ca²⁺. Interestingly, we observed that neither Ca²⁺ nor Glu was able to induce any significant increase in ATP content on their own. The increase in ATP synthesis occurred exclusively when cells were exposed to Glu in the presence of extracellular Ca²⁺ (Fig. 8).
The results of this study show that Glu (0.5–1 mM) was able to increase ATP synthesis in SH-SY5Y (neuroblastoma) and C6 (glioma) cell lines used as model of neuronal and non-neuronal cells, respectively. Both plasma membrane NCX and EAAT activities are required to elicit this metabolic response. This experimental evidence expanded our previous study in which we demonstrated a key role of the mitochondrial NCX and EAAT in Glu-induced ATP synthesis (Magi et al., 2012) and strengthened the earlier postulated role of Glu in brain energy metabolism (McKenna et al., 1996; Olstad et al., 2007).

It is well known that as a neurotransmitter, the released Glu exerts its signaling function by interacting with specific receptors until it is removed from the extracellular fluid by the rapid uptake operated by EAATs (Kanai et al., 1993; Danbolt, 2001). The Glu taken up from the cells can be used as intermediary metabolite for ATP production (Hertz and Hertz, 2003; Panov et al., 2009); in this regard, a role for the EAAT-dependent uptake in brain cell energy metabolism has been also hypothesized (Sonnewald et al., 1997; Genda et al., 2011; Bauer et al., 2012), but at present, scarce information is available on the role of EAATs in the metabolic response to Glu. Our results clearly showed that EAATs mediate Glu entry into the cells, leading to ATP production, since the nontransportable EAAT inhibitor DL-TBOA (Shimamoto et al., 1998; Anderson et al., 2001; Shigeri et al., 2004; Montiel et al., 2005) completely prevented the metabolic response to Glu (Fig. 1, A and B). EAATs are Na⁺-dependent transporters (Tzingounis and Wadiche, 2007); thus, consistent with the electrogenicity of EAAT Glu-uptake, significant plasma membrane depolarization is expected to occur as a consequence of the Na⁺ entry. In line with this idea, we observed a Glu-induced and DL-TBOA–inhibited plasma membrane depolarization (Fig. 2). Because of the EAAT Na⁺ dependency, a mechanism is required to preserve the driving force provided by the transmembrane Na⁺ gradient after Glu entry. For this reason, we tested the hypothesis that the plasma membrane NCX proteins could sustain the activity of EAATs by extruding the Na⁺ ions flowing into the cells with Glu. The blockade with specific inhibitors, such as KB-R7943 or SN-6 (Watanabe et al., 2006; Niu et al., 2007), completely prevented plasma membrane depolarization and a Glu-mediated ATP increase in both SH-SY5Y and C6 cells (Figs. 1, C and D, and 2, A–D), confirming that NCX, by operating on the reverse mode, can restore the Na⁺ gradient and consequently sustain EAAT activity. If NCX is working on the reverse mode, then the Na⁺ extrusion from the cytoplasm should be coupled to a Ca²⁺ entry, and exposure to Glu is expected to elicit an increase in [Ca²⁺]cyt. Confocal video imaging experiments with Fluo-4 AM substantiated this hypothesis (Fig. 3), providing further evidence for a key role of NCX in the Glu-induced ATP synthesis. Notably, such Ca²⁺ response that is due to the reverse operation mode of NCX was completely counteracted by KB-R7943 or SN-6 (Fig. 3). Obviously, DL-TBOA was also able to block the [Ca²⁺]cyt increase induced by Glu (Fig. 3). In this regard, Rojas et al.
(2013) demonstrated that in rat cerebellar type 1 astrocytes, the intracellular Ca\(^{2+}\) signal induced by physiologic concentration of the excitatory amino acid Glu and aspartate is the result of the Na\(^+\) entry through EAAT, which activates the reverse mode of NCX leading to Ca\(^{2+}\) entry (Rojas et al., 2013). It is also interesting to note that previous morphologic observations showed that both EAAT (Danbolt, 2001) and NCX (Minelli et al., 2007) localized in the terminal processes of astroglial cells.

We recently showed that EAATs are also expressed within mitochondria in various tissues (i.e., brain, heart) and cell lines (Magi et al., 2012). Such subcellular localization has a functional relevance since these transporters contribute to the Glu-stimulated ATP synthesis (Magi et al., 2012). Notably, the mitochondrial EAAT-dependent Glu entry route is regulated by mitochondrial NCX (Magi et al., 2012). In particular, we reported that this mechanism relies on the selective interaction between a specific EAATs subtype—EAAC1—and a specific NCX subtype—NCX1. Based on these findings, we tested the hypothesis that EAAC1 (the main EAAT isoform expressed by our cell lines (Magi et al., 2012) and NCX1 could also interact at plasma membrane level. We conducted coimmunoprecipitation studies in both SH-SY5Y and C6 cell isolated membrane fractions and, as shown in Fig. 4, we confirmed that NCX1 and EAAC1 associate even on the cell surface as they do within mitochondria.

Collectively, these findings represent one of the main strengths of this study, supporting the idea of a general mechanism in which cooperation between NCX1 and EAAC1 sustains brain energy metabolism, cooperation that can be especially relevant when ATP production is critically compromised, such as in ischemia. In fact, during an ischemic insult, cells massively release Glu, which in turn can lead to cell death immediately after the ischemia, but it might also be essential for the recovery of metabolic functionality in later stages (Ikonomidou and Turski, 2002). Effectively, Glu can participate in the recovery of energy production being used as an intermediary metabolite for ATP synthesis, especially when the oxygen tension is not so low as to abolish the oxidative metabolism, as observed in the ischemic penumbra and in the poststroke recovery phases. The role of substrates as an alternative to glucose in supporting neuronal activity during and after hypoglycemia was recently explored in vitro and in vivo by using NMR, spectroscopy, and metabolic modeling (Choi et al., 2001; Criego et al., 2005; Oz et al., 2009; Rao et al., 2010; Amaral et al., 2011). In this regard, Sutherland et al. (2008) investigated how metabolism was processed in the rat brain during and after recovery from profound hypoglycemia. They provided evidence of a time-dependent increase in aspartate in parallel to a decrease in Glu/glutamine levels and suggested that Glu, via aspartate aminotransferase, is the primary source of carbon when
glucose-derived pyruvate is unavailable (Sutherland et al., 2008). A substantial net consumption of Glu during hypoglycemia was also documented by Rao et al. (2010) by monitoring the neurochemical profile in the hippocampus of 14-day-old rats.

To explore further the physiologic importance of the EAAC1-NCX1 relationship, we tested the possibility that a functional association between NCX1 and EAAC1 could be involved in the metabolic response to Glu not only in brain but also in the heart. Early evidence suggests that in cardiac tissue, Glu exerts a protective action in ischemia by sustaining energy metabolism (Pisarenko et al., 1995). More recently, a putative protective role of Glu in myocardial infarction has also been postulated (Sivakumar et al., 2008, 2011). However, to the best of our knowledge, no molecular mechanisms have been proposed. To this aim, we decided to use as cardiac model two cell clones, namely H9c2-WT (with no detectable endogenous NCX1 expression) and H9c2-NCX1 (constitutively expressing NCX1). Since both H9c2-WT and H9c2-NCX1 express GLAST, GLT-1 and EAAC1, these cell lines gave us the chance to specifically evaluate the importance of NCX1 in Glu-response. In line with the results obtained under NCX pharmacological blockade, in H9c2-WT Glu failed to induce any detectable response in terms of ATP synthesis, plasma membrane depolarization, and [Ca\textsuperscript{2+}]\textsubscript{cyt} increase (Figs. 5B, 6A, and 7A) However, these DL-TBOA and SN-6 sensible responses were restored in H9c2-NCX1 cells (Figs. 5, B–D, 6, A and C, and 7, A and C), where we also detected the specific EAAC1-NCX1 physical interaction (Fig. 5A).

One may speculate that the increase in ATP content could be ascribed to the increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} (Denton, 2009) occurring as a consequence of the hypothesized NCX reverse mode of operation. However, we observed that a rise in [Ca\textsuperscript{2+}]\textsubscript{cyt} was unable on its own to increase ATP levels in absence of Glu. Otherwise, Ca\textsuperscript{2+} plays a fundamental role, given that in absence of extracellular Ca\textsuperscript{2+}, Glu failed to induce any significant increase in ATP content (Fig. 8).

Therefore the following mechanism can be suggested: NCX activity maintains Na\textsuperscript{+} gradient allowing Glu and Na\textsuperscript{+} ions to enter into the cells. Moreover, the increase in [Na\textsuperscript{+}], induces NCX-reverse mode, leading to [Ca\textsuperscript{2+}]\textsubscript{cyt} increase. Such increase can be buffered by mitochondria, stimulating Ca\textsuperscript{2+}-dependent dehydrogenases (Denton, 2009). This effect, in the presence of Glu as substrate, may contribute to the increase in ATP synthesis. Indeed, once in the cytoplasm, Glu can be taken up by mitochondria, where EAAC1 coexist with NCX1 in a macromolecular complex as we previously described (Magi et al., 2012). Here NCX1 operates in the reverse mode to re-establish the Na\textsuperscript{+} gradient across the mitochondrial membrane, further increasing mitochondrial Ca\textsuperscript{2+} concentration and Ca\textsuperscript{2+}-dependent dehydrogenases activity, with a concomitant increase in ATP synthesis.

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**Fig. 8.** Effect of Ca\textsuperscript{2+} and Glu on ATP synthesis. ATP production in SH-SY5Y (A), C6 (B), and H9c2-NCX1 (C) after 1-hour exposure to Glu in both the presence and absence of extracellular Ca\textsuperscript{2+}. ATP production was also evaluated in absence of Glu in a Na\textsuperscript{+}-free extracellular solution able to evoke an increase in [Ca\textsuperscript{2+}]\textsubscript{cyt}. For all the three cell lines analyzed, the increase in ATP synthesis occurred exclusively when cells were exposed to Glu in the presence of extracellular Ca\textsuperscript{2+}. Each bar represents the mean of six different experiments for SH-SY5Y cells and of 10 different experiments for C6 and H9c2-NCX1 cells. *P < 0.001 versus any other group (A and B); *P < 0.001 versus Ctrl (control) standard buffer solution (s.b.s.), Ctrl 0Ca\textsuperscript{2+}, Ctrl 0Ca\textsuperscript{2+} + Glu; *P < 0.05 versus 0Na\textsuperscript{+} (C).
In conclusion, we provide evidence that the EAAC1-NCX1 dependent influx pathway participates to the Glu-dependent metabolic response of neuronal and non-neuronal cells. The EAAC1-NCX1 interplay could have important implications, especially in pathologic conditions. Future studies will try to address this hypothesis.

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