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SVCT2 Overexpression in Neuroblastoma Cells Induces Cellular Branching that is Associated with ERK Signaling

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Abstract Expression of the sodium and ascorbic acid (AA) cotransporter SVCT2 is induced during the period of cellular arborization and synaptic maturation of early postnatal (P1-P5) rat cerebral neurons. The physiological importance of the transporter for neurons is evidenced by the lethality and delayed neuronal differentiation detected in mice with ablation of SVCT2. The mechanism(s) involved in these defects and the role of SVCT2 in neuronal branching have not been determined yet. To address this, we used lentiviral expression vectors to increase the levels of SVCT2 in N2a cells and analyzed the effects on neurite formation. Expression of a fusion protein containing the human SVCT2wt and EYFP induced an increase in the number of MAP2+ neurites and filopodia in N2a cells. Overexpression of SVCT2 and treatment with AA promoted ERK1/2 phosphorylation. Our data suggest that enhanced expression of the high affinity AA transporter SVCT2, which tightly regulates intracellular AA concentrations, induces neuronal branching that then activates key signaling pathways that are involved in the differentiation and maturation of cortical neurons during postnatal development.

Keywords SVCT2 · Vitamin C · SVCT1 · Filopodia · Branching · N2a cells

Abbreviations

AA	Ascorbic acid
DMEM	Dulbecco's modified Eagle's medium
EYFP	Enhanced yellow fluorescent protein
FBS	Fetal bovine serum
GLUTs	Glucose transporters
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
SDS	Sodium dodecyl sulfate
SVCT	Sodium and ascorbic acid cotransporter

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Introduction

Vitamin C is involved in the differentiation of precursor cells from the mesencephalon and other regions of the brain [1–3]. Vitamin C concentrations in the brain exceed those in the blood by 10-fold due to uptake of ascorbic acid (AA), the reduced form of vitamin C, by cotransporters of sodium and AA, SVCT1, and SVCT2 [4–7]. SVCT1 is a low affinity/high capacity transporter, and its expression is restricted to the epithelia of the intestine, liver, and kidney [5]. By contrast, SVCT2 is a high affinity/low capacity transporter that is widely expressed throughout the brain, especially in neurons, microglia, the choroid plexus, and hypothalamic tancytes [5, 7–12].

The functional activity of full-length SVCT1 and SVCT2 (SVCT1wt and SVCT2wt) is regulated through coexpression

of short isoforms that are not directly involved in AA uptake [12, 13]. Recently, we demonstrated that a short isoform of SVCT2 (SVCT2sh) is expressed in cortical neurons during postnatal development of the mouse brain and that it oligomerizes with SVCT2wt to regulate AA uptake [12]. During embryonic development of the cerebral cortex, SVCT2 mRNA has been detected in the ventricular and subventricular zones, where radial glial cell bodies are localized [14]. Knockout animals have been crucial for understanding the role of SVCT2 in perinatal survival [15], and the lethality of SVCT2 knockout is due to defects in neuronal differentiation and maturation [16]. Moreover, we recently showed that SVCT2 is induced in the neurons of the internal layers (5 and 6) of the cortex during the first 2 weeks of postnatal development, which coincides with the initiation of the morphological arborization and synaptic maturation of cortical neurons [12]. Because the concentration of AA does not drastically change during the first days of postnatal development [17], the expression levels and cellular membrane localization of SVCT2 might be a key factor that regulates neuronal differentiation and maturation. However, no studies have addressed whether overexpression of SVCT2 and/or AA supplementation promotes neuronal differentiation, and what, if any, signaling pathway is activated by enhanced AA uptake driven by SVCT2 overexpression in these cells. Moreover, the effectiveness of overexpression of the low affinity SVCT1 as a means to further increase the intracellular concentration of vitamin C in neural cells remains unknown.

Here, we provide the first demonstration that SVCT2 overexpression in N2a (N2a) cells induces a differentiated phenotype that is characterized by the development of filopodia and microtubule-associated protein (MAP2)-positive processes. However, AA supplementation did not promote any further increase in N2a differentiation. In contrast, SVCT1 overexpression with or without AA supplementation did not completely reproduce the SVCT2-induced effects. Finally, we show that AA uptake induces MAPK-ERK1/2 phosphorylation in SVCT2-overexpressing N2a cells, which is in agreement with the pivotal role of this signaling pathway in neuronal arborization and synaptogenesis during the development of the CNS.

Materials and Methods

Transient Expression of SVCT Isoforms in n2a Cells N2a cells were seeded on sterile coverslips in 12-well plates at a density of 2×10^5 cells/well and were transiently transfected using Lipofectamine Reagent (Invitrogen, Rockville, MD, USA). Plasmids were added (0.5 μg/well) and included the following: pEYFP-N1/hSVCT2wt, pEYFP-N1/hSVCT2sh, pEYFP-N1/hSVCT1, pEYFP-N1, and pcDNA3.1/hGLUT1.

Additionally, a shRNA of 64 bp, hSVCT2-shRNA, was designed to inhibit the overexpression of human SVCT2wt (NM_005116) after it was cloned into the FuxH1-Off-EGFP (restriction sites AscI and PacI) expression vector. shRNA expression is regulated by the H1 promoter and enhanced green fluorescent protein (EGFP) expression under the CMV promoter. A sequence of 64 bp was also cloned into the same vector to inhibit expression of *E. coli* β-galactosidase, producing the control, eβGal-shRNA. In cotransfection assays, 0.4 μg of phSVCT2-shRNA and pEYFP-N1/hSVCT2wt or 0.4 μg of peβGal-shRNA and pEYFP-N1/hSVCT2wt was added per well. The medium was changed 2.5 h after the transfection, and the cells were examined by microscopy and used for all analyses 24 h after the transfection. In parallel, cells were treated with 200 μM AA, 2.5 h and 18 h after the transfection. For scanning electron microscopy, the cells were fixed with 4 % paraformaldehyde for 30 min and analyzed using an Etec Autoscan SEM (Etec Corp., Hayword, CA, USA).

Cells Culture and Immunocytochemistry N2a mouse neuroblastoma cells (CCL-131) were obtained from American Type Cell Collection (ATCC, Rockville, MD, USA) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with F12 nutrient mixture (Life Technologies, Carlsbad, CA, USA), 10 % fetal bovine serum (FBS), penicillin, and streptomycin. For immunocytochemistry, N2a cells were grown on coverslips, fixed with 4 % paraformaldehyde for 30 min at 22 °C, washed with phosphate-buffered saline (PBS), and incubated in PBS containing 1 % bovine serum albumin (BSA) and 0.2 % triton X-100 for 5 min at 22 °C. The cells were incubated overnight at 22 °C with the following antibodies: anti-SVCT2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MAP2 (1:50; Chemicon, Temecula CA, USA) or anti-βIII-tubulin (1:3000; Promega, Madison, WI, USA). The cells were then incubated for 2 h with Cy3 conjugated affinity purified donkey anti-goat IgG or Cy3 conjugated affinity purified donkey anti-mouse IgG (both 1:200; Jackson ImmunoResearch, West Grove, PA, USA) at 22 °C. In addition, the cells were incubated with TOPRO-3 iodide (1:1000; Life Technologies) for nuclear staining.

Quantification of Filopodia and Processes Quantification of filopodia and processes was performed with a Zeiss Axioplan 2 fluorescence microscope coupled to a Nikon DXM 1200 F digital camera for image capture. For each sample, 100 transfected cells were counted, and the percentage that exhibited processes or filopodia was determined. The criteria used to identify cellular processes and filopodia in the N2a cells were based on the studies of Haapasalo et al. [18]. Briefly, a cellular process was defined as an extension that was longer than half the diameter of the cell body, and filopodia were defined as cellular extensions that were shorter than processes.

Lentiviral Production and Transduction of N2a Cells
High-titer human immunodeficiency virus type 1 (HIV-1)-derived lentiviral vector particles that were pseudotyped with the vesicular stomatitis virus G protein (VSV-G) were produced by transient cotransfection of 293 T cells. The four-plasmid transfection was performed as described previously [19] using the self-inactivating (sin) gene transfer construct pRRLsin.PPTs.hCMV.hSVCT2-EYFPpre, which encodes enhanced yellow fluorescent protein as a reporter gene; pCMVdR8.74, a multiple attenuated packaging construct that contains the RRE sequence that codes for the HIV-1 gag and pol genes; the pRSV-Rev construct that encodes the Rev protein; and pMD2.VSVG, which produces the vesicular stomatitis virus G protein. Briefly, human kidney 293 T cells (1.8×10^7) were plated on 15 cm plates and transfected by calcium phosphate DNA precipitation [20]. Conditioned medium was harvested 62 h after transfection and filtered through 0.45-mm filters. The vector preparation titers were determined by transducing 293 T and Cos-7 cells with serial dilutions of vector supernatants. N2a cells were grown on coverslips and incubated with conditioned medium containing the lentivirus (dilution 1/1) at 37 °C for 14–16 h. The medium was replaced 24 h after the transduction and maintained for another 24 h. Finally, the cells were used for immunocytochemistry or Western blot analyses.

Western Blot Analysis The cells from the 6-well plates were used to prepare total protein extracts by homogenizing transfected N2a cells in 0.3 mM sucrose, 3 mM DTT, 1 mM EDTA, 1.0 mg/mL PMSF, 1 mg/mL of pepstatin A, 2 mg/mL of leupeptin, and 2 mg/mL of aprotinin. To examine ERK1/2 phosphorylation, N2a cells were grown normally for 72 h, maintained in FBS-free medium for 6 h, and then treated with vitamin C for dose–response and time course experiments. The cells were incubated in 70 μL lysis buffer containing 25 mM Tris–HCl (pH 7.4), 25 mM NaCl, 1 % triton X-100, 0.1 % SDS, 500 μM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$, 10 μg/mL of aprotinin, 10 μg/mL of pepstatin A, 10 μg/mL of leupeptin, and 25 nM okadaic acid at 4 °C. Cell lysates were scraped from the culture dishes, incubated for 10 min on ice, and centrifuged at $8000 \times g$ for 15 min at 4 °C. The protein concentration of the supernatant was determined using the Bradford method, and 100 μg of protein was suspended in a Laemmli buffer, separated via 10 % SDS-PAGE, and transferred to PVDF membranes (Merck Millipore Corp., Darmstadt, Germany). The membranes were blocked at room temperature for 1 h in Tris-buffered saline containing 3 % fat-free milk, with or without 0.05 % Tween 20 and were then incubated overnight at 4 °C with an anti-GFP antibody (1:2000; Santa Cruz Biotechnology), which cross-reacts with enhanced yellow fluorescent protein (EYFP). After the membranes were washed with Tris-buffered saline, they were incubated with the secondary antibody for 2 h at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence reagents according to the manufacturer's instructions (PerkinElmer, Waltham, MA, USA). The films were scanned, and the image analysis program Scion (NIH) was used for densitometric analysis of the bands. To correct for loading, membranes were stripped in ReBlot Plus Mild Antibody Striping Solution (Merck Millipore Corp.) at room temperature for 30 min and reprobed with an antiactin antibody (1:1500; Santa Cruz Biotechnology).

Vitamin C Uptake N2a cells that were transfected with hSVCT1-EYFP were carefully selected under the microscope to ensure that only plates with uniformly growing cells were used. The cells from the 12-well plates were used to estimate the average number of cells in each well. Briefly, the cells were washed with incubation buffer (15 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$ and 0.8 mM $MgCl_2$) and incubated in the same medium for 10 min at 37 °C. Uptake assays were performed in 500 μL of incubation buffer containing a final concentration of 100 μM 1-^{14}C-L-AA (specific activity of 4 mCi/mmol; DuPont-NEN, Boston, MA, USA) and 0.1 mM DTT. The uptake was stopped by washing the cells twice with ice-cold incubation buffer consisting of 0.8 mM $HgCl_2$. The cells were homogenized in 0.5 mL of lysis buffer (10 mM Tris–HCl, pH 8.0 and 0.2 % SDS) and assayed by liquid scintillation spectrometry to quantify the incorporated radioactivity in counts per minute (CPM).

Statistical Analysis The data represent the mean±SD of three independent experiments, and each determination was performed in triplicate. Statistical comparisons between two or more groups were carried out using analysis of variance (ANOVA), followed by Bonferroni post tests. $P < 0.001$ was considered significant.

Results

Overexpression of SVCT2 Induces a Differentiated Phenotype in N2a Cells To overexpress human SVCT2 in the N2a cell line, we used the vector pEYFP-N1/hSVCT2wt, which is conjugated to enhanced yellow fluorescent protein (Fig. 1). Non-transfected cells were stained with an anti-βIII-tubulin antibody and exhibited the typical spherical morphology of N2a cells. A single cellular process was observed in some cells (Fig. 1a); this type of process was similar to that observed using the empty vector for EYFP (Fig. 1b, c). However, SVCT2 overexpression induced a dramatic morphological change in more than 70 % of the transfected cells. This change was characterized by filopodia formation and process sprouting, as observed by confocal microscopy (Fig. 1d, e,
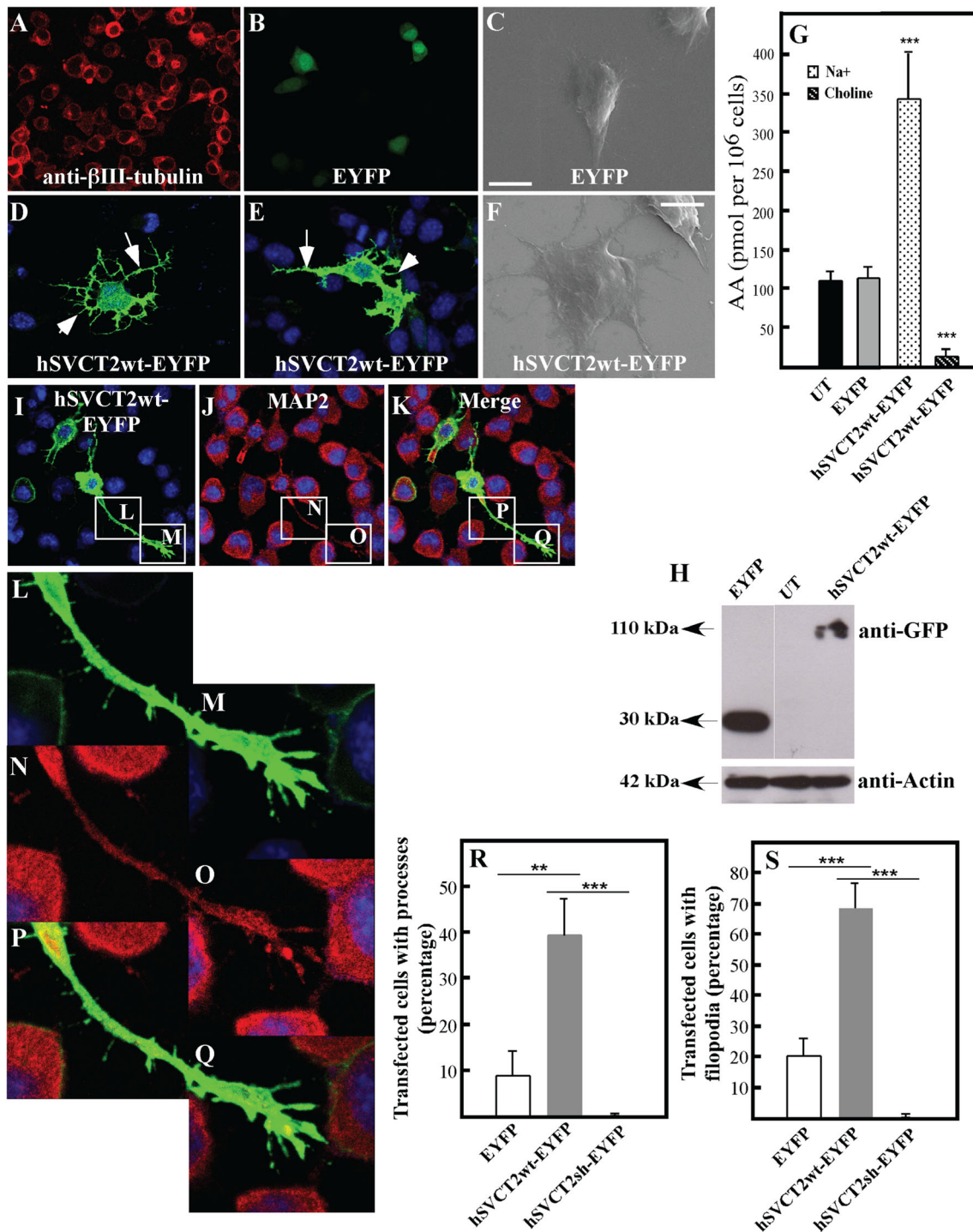
Fig. 1 Overexpression of functional SVCT2 promotes the formation of branches in N2a cells. Confocal microscopy analysis of βIII-tubulin expression in non-transfected cells (Cy3, *red channel*) (**a**). Confocal microscopy (**b**, **d**, **e**) and scanning electron microscopy (*SEM*) (**c**, **f**) analysis of cells expressing enhanced yellow fluorescence protein (*EYFP*) or the human full-length isoform of SVCT2 linked to EYFP (hSVCT2wt-EYFP). hSVCT2wt-EYFP overexpression generated morphological changes characterized by the outgrowth of filopodia (*arrowhead*) and processes (*arrows*) (**d**, **e**). Uptake of 100 μM AA was analyzed in the presence of NaCl or choline at 37 °C in non-transfected and EYFP- or hSVCT2wt-EYFP-overexpressing cells (**g**). Western blot analysis of SVCT2 overexpression using anti-GFP antibodies in total protein extracts from non-transfected and EYFP- or hSVCT2wt-EYFP-overexpressing cells (**h**). Immunofluorescence analysis with an anti-MAP2 antibody (Cy3, *red channel*) in hSVCT2wt-EYFP-overexpressing cells (*green channel*). Nuclei were stained with TOPRO-3 (*blue channel*) (**i–q**). Semiquantitative analysis of cells with processes and filopodia after overexpression of EYFP, hSVCT2wt-EYFP, or the human short-length isoform of SVCT2 (hSVCT2sh-EYFP) (**r**, **s**). Data represent the mean±SD of three independent experiments. **$p < 0.01$; ***$p < 0.001$. Scale bars: C, F, 10 μm

arrowheads and arrows, respectively) and scanning electron microscopy (Fig. 1f). Functional analysis demonstrated a 3-fold increase in sodium-dependent L-^{14}C-AA uptake in the SVCT2-overexpressing cells compared with the non-transfected and EYFP-overexpressing cells (341 ± 71 pmol per 10^6 cells vs. 109 ± 16 pmol per 10^6 cells and 113 ± 24 pmol per 10^6 cells, respectively) (Fig. 1g). SVCT2 overexpression was also analyzed by Western blot analysis using a specific antibody against EYFP that detected a single band of 110 kDa, which corresponds to the fusion protein SVCT2wt-EYFP, compared with a band of 30 kDa, which corresponds to the EYFP protein alone (Fig. 1h). Immunocytochemical analysis of the cytoskeletal neuronal marker MAP2 showed a homogeneous distribution in the cytosol of non-transfected N2a cells, and its redistribution into processes after SVCT2 overexpression (Fig. 1i–q). The number of SVCT2-transfected cells that exhibited filopodia and process outgrowth was quantified 24 h after the transfection (Fig. 1r, s). This showed that approximately 40 % of the SVCT2-transfected cells exhibited processes (Fig. 1r), and nearly 70 % had filopodia (Fig. 1s). Interestingly, overexpression of the short form of human SVCT2 (hSVCT2sh-EYFP) significantly inhibited process and filopodia formation (Fig. 1r, s), and the cells showed the typical spherical morphology (data not shown). These results indicate that full-length SVCT2 overexpression in N2a cells induces a differentiated phenotype that is characterized by filopodia formation and process outgrown.

The N2a Cell Differentiation that is Associated with SVCT2 Overexpression is Dependent on SVCT2 Localization and Function in the Plasma Membrane Using specific shRNA overexpression, we conducted a competition experiment to further evaluate the dependence of the morphological changes that were observed in the N2a cells on SVCT2 overexpression. Confocal analysis of control cells that coexpressed the peβGal-shRNA and pEYFPN1/SVCT2wt vectors revealed the presence of many filopodia and a long principal process (Fig. 2c) in these cells; this long process was similar to that observed in N2a cells following SVCT2 overexpression (Fig. 2b). However, the cells that coexpressed phSVCT2-shRNA and pEYFPN1/SVCT2wt maintained a spherical shape and the absence of filopodia and processes (Fig. 2d), similar to the cells that overexpressed EYFP (Fig. 2a). The necessity of AA uptake for the generation of the morphological changes that were observed in the N2a cells that overexpressed SVCT2 was analyzed using two experimental strategies. The first approach consisted of incubating SVCT2-overexpressing cells with a specific antibody against SVCT2 that blocks AA transport, as previously reported [21]. This condition produced spherical N2a cells; the shape of these cells was similar to that of the EYFP-transfected cells (Fig. 2e, f, a, respectively). In the second experimental approach, SVCT2-overexpressing cells were incubated with
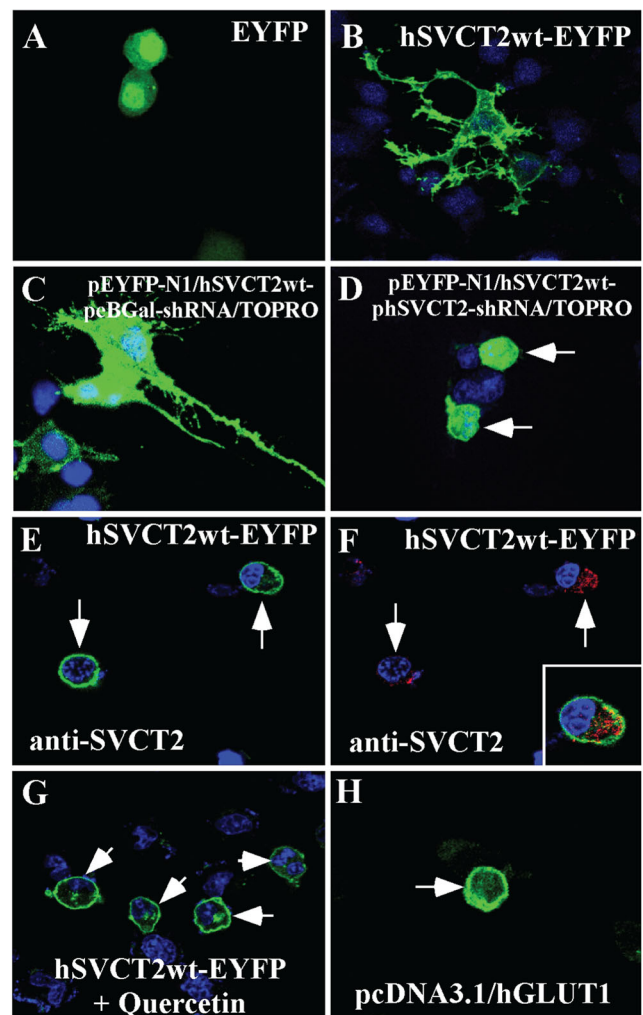

Fig. 2 The morphological changes induced by SVCT2 overexpression are dependent on SVCT2 localization to the plasma membrane and its incorporation of ascorbic acid. Confocal microscopy analysis of cells that overexpressed **a** EYFP (*green channel*), **b** phSVCT2wt-EYFP (*green channel*), **c** phSVCT2wt-EYFP+peβGal-shRNA, **d** phSVCT2wt-EYFP+phSVCT2-shRNA (*arrows*), or **e**, **f** phSVCT2wt-EYFP (*green channel*) overexpressed for 24 h and coincubated with anti-SVCT2 antibody (*arrows*). The samples shown in **e** and **f** were additionally incubated with a Cy3-labeled secondary antibody (*red channel* shown in **f**). Inset in (**f**), merged image of the cells shown in **e** and **f**. **g** Confocal microscopy analysis of cells overexpressing phSVCT2wt-EYFP (*green channel*, *arrows*) in the presence of 300 µM quercetin, which was added after transfection. **h** Confocal microscopy analysis of cells overexpressing the human hexose transporter GLUT1 (*green channel*, *arrow*). Nuclei were stained with TOPRO-3 (*blue channel*)

300 µM quercetin, an inhibitor of SVCT2 function [14], for 24 h. Again, we observed cells that were spherical in shape and that lacked filopodia and processes (Fig. 2g). A similar result was obtained when GLUT1 was overexpressed in these cells (Fig. 2h). Altogether, our data indicate that functional SVCT2 is required to induce N2a differentiation.

AA Supplementation Does Not Enhance the Differentiation Effect of SVCT2 Overexpression in N2a Cells Because

the culture medium contained low concentrations of AA, we decided to evaluate whether increasing the availability of AA would enhance the morphological changes that were induced by SVCT2 overexpression. Cells that overexpressed EYFP, EYFP-hSVCT2wt, or EYFP-hSVCT2sh were supplemented twice with 200 μM AA and maintained for 24 h (Fig. 3a). Neither microscopic nor quantitative analyses demonstrated significant morphological differences between the cells that overexpressed the control and fusion proteins with or without AA supplementation (Fig. 3b–i). These results indicate that AA supplementation does not increase the morphological

changes that are induced by SVCT2 overexpression in N2a cells.

Increasing Intracellular Vitamin C Content Via the Overexpression of SVCT1 and AA Supplementation Does Not Completely Reproduce the Morphological Changes Induced by SVCT2 Overexpression

We next studied whether overexpression of SVCT1, a low affinity AA transporter that has a higher transport capacity at 200 μM AA than does SVCT2, would reproduce SVCT2-induced filopodia and process outgrowth. We used the vector pEYFPN1/hSVCT1 to

Fig. 3 Vitamin C supplementation does not increase the morphological changes induced by SVCT2 overexpression in N2a cells. The experimental protocol for AA supplementation in N2a cells (a). Confocal microscopy analysis of cells overexpressing EYFP, hSVCT2sh-EYFP, or SVCT2wt-EYFP in the absence (b–d) or presence (e–g) of 200 μM ascorbic acid. Semiquantitative analysis of cells with processes (h) and filopodia (i) after the overexpression of EYFP, hSVCT2wt-EYFP, or hSVCT2sh-EYFP and incubation in the absence or presence of 200 μM ascorbic acid. Data represent the mean±SD of three independent experiments. ns: not statistically significant
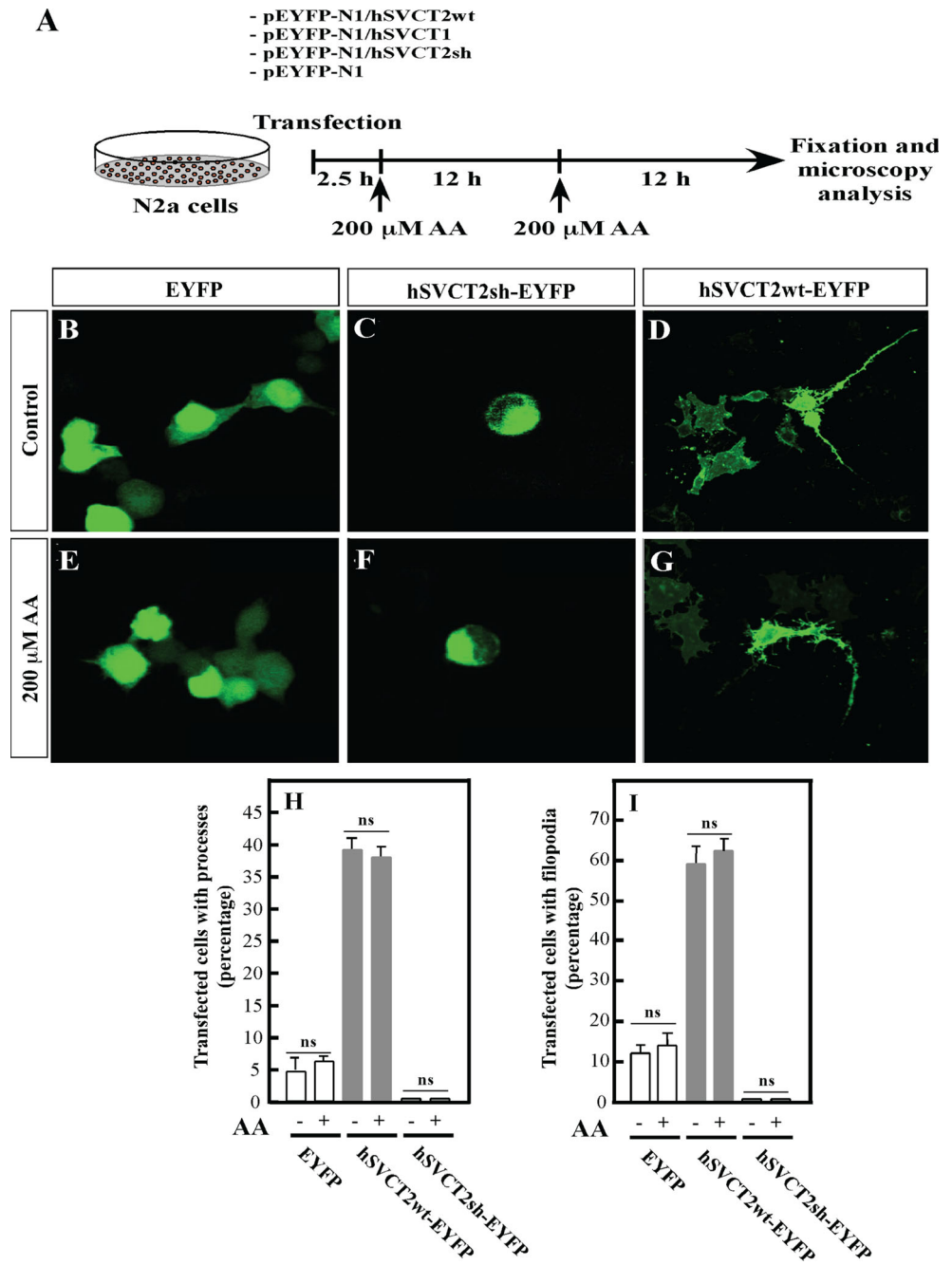
overexpress the human SVCT1 isoform conjugated to EYFP in the N2a cell line (Fig. 4). Total protein extracts from the pEYFP-N1/hSVCT1-transfected N2a cells were probed with a specific antibody against SVCT1, and a single band with an
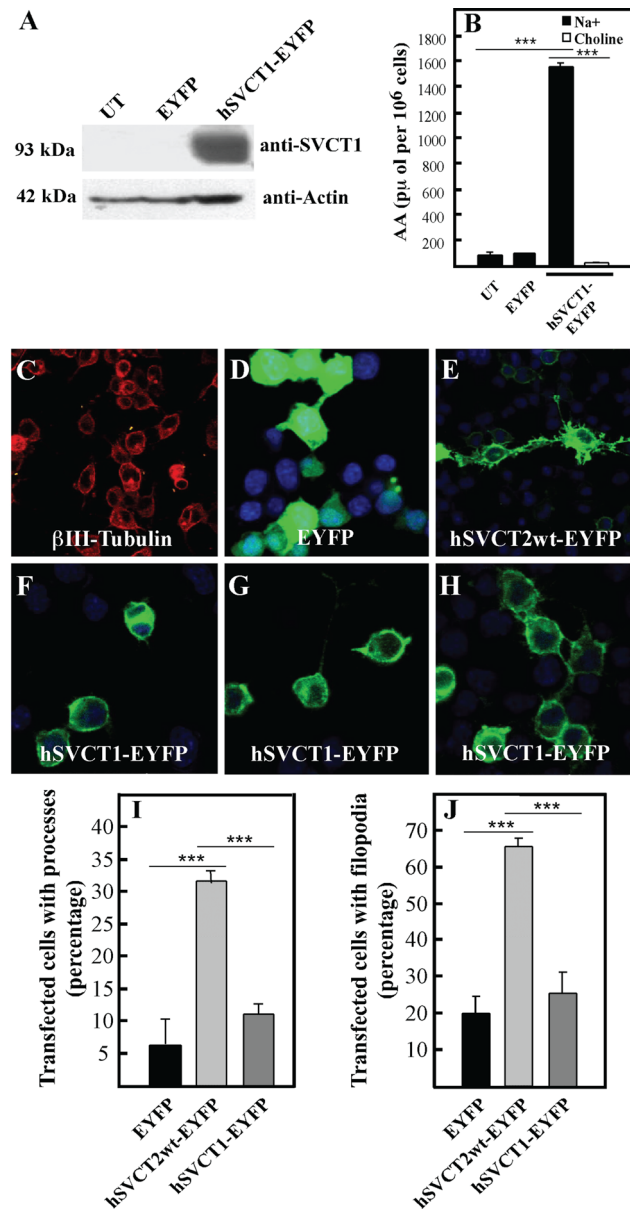

Fig. 4 SVCT1 overexpression does not reproduce the differentiation of N2a cells observed after SVCT2 overexpression. Western blot analysis of SVCT1 overexpression with an anti-SVCT1 antibody in total protein extracts from non-transfected or EYFP- and hSVCT1-EYFP-overexpressing cells (a). Uptake of 100 μM AA was studied in the presence of NaCl or choline at 37 °C in non-transfected and EYFP- or hSVCT1wt-EYFP-overexpressing cells (b). βIII-tubulin immunostaining in non-transfected cells (Cy3, *red channel*) (c). **d–h**. Confocal microscopy analysis of cells overexpressing EYFP (**d**), hSVCT2wt-EYFP (**e**), or hSVCT1-EYFP (**f–h**). Nuclei were stained with TOPRO-3 (*blue channel*) (**d–h**). Semiquantitative analysis of cells with processes (**i**) and filopodia (**j**) after the overexpression of EYFP, hSVCT2wt-EYFP, or hSVCT1-EYFP. Data represent the mean±SD of three independent experiments. ***$p<0.001$

apparent molecular mass of 93 kDa was observed (Fig. 4a). Moreover, functional studies revealed that the L-^{14}C-AA uptake of the SVCT1-overexpressing cells was 15-fold higher than that of the non-transfected or EYFP-overexpressing cells (1564±20 pmol per 10^6 cells vs. 91±19 pmol per 10^6 cells and 99±0.3 pmol per 10^6 cells, respectively) (Fig. 4b). However, not all of the SVCT1-overexpressing N2a cells showed the morphological changes (Fig. 4f–h) that were previously observed following SVCT2 overexpression (Fig. 4e); instead, these cells maintained a phenotype that was similar to that of the control cells (Fig. 4c, d). Quantification of filopodia and process outgrowth after 24 h of transfection (Fig. 4i, j) showed a similar percentage of cells with each morphological feature after transduction with SVCT1 (filopodia 23±4 %; processes 12±2 %) or control vector (filopodia 20±3 %; processes 7± 4 %). In contrast, both values were 3-fold higher SVCT2-overexpressors (filopodia 66±3 %; processes 32±2 %). These results underline that only SVCT2, but not the low affinity/ high capacity AA transporter SVCT1, is capable of inducing morphological arborizations in N2a cells.

The reduced effects of SVCT1 on neuronal differentiation could be caused by the inefficient transport of AA from the low culture medium, which was low in AA levels. As expected, supplementation of AA in the culture medium of N2a cells overexpressing either EYFP or hSVCT2-EYFP did not impact on the morphological changes (Fig. 5b, c, e, f). Moreover, cells overexpressing hSVCT1-EYFP showed the outgrowth of a few short processes and filopodia after addition of AA (Fig. 5d–g, arrows and arrowhead, respectively). Quantification analysis showed that overexpression of hSVCT1-EYFP in combination with AA supplementation significantly increased the percentage of cells with processes and filopodia (18±2 and 43±1 %, respectively) compared with control cells (11±3 and 22±9 %, respectively) (Fig. 5h, i). However, these values were still lower than those obtained with SVCT2 overexpression alone. These results indicate that the differentiation of N2a cells that was induced by overexpression of SVCT2 was partially emulated by hSVCT1 overexpression but only after supplementation with 200 μM AA.

The Combination of SVCT2 Overexpression and AA Treatment Increases ERK1/2 Phosphorylation in N2a Cells Various reports have shown that vitamin C can activate ERK1/2 in different types of peripheral cells; this activation generates diverse cellular responses, such as proliferation, differentiation, and apoptosis [22–24]. However, it is unknown whether AA can promote the phosphorylation of ERK1/2 in cells of a neuronal lineage and whether activation of this pathway could promote AA-mediated neuronal differentiation. We evaluated the ability of AA to induce ERK1/2 phosphorylation in N2a cells that overexpressed hSVCT2. Due to the low transfection efficiency obtained with Lipofectamine reagent, we used hSVCT2-EYFP-expressing lentivirus to increase the

Fig. 5 Ascorbic acid supplementation with SVCT1 overexpression does not completely reproduce the morphological changes induced by SVCT2 overexpression in N2a cells. The experimental protocol for AA supplementation in N2a cells (**a**). Confocal microscopy analysis of cells overexpressing EYFP, hSVCT2wt-EYFP, or hSVCT1-EYFP in the absence (**b**–**d**) or presence (**e**–**g**) of 200 μM ascorbic acid. *Arrows*, cell processes (**g**). *Arrowhead*, filopodia (**g**). Semiquantitative analysis of cells with processes (**h**) and filopodia (**i**) after the overexpression of EYFP, hSVCT2wt-EYFP, or hSVCT1-EYFP and incubation in the absence or presence of 200 μM ascorbic acid. Data represent the mean±SD of three independent experiments. *ns* not statistically significant. Scale bars: B–G, 20 μm
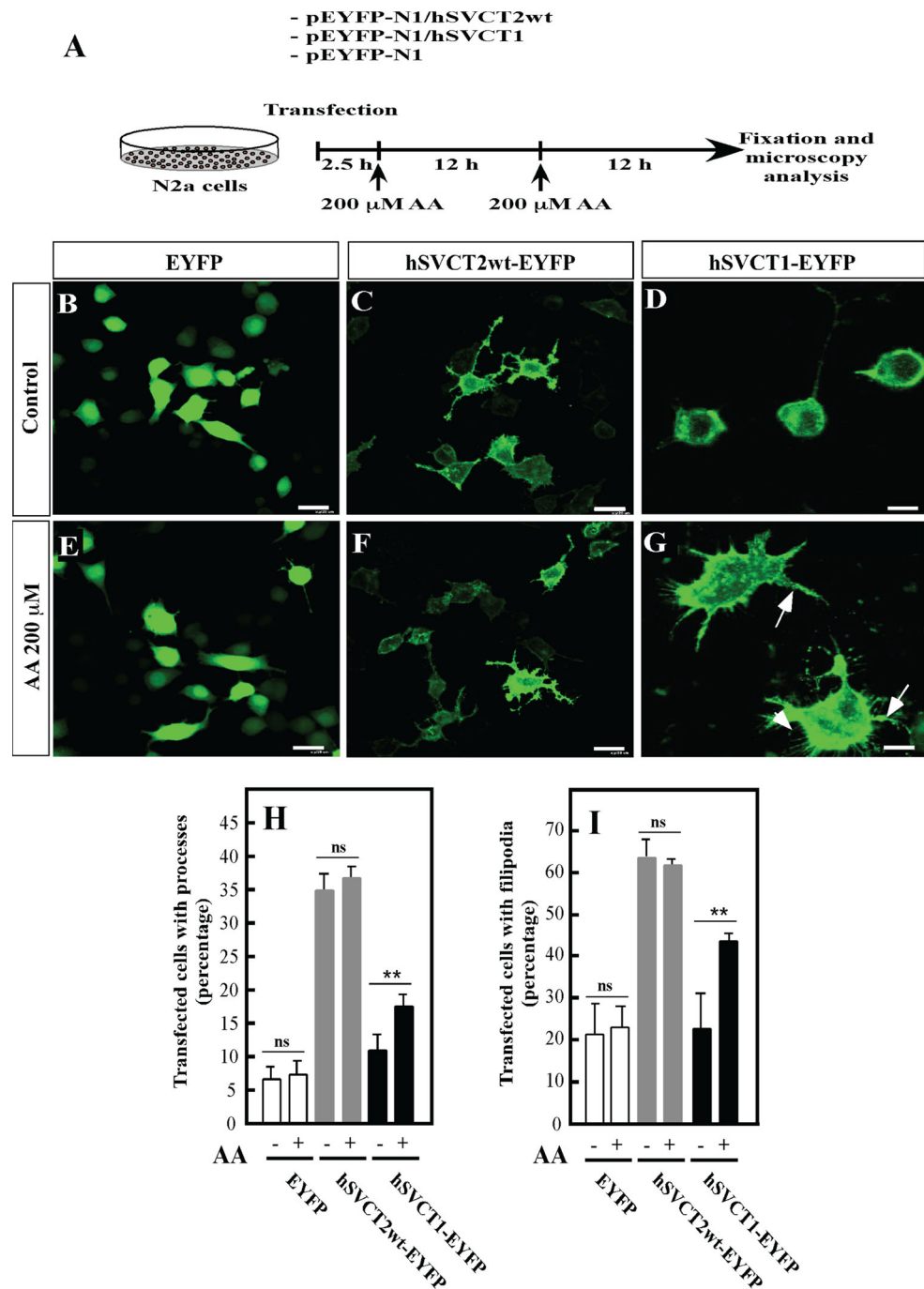
percentage of cells that overexpressed SVCT2 to enhance the possibility of detecting changes in ERK1/2 phosphorylation (Supplementary Figure 1). To evaluate the effects of AA on ERK1/2 activation, growth-arrested control (non-transduced or EGFP-transduced) or SVCT2-overexpressing cells were analyzed at different times (Fig. 6a). ERK1/2 phosphorylation was increased 10-fold at 15 min in SVCT2-overexpressing cells (Fig. 6d). This increase was sustained for 120 min (Fig. 6e). In contrast, no changes in ERK1/2 phosphorylation were detected in control cells (Fig. 6b, c). We also determined

the concentration dependence for AA-mediated ERK1/2 activation in the control and SVCT2-overexpressing cells using a similar viral transduction and growth arrest protocol after the cells were maintained for another 15 min in PBS (control) or in 15, 50, and 150 μM AA (Fig. 7a). The degree of ERK1/2 phosphorylation was not altered in the non-transduced and EGFP-transduced control cells at any AA concentration tested (Fig. 7b, c). However, the N2a cells that were transduced with the SVCT2 lentivirus and treated with 15–150 μM AA showed a 10-fold increase in ERK1/2 phosphorylation
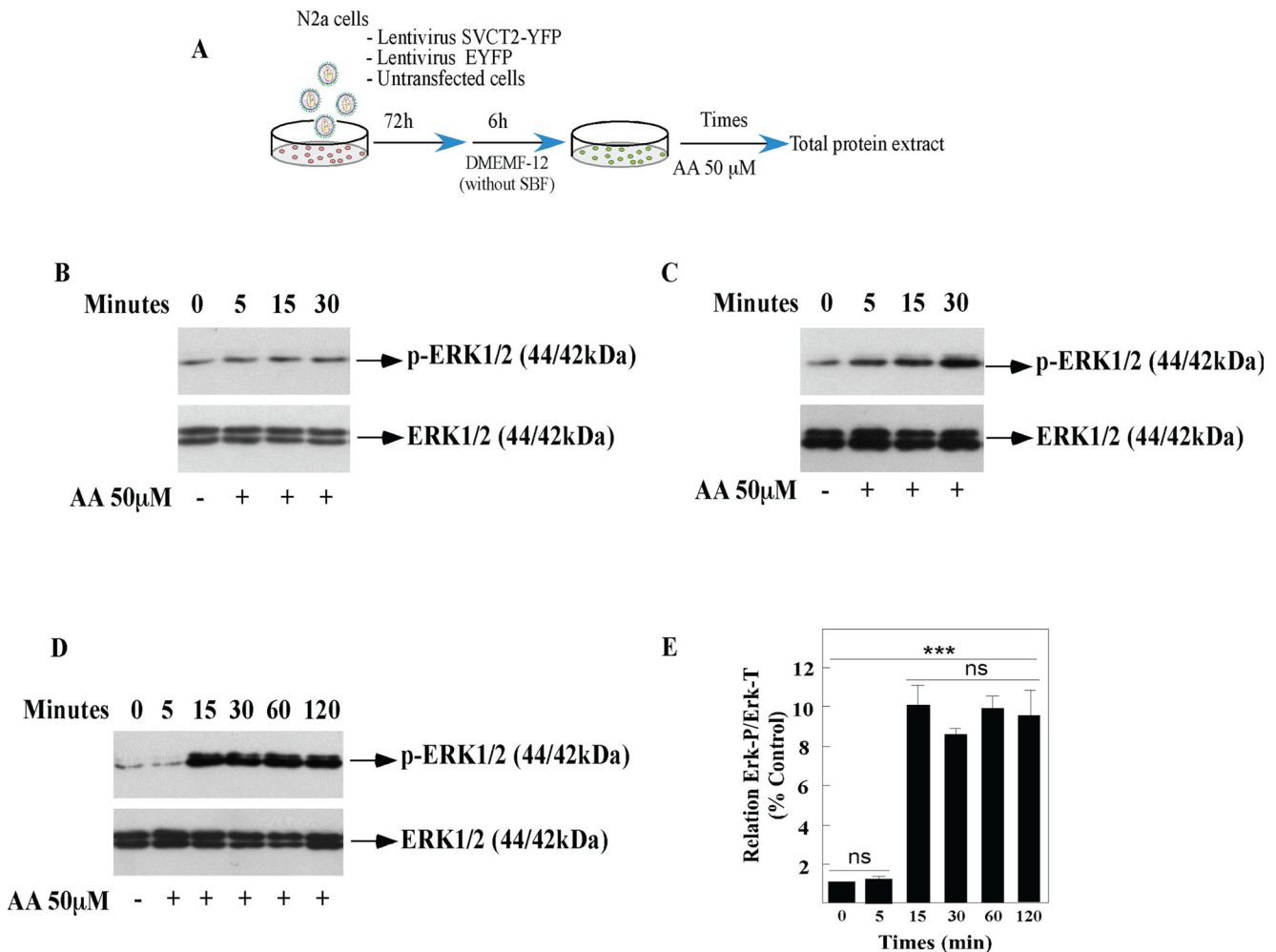
Fig. 6 Ascorbic acid stimulates ERK1/2 phosphorylation in SVCT2 overexpressing N2a cells. The experimental protocol for lentiviral transduction and AA supplementation in N2a cells (**a**). Cells were left non-transduced (*control*) or were transduced with different lentiviral particles and maintained for 72 h, after which they were growth-arrested for 6 h and stimulated with 50 μM AA for different periods of time. Western blot analysis of phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 in protein extracts from non-transduced (**b**), EGFP-transduced (**c**), and hSVCT2-EYFP-transduced (**d**) cells. The membranes were probed with anti-pERK1/2 and total ERK1/2 antibodies (*loading control*). Semiquantitative analysis of ERK1/2 phosphorylation levels in cells overexpressing SVCT2. A 10-fold increase in the levels of ERK1/2 phosphorylation was observed after 15 min and for up to 120 min of treatment with 50 μM AA. No differences in the phosphorylation of ERK1/2 were detected in the non-transduced or EGFP-transduced cells. The results were normalized by arbitrarily setting the densitometry value of the control cells (time=0) to 1. Data represent the mean±SD of three independent experiments. ***$p < 0.001$

(Fig. 7d, f). Taken together, these results demonstrate that overexpression of SVCT2 in combination with AA supplementation induces phosphorylation of ERK1/2 in N2a cells. ERK1/2 activation could mediate the morphological changes and differentiation effect observed after SVCT2 overexpression in vitro.

Discussion

Using the N2a cell line, we aimed to elucidate the participation of SVCT2 in the differentiation of neuronal cells and also to separate the effects of SVCT2 overexpression and function from AA supplementation. Moreover, we also overexpressed

SVCT1 in the presence of AA supplementation to evaluate the effects of high intracellular AA concentrations on neuronal differentiation. We provided the first demonstration that over-expression of SVCT2 induced the differentiation of N2a cells and that this effect required low extracellular levels of AA. Finally, we observed that AA uptake by the N2a cells induced activation of the ERK1/2 protein kinase pathway, a key signaling cascade that is involved in neuronal branching and synaptogenesis during CNS development [25–27].

Previous reports have shown that vitamin C enhances the differentiation of various cell types [28–32]. However, only MC3T3-E1 osteoblast cells have been shown to differentiate after SVCT2 overexpression in vitro [30]. N2a is a mouse neural crest-derived cell line that has been extensively used
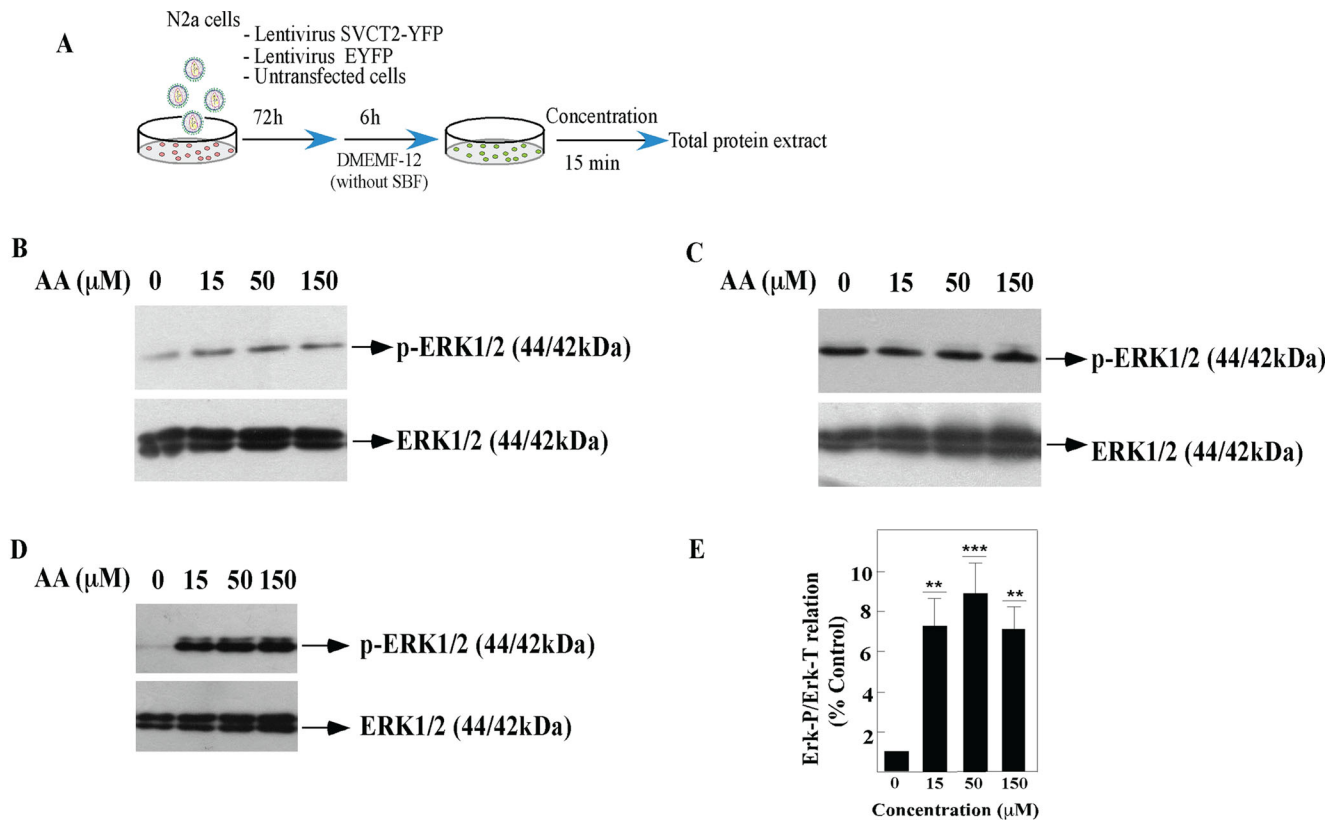
Fig. 7 The effect of AA on ERK activation is rapidly saturated in SVCT2 overexpressing Neuro2a cells. The experimental protocol for lentiviral transduction (**a**). Cells were left non-transduced (*control*) or were transduced with different lentiviral particles and maintained for 72 h, after which they were growth-arrested for 6 h and stimulated with different doses of AA for 15 min. Western blot analysis of phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 in protein extracts from non-transduced (**b**), EGFP-transduced (**c**), and hSVCT2-EYFP-transduced (**d**) cells. The membranes were probed with anti-pERK1/2 and total ERK1/2 antibodies (*loading control*). **e**.

Semiquantitative analysis of ERK1/2 phosphorylation levels in cells overexpressing SVCT2. A 10-fold increase in the levels of ERK1/2 phosphorylation was observed after 15 min of treatment with 50 μM AA, and this increase was maintained for concentration of up to 150 μM. No differences in the phosphorylation of ERK1/2 were detected in the non-transduced or EGFP-transduced cells. The results were normalized by arbitrarily setting the densitometry value of the control cells (time=0) to 1. Data represent the mean±SD of three independent experiments. **$p<0.01$; ***$p<0.001$

to study differentiation into dopaminergic neurons, which is characterized by a dramatic morphological change that involves the outgrowth of filopodia and processes [18, 33]. Our data demonstrated that overexpression of SVCT2 strongly increased the number of filopodia and processes, and in turn, enhanced the differentiation of N2a cells. Furthermore, the localization and function of SVCT2 at the plasma membrane was a key event for the in vitro differentiation of N2a cells, and treatment with quercetin or an anti-SVCT2 antibody inhibited this process. Interestingly, both treatments inhibited SVCT2 function in N2a cells, HN33.11 cells, rat cortical neurons [14], and pyramidal neurons from hippocampal region CA1 [21]. Together, these data demonstrate that N2a cell differentiation in vitro is dependent on overexpression of the high affinity/low capacity AA transporter SVCT2 and on uptake of the reduced form of vitamin C.

AA concentrations are not drastically changed during the first days of postnatal development [17], which is when cortical neurons differentiate and arborize to establish the synaptic

network [34]. Given that SVCT2 has an apparent Km of 20 μM for AA, it is expected that its transport activity is completely saturated in the context of the vitamin C concentrations that are present in the postnatal cerebral cortex (200–400 μM, 5 μmol/g tissue). Therefore, if vitamin C is important for the differentiation process, its uptake will be basically dependent on the expression levels of SVCT2 rather than on extracellular variations in vitamin C in the cortex. In parallel, overexpression of the low affinity/high capacity AA transporter SVCT1, which has an apparent Km of 180 μM, allowed us to enhance the intracellular concentrations of AA [35]. However, the differentiation effect was only partial and required supplementation with 200 μM AA. We conclude that the higher intracellular levels of AA that are generated after overexpression of SVCT1 do not reproduce the conditions that are required to induce the differentiation effects observed after SVCT2 overexpression. Hence, we postulate that neuronal cells tightly regulate intracellular levels of vitamin C to generate the specific differentiation effects required for neuronal maturation.

To evaluate the intracellular signaling events that are triggered after AA uptake in neuronal cells that overexpress SVCT2, we analyzed the phosphorylation level of ERK1/2 kinases, which are involved in the differentiation and maturation of neurons of the CNS [25–27]. Previous studies have shown that AA induces the phosphorylation and activation of ERK1/2, which is followed by cellular responses such as apoptosis and differentiation in non-neuronal cell lines [22, 24, 36, 37]. AA promotes osteogenic differentiation of progenitor cells from the periodontal ligament (PDL) and also differentiation of ATDC5 chondrogenic cells, through activation of ERK1/2 [36–38]. Thus, the final effects induced by AA and the consequent activation of ERK1/2 are cell type-dependent.

Our results indicate that in N2a cells, ERK1/2 activation may participate in the differentiation phenomenon that is induced after SVCT2 overexpression and AA uptake. ERK1/2 kinases have been involved in the long-term potentiation (LTP) of synaptic plasticity, remaining active for several hours to enhance the expression of immediate early genes [39–41]. This activation may be responsible for the increased expression of differentiation-, maturation-, and neurotransmission-related genes that has been reported in rat stem cells and mesencephalic precursors following treatment with physiological concentrations of AA [42, 43]. These data suggest that AA and SVCT2 may be important molecules for the potentiation of synaptic plasticity by inducing the expression of certain genes through ERK1/2 activation. However, AA induced ERK1/2 phosphorylation after 15 min, which suggests an indirect action of AA over these kinases. In this context, it has been demonstrated that free radicals, such as NO_2 and $O_2^{\cdot-}$, can act together with AA to promote Ras activation through GDP/GTP exchange. In turn, activated Ras (bound to GTP) interacts with and activates Raf-1 [44]. Raf-1 is a serine/threonine kinase that phosphorylates MEK, which in turn phosphorylates and activates ERK1/2, another serine/threonine kinase that phosphorylates a wide variety of proteins, and among them, the transcription factor CREB regulates the expression of neuronal and synaptic plasticity genes [45].

AA uptake and SVCT2 overexpression may mediate the abovementioned signaling mechanism in neuronal cells. To date, it has only been reported that overexpression of SVCT2 in MC3T3-E1 preosteoblastic cells stimulates osteoblastic differentiation and mineralization [30]. Although no study has addressed the effect of SVCT2 overexpression on the differentiation process of neuronal cells, we have recently demonstrated that SVCT2 expression is strongly increased in transient neural precursors present in the ventricular neurogenic niche [3]. It is also strongly expressed in cortical neurons at 1–5 days postnatal development, which activate mechanisms of arborization and synaptic connectivity [12]. Hence, we propose that neuronal differentiation and maturation requires a strong increase of SVCT2 expression.

In conclusion, our results indicate that SVCT2 expression and function at the plasma membrane are key events in the induction of the neuronal differentiation of N2a cells and that intracellular AA stimulates ERK1/2 phosphorylation, which could be the signaling pathway that mediates the in vitro differentiation effect.

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