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Plasma Membrane and Vesicular Glutamate Transporter Expression in Chromaffin Cells of Bovine Adrenal Medulla

A.M. Oliván, R. Pérez-Rodríguez, C. Roncero, C. Arce, M.P. González, and M.J. Oset-Gasque*

Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain

The study of the functional expression of glutamate signaling molecules in peripheral tissues has received relatively little attention. However, evidence is increasing for a role of glutamate as an extracellular signal mediator in endocrine systems, in addition to having an excitatory amino acid neurotransmitter role in the CNS. Chromaffin cells are good models of catecholaminergic neurons, in which previous work from our group demonstrated the existence of both functional glutamate receptors and specific exocytotic and nonexocytotic glutamate release. In this work, the presence of specific plasma membrane (EAATs) and vesicular glutamate (VGLUTs) transporters has been investigated by using confocal microscopy, flow cytometric analysis, Western blot, and qRT-PCR techniques. We found specific expression of EAAT3, EAAT2, VGLUT1, and VGLUT3 in about 95%, 65%, 55%, and 25%, respectively, of the whole chromaffin cell population. However, chromaffin cells do not express VGLUT2 and have a very low expression of EAAT1. VGLUTs are localized mainly in the membrane fraction, and EAATs share their subcellular location between membrane and cytosolic fractions. Their estimated molecular weights were about 70 kDa for EAAT2, about 65 kDa for EAAT3, about 50 kDa for VGLUT1, and about 60 kDa for VGLUT3. RT-qPCR techniques confirm the expression of these glutamate transporters at the mRNA level and show a different regulation by cytokines and glucocorticoids between VGLUT1 and -3 and EAAT2 and -3 subfamilies. These interesting results support the participation of these glutamate transporters in the process of glutamate release in chromaffin cells and in the regulation of their neurosecretory function in adrenal medulla.

Key words: glutamate; excitatory amino acid transporters; glutamate release; cell death; chromaffin cells; adrenal medulla

The dicarboxylic amino acid glutamate is recognized as the major excitatory neurotransmitter in the central nervous system (CNS) of mammals, having important pathophysiological functions in synaptic plasticity and excitotoxicity (Platt, 2007). The role of glutamate as an excitatory neurotransmitter in mammals CNS has gained increasing support since the successful cloning of a great number of genes that encode the signaling machinery required for its neurocrine function in brain synapses. This machinery includes glutamate receptors as detection signals, plasma membrane glutamate transporters (excitatory amino acid transporters; EAATs) as finishing signals, and vesicular transporters (VGLUTs) as exocytotic releasing signals. EAATs are high-affinity Na^+-coupled glutamate transporters, which are members of the solute carrier family 1 (SLC1). There are five isoforms (EAAT1–EAAT5) of these transporters, both in neurons (EAAT3 or EAAC1 and EAAT4) and in astrocytes (EAAT1 or GLAST and EAAT2 or GLT1), EAAT5 being located in photoreceptor cells (for review see Kanai and Hediger, 2004). So far, three VGLUTs isoforms, which belong to the type I phosphate transporter family (also referred to as the *SLC17 family*), have been described (Hisano, 2003). The first characterized vesicular glutamate transporter, VGLUT1, is present in excitatory neurons of brain and cerebellum cortex, hippocampus, and thalamus; VGLUT2 in subcortical glutamatergic neurons between thalamus and spinal medulla; and the most recently cloned, VGLUT3, discretely distributed in brain, both in excitatory and in inhibitory neurons, as well as in cholinergic and monoaminergic

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*Correspondence to: M.J. Oset-Gasque, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, Complutense University of Madrid, E-28040 Madrid, Spain. E-mail: mjoset@farm.ucm.es

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neurons and glial cells (Takamori, 2006; Liguz-Lecznar and Skangiel-Kramska, 2007).

Until now, very little attention has been paid to the functional expression of glutamate transporters in peripheral nervous system and in nonneural tissues. Recent findings from molecular biology show new functions for glutamate as an extracellular signaling molecule in endocrine, autocrine, and/or paracrine tissues, such as pancreas, intestine, testis, placenta, kidney, and bone, as well as adrenal and pineal glands (Danbolt, 2001; Hinoi et al, 2004).

With regard to glutamate's function in chromaffin cells from adrenal medulla, neuroendocrine cells sharing a common embryologic origin with neurons and models of sympathetic postganglionic neurons, our research group demonstrated that this neurotransmitter may be involved in the regulation of catecholamine (CA) secretion, by interacting specifically with different glutamate receptor subtypes: NMDA, AMPA, KA, and t-ACDP (González et al., 1998; Arce et al., 2004). Thus, the role of glutamate as a potential neurosecretory regulator in this tissue has been established.

Several authors have suggested that adrenal glands receive sympathetic afferents and glutamatergic innervations along with their typical cholinergic innervations (Parker et al., 1993; Pyner and Coote, 1995). On top of this evidence, glutamatergic innervations to sympathetic adrenal neurons have also been found (Llewellyn-Smith et al., 1992, 1995). Moreover, our most recent work shows that chromaffin cells release glutamate, after stimulation with different secretagogues, by two processes, one exocytotic and Ca^{2+}-dependent and the other non-exocytotic, Ca^{2+}-independent, and probably mediated (as in neurons) by reversion of electrogenic transporters of plasma membrane (Romero et al, 2003). Thus, the question arises of whether specific plasma membrane and/or vesicular transporters are expressed in chromaffin cells and could mediate glutamate release by adrenal chromaffin cells.

The aim of this work was to study whether chromaffin cells express any vesicular and/or plasma membrane glutamate transporters and, thus, obtain additional evidence to support our previous results on the existence of both processes of glutamate release in chromaffin cells and a role for glutamate in the regulation of catecholamine secretion by chromaffin cells.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium, fetal calf serum, HEPES, and RNAse A were from Gibco BRL, U.K., and collagenase from *Clostridium histolyticum* (EC 3.4.4.19) was supplied by Boehringer Mannheim S.A. (Barcelona, Spain). Antibiotics, cytosine arabinoside, 8-fluoro-deoxyuridine (FDU), neutral red, and propidium iodide were from Sigma (Madrid, Spain). Amphotericin B was from ICN Ibérica S.A. (Barcelona, Spain). All other chemicals were reactive-grade products from Merck (Darmstadt, Germany). Rabbit anti-VGLUT1, anti-VGLUT2, and anti-VGLUT3 polyclonal antibodies were from Synaptic Systems (Göttingen, Germany); monoclonal mouse anti-EAAT3 antibody and polyclonal guinea pig anti-EAAT1 and anti-EAAT2 antibodies were from Chemicon (Temecula, CA). Mouse anti-β-actin, antisynaptophysin, and antityrosine hydroxylase monoclonal antibodies; rabbit antiglutamate decarboxylase 65/67 ($GAD_{65/67}$), antiphenyl ethanolamine-N-methyltransferase (PNMT) polyclonal antibodies, and anti-rabbit IgG TRITC, anti-mouse IgG FITC, or anti-guinea pig Cy3 or FITC conjugates secondary antibodies were from Sigma (St. Louis, MO). Other reagents were from Sigma or Boehringer (Mannhein, Germany).

Chromaffin Cell Primary Culture

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Pérez-Rodríguez et al. (2009). Cell viability and purity checking and cell plating in Petri dishes or on coverslips were performed as described elsewhere (Vicente et al., 2006), and cells were used 3–7 days after plating.

Synaptosomal Preparation From Cerebral Cortex

Synaptosomes from rat cerebral cortices were prepared and purified on discontinuous Percoll gradients (Amersham Biosciences, Uppsala, Sweden), as previously described (Millán et al., 2002).

Inmunocytochemistry and Confocal Microscopy

For immunocytochemical analysis, chromaffin cells or synaptosomes (used as positive controls) were plated on poly-lysine-coated glass coverslips. Cells (seeded at a density of 3×10^5/coverslip) were fixed for 2 min in ice-cold 1:1 acetone-methanol mixture (v/v) and washed twice with PBS. After blocking in PBS with 3% BSA/0.1% Tx-100 for 1 hr, the preparations were incubated with primary antibodies (rabbit anti-VGLUT1, anti-VGLUT2, and anti-VGLUT3 polyclonal 1:500; mouse anti-EAAT3 and antisynaptophysin monoclonal 1:500; and guinea pig anti-EAAT1 and anti-EAAT2 polyclonal 1:500) in PBS with 3% BSA for 1 hr at room temperature. After washing in PBS with 0.1% Tx-100 for 1 hr, secondary antibodies were added (FITC-labeled goat anti-mouse IgG, TRITC-labeled goat anti-rabbit IgG or Cy3-labeled goat anti-guinea pig IgG at 1:500 in PBS with 3% BSA) and incubated for another 1 hr. To study vesicular localization of VGLUTs or colocalization of plasma membrane and vesicular glutamate transporters, the cells were double immunostained with antibodies against one vesicular glutamate transporter and with antibodies against synaptophysin (monoclonal mouse antisynaptophysin), a marker of synaptic vesicles. The coverslips were mounted with Prolong Antifade in 50% glycerol in PBS containing 2.5% DABCO, and digital images were taken with a Leica confocal microscope and analyzed with Leica confocal software. Negative controls were made by incubating chromaffin cells or synaptosomes with secondary antibodies in the absence of primary antibodies. Thus, nonspecific signals could be detected.

Immunocytochemical Flow Cytometry Analysis

The quantification of glutamate transporter expression in chromaffin cells was performed in a Becton Dickinson FACScan Flow Cytometer (Becton-Dickinson, San Jose, CA). Basal or stimulated chromaffin cells (10^6/condition) were washed twice with PBS, harvested with trypsin-EDTA (0.25% trypsin, 1 mM EDTA), and pelleted by centrifugation at 2,000*g* for 5 min. After washing with ice-cold PBS, cells were fixed with 1 ml of 70% (v/v) methanol at −20°C for 2 min, centrifuged, and washed again with PBS. Immunostaining was performed by incubating cells with primary antibodies (1/100) in PBS with 3% BSA, for 1 hr at 37°C. After washing in PBS, secondary antibodies were added (FITC-labeled goat anti-mouse or anti-guinea pig or TRITC-labeled goat anti-rabbit IgGs) at 1:200 in PBS with 3% BSA and incubated for another 1 hr. Cells were finally centrifuged, washed, resuspended in cold PBS, and analyzed by flow cytometry.

Subcellular Fractionation of Chromaffin Cells: Cytosolic and Membrane Extract Preparations

Chromaffin cells (5×10^6), washed twice with cold PBS, were resuspended in a lysis buffer containing 250 mM sucrose, 25 mM Tris/HCl, pH 6.8, 1 mM EDTA, 0.05% digitonin, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin. Samples were centrifuged at 13,000*g* for 3 min at 4°C. Supernatants were taken and considered as cytosolic fractions. The pellets (containing the membrane fractions) were extracted with 40 mM HEPES, pH 7.6, 0.5 M EDTA, 1 M KCl, 5% glycerol, 0.2% Tx-100, 5 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin and centrifuged at 13,000*g* for 3 min at 4°C. Supernatant was taken and considered as membrane fraction. Proteins in both cytosolic and membrane fractions were measured with the Bio-Rad (Hercules, CA) protein reagent.

Western Blot Analysis

Proteins from cytosolic and membrane factions were boiled in Laemmli sample buffer (200 mM Tris-HCl, 10% glycerol, 6% SDS, 4% β-mercaptoethanol, 2 mM EDTA, 0.02% bromophenol blue, pH 6.8), and equal amounts of protein (25–75 μg) were size-fractionated in a 10% acrylamide gel; transferred to a PVDF membrane; and, after blocking with 5% nonfat dry milk, incubated with the corresponding primary antibodies and visualized according to the ECL Western Blotting detection system (Amersham) as described by Vicente et al. (2002). Different exposure times were performed with each blot to ensure the linearity of the band intensities. Band intensities were measured on a densitometric scanner and normalized with respect to β-actin expression.

RT-PCR Analysis

An RNeasy Mini Kit was used for total RNA isolation. Reverse transcription (RT) was carried out for 1 hr at 55°C with oligodeoxythymidylate primer using 5 μg total RNA from each sample for complementary DNA synthesis. Semiquantitative and real-time quantitative PCR was performed in order to determine the levels of EAATs and VGLUTs and housekeeping GAPDH mRNAs by using the specific primers listed in Table I synthesized by Sigma-Aldrich.

TABLE I. Specific Primers

Primer	Sequence
GA3PDH forward	5′-CACAGTCAAGGCAGAGAACG-3′
GA3PDH reverse	5′-TACTCAGCACCAGCATCACC-3
EAAT1 forward	5′-GGTCACTGCAGTCATTGTGG-3′
EAAT1 reverse	5′-CACCAGCATCTGTAGCATCC-3′
EAAT2 forward	5′-TGCTGGACAGAATGAGAACG-3′
EAAT2 reverse	5′-TCGGTGCTGAGAGTCAATGG-3′
EAAT3 forward	5′-GGAGAAGCTCTCCAAGAAGG-3′
EAAT3 reverse	5′-CTCATTGTCAAGTGCTGTGG-3′
VGLUT1 forward	5′-TGGCCTCATACACGGTTCC-3′
VGLUT1 reverse	5′-GTGGAGGTAGCCACAATAGC-3′
VGLUT2 forward	5′-TTAGCTGGCATTCTTGTGC-3′
VGLUT2 reverse	5′-CAGGACTCTCGTAGGACACC-3′
VGLUT3 forward	5′-CTGCGTCATGTGTGTCAGG-3′
VGLUT3 reverse	5′-GGAGGTTGTAGCCAGTCTGC-3′

Real-time PCR. The SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the 7900 HT Fast Real-Time PCR system (Applied Biosystems) were used for detecting the real-time qPCR products of reverse-transcribed cDNA samples, according to the manufacturer's instructions. qPCR conditions were 95°C (10 min) followed by 40 cycles of 15 sec at 95°C and annealing for 1 minute at 60°C. Three independent quantitative PCR assays were performed for each gene and measured in triplicate. Three non-template controls (NTCs) were run for each qPCR assay, and genomic DNA contamination of total RNA was controlled using RT minus controls (samples without the reverse transcriptase).

Semiquantitative PCR. Conventional PCR amplifications were conducted in a 25 μl solution containing 1× PCR buffer, 0.2 mM dNTP mix, 1.5 mM magnesium chloride, 400 nM of each primer, 1 U DNA polymerase, and 2 μl cDNA template, corresponding to 5 μg total RNA in a 20 μl final volume. Negative control of amplification was performed with 2 μl water instead of cDNA template. Amplification conditions were 2 min at 95°C, 11 cycles of 30 sec at 95°C, 30 sec at 61°C, decreasing 0.5°C every cycle, and 20 sec at 72°C, followed by 23 cycles of 30 sec at 95°C, 30 sec at 55.5°C, and 20 sec at 72°C, and a final extension of 2 min at 72°C. Reactions were carried out in a thermal cycler. Ten microliters of the PCR products was resuspended in 6× loading buffer (30% glycerol, 0.5 μg/ml BrEt) and electrophoresed through 1.5% agarose in 0.5× TBE buffer (45 mM Tris-borate; 1 mM Na_2EDTA, pH 8.0) with 0.5 μg/ml BrEt for 1.5 hr.

Statistical Analysis

Data were expressed as mean ± SEM values of three or four independent experiments with different cell batches, each performed in duplicate or triplicate. Statistical comparisons were assessed by using one-way ANOVA (Scheffe's F test), followed in some instance by a two-way ANOVA test. Differences were accepted as significant at $P \leq 0.05$.

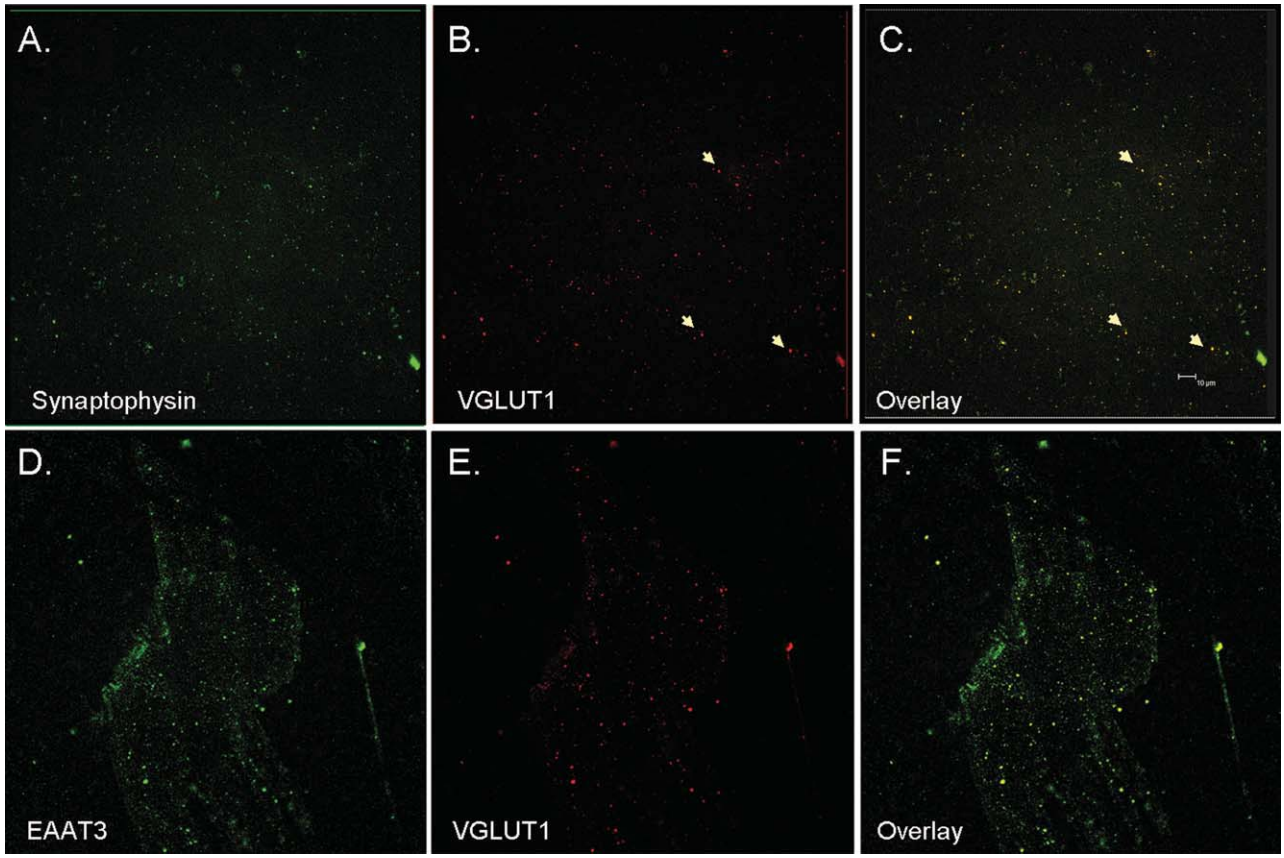

Fig. 1. Expression of VGLUT1 and EAAT3 in cerebral rat cortical synaptosomes determined by immunocytochemistry and confocal microscopy. Synaptosomes were fixed on polylysine-coated coverslips, and confocal images of double-stained nerve terminals were obtained by performing immunocytochemistry using antibodies against the vesicular glutamate transporter VGLUT1 (**B,E**) and the plasma membrane transporter EAAT3 (**D**). Specific primary antibody against synaptophysin was used to label synaptic vesicles (**A**). Nerve terminals were visualized with FITC filters (green) for synaptophysin (A) and EAAT3 (D) and with TRITC filters (red) for VGLUT1 (B,E) in a multispectral confocal microscopy Leica TCS-SP2-AOBS with a ×40 objective. Merged panels are shown in **C,F**. Scale bar = 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS

Immunocytochemical Studies and Confocal Microscopy Reveal the Expression of Different Plasma Membrane (EAATs) and Vesicular (VGLUTs) Glutamate Transporters in Chromaffin Cells

To ensure the specific microscopic detection of fluorescent signals for glutamate transporters in chromaffin cells, previous immunocytochemical staining experiments for VGLUT1 and EAAT3 were performed in synaptosomes. In these experiments, a lower concentration of nerve terminals was attached to the polylysine-coated coverslips to facilitate visualization as described by Millan et al. (2002). Synaptosomes were labeled with antiserum against the vesicular protein synaptophysin, and glutamatergic nerve terminals were identified with antisera against the vesicular glutamate transporter VGLUT1 and the neuronal plasma membrane transporter EAAT3 (Fig. 1). Among the synaptophysin-containing particles (521 particles from three fields), VGLUT1 was detected in 59.8% ± 2.7% (mean ± SEM) of the terminals (Fig. 1A–C), a proportion very similar to that detected by Millan et al. (2002). Among the EAAT3-immunostained synaptosomes (582 particles from three fields) VGLUT1 was detected in 38.9% ± 2.4% (mean ± SEM) of the terminals (Fig. 1D–F).

Once we had ensured the accuracy of our immunochemical staining, we performed the same immunocytochemistry experiments in chromaffin cells, beginning with VGLUT1 and EAAT3 expression, given their known specific expression in neurons, and taking into account the neural origin of chromaffin cells. We used synaptophysin immunolabelling to ensure specific staining of the chromaffin cells. Results in Figure 2 show that, as in the case of synaptosomes, there is a specific immunolabelling for VGLUT1 in about 60–70% of chromaffin cells stained with synaptophysin (Fig. 2A–C), whereas specific immunostaining for EAAT3 was shown in almost the whole population of chromaffin cells (Fig. 2D). Figure 2E,F shows that the great majority of EAAT3-immunollabelled chromaffin cells were also specifically stained for VGLUT1, although with differences

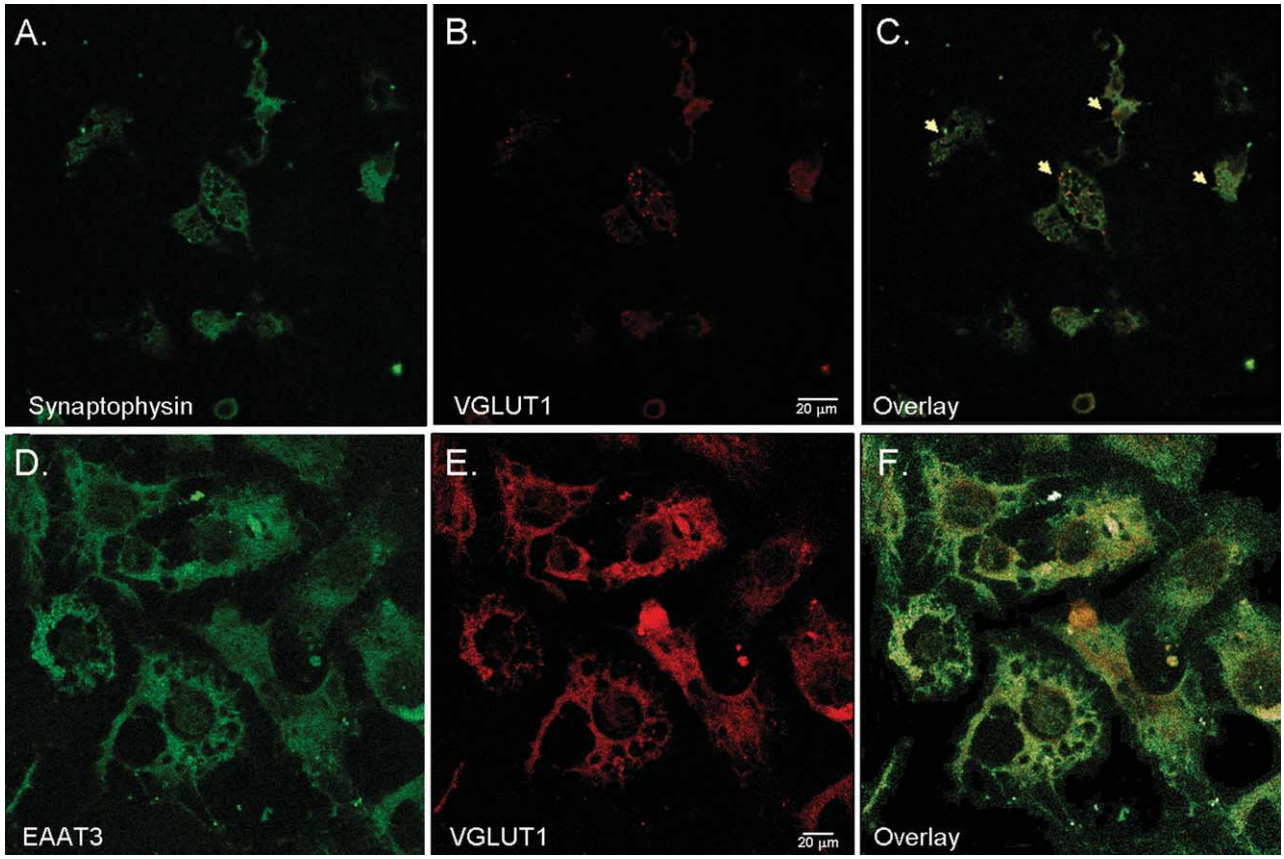

Fig. 2. Expression of VGLUT1 and EAAT3 in bovine chromaffin cells by immunofluorescence confocal microscopy. Chromaffin cells (3×10^5/coverslip) were fixed on polylysine-coated coverslips, and confocal images of double-stained cells were obtained by immunocytochemistry using antibodies against the vesicular glutamate transporter VGLUT1 (red; **B,E**) and the plasma membrane transporter EAAT3 (green; **D**). Antibody against synaptophysin was used to label secretory vesicles (green; **A**). Cells were visualized with FITC filters for synaptophysin (A) and EAAT3 (D) and with TRITC filters for VGLUT1 (B,E) in a multispectral confocal microscopy Leica TCS-SP2-AOBS with a ×40 objective. Merged panels are shown in **C,F**. Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

regarding the intensity of immunollabeling in different cells. These results suggest the colocalization of both neuronal glutamate transporters in the same population of chromaffin cells. To determine whether other vesicular or plasma membrane glutamate transporters were expressed in chromaffin cells, additional immunocytochemical experiments were performed for VGLUT2 and VGLUT3 as well as for EAAT1 and EAAT2. Results in Figure 3 indicate that, although no specific immunoreactivity was observed in the case of VGLUT2 (Fig. 3A–C), there was a specific immunolabelling for VGLUT3 in about 20–30% of chromaffin cells (Fig. 3D–F); however, specific immunolabelling for VGLUT3 was observed not only in chromaffin (i.e., cell 1) but also in cells not immunolabelled for synaptophysin (i.e., cell 2), which indicates that cells other than chromaffin could also express VGLUT3. In addition, a very weak immunolabelling was observed for EAAT1 (Fig. 3G–I) in a small number of chromaffin cells, whereas immunoreactivity for EAAT2 was stronger and was observed in the great majority of chromaffin cell population immunolabeled with synaptophysin (Fig. 3J–L). Thus, taken together, these results seem to indicate that chromaffin cells preferably express VGLUT1 and EAAT3, followed by VGLUT3 and EAAT2.

Quantification of the VGLUT and EAAT Expression in Chromaffin Cells by Flow Cytometry Techniques

To quantify, in a more accurate way, the cell subpopulation that expresses each glutamate transporter, we carried out quantitative studies by flow cytometry. To ensure the accuracy of the technique used for labeling specific chromaffin cell phenotypes, we examined the number of total catecholaminergic, adrenergic, and GABAergic chromaffin cells by using specific antibodies against tyrosine hydroxylase (TH), the limiting enzyme in catecholamine biosynthesis; phenyl ethanolamine N-methyl transferase (PNMT), the specific marker for adrenergic cells; and glutamate descarboxylase (GAD), the limiting enzyme for GABA biosynthesis. Results indicate proportions of 95.6% ± 2.8%, 75.9% ± 1.7%, and

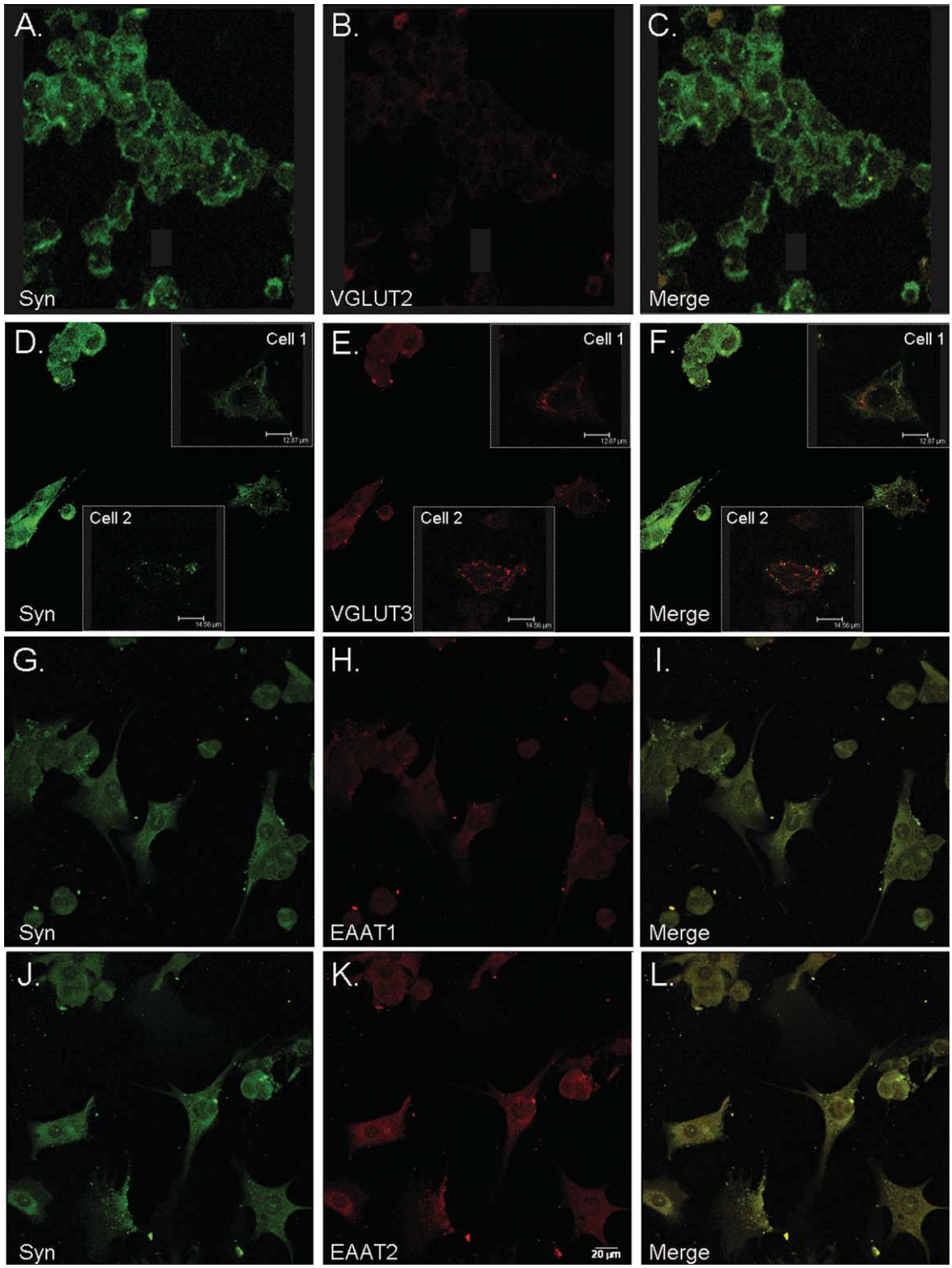

Fig. 3. Expression of VGLUT2 and -3 and EAAT1 and -2 in bovine chromaffin cells determined by immunofluorescence confocal microscopy. Expression of VGLUT2 (B) and -3 (E) and EAAT1 (H) and -2 (K) was examined by confocal microscopy as indicated in Figure 2. Double immnolabeling for each of these GLTs (red) and synaptophysin (green) indicated the absence of expression of VGLUT2 (**A–C**), the expression of VGLUT3 (**D–F**), the poor immunolabeling for EAAT1 (**G–I**), and the prominent immunolabeling for EAAT2 (**J–L**) in secretory vesicles immunolabeled for synaptophysin (D,E). Merged panels are shown in C,F,I,L. In F, cell 1 and cell 2 are examples of cells in which VGLUT3 signals colocalize (cell 1) or not (cell 2) with synaptophysin signals. Images are representative of five or six fields analyzed in three experiments performed with different cell batches. Scale bar = 20 µm.

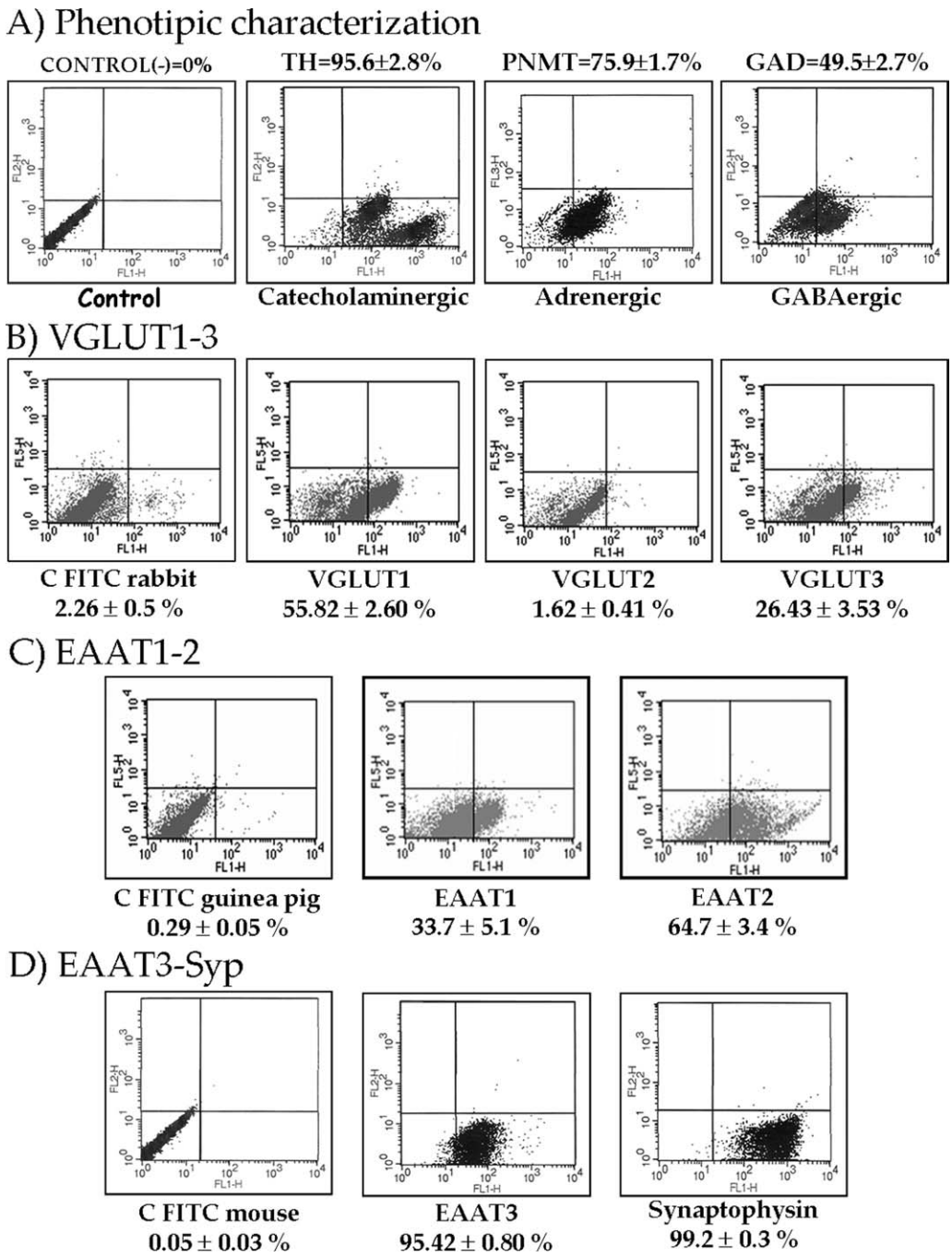

Fig. 4. Expression of glutamate transporters in chromaffin cells assessed by immunocytochemical flow cytometry techniques. Cells were harvested with trypsin-EDTA, pelleted, and fixed. Immunostaining was performed by incubation with primary antibodies against GLTs and secondary antibodies (FITC-labeled goat anti-rabbit for VGLUTs, anti-guinea pig for EAAT1-2, and anti-mouse for EAAT3 IgGs) and analyzed by flow cytometry as indicated in Materials and Methods. Figures represent plots of log fluorescence vs. cell count from flow cytometry. **A**: Phenotypic characterization of chromaffin cells by flow cytometry by using mouse anti-TH and anti-rabbit PNMT and anti-GAD antibodies. **B**: VGLUTs expression in different proportions by chromaffin cells. **C**: EAAT1 and EAAT2 expression in different proportions by chromaffin cells. **D**: EAAT3 expression in about the whole chromaffin cell population. Data indicate the percentage of expression (mean ± SEM) for different GLTs obtained from three separate experiments, from different cell batches, each performed in triplicate.

49.5% ± 2.7% of catecholaminergic, adrenergic, and GABAergic cells, respectively (Fig. 4A), proportions that are in the rank order of those observed in our previous studies (Oset-Gasque et al., 1998; Castro et al., 2003). Flow cytometry results of chromaffin cells immunolabelled with anti-VGLU1-3 transporters show that

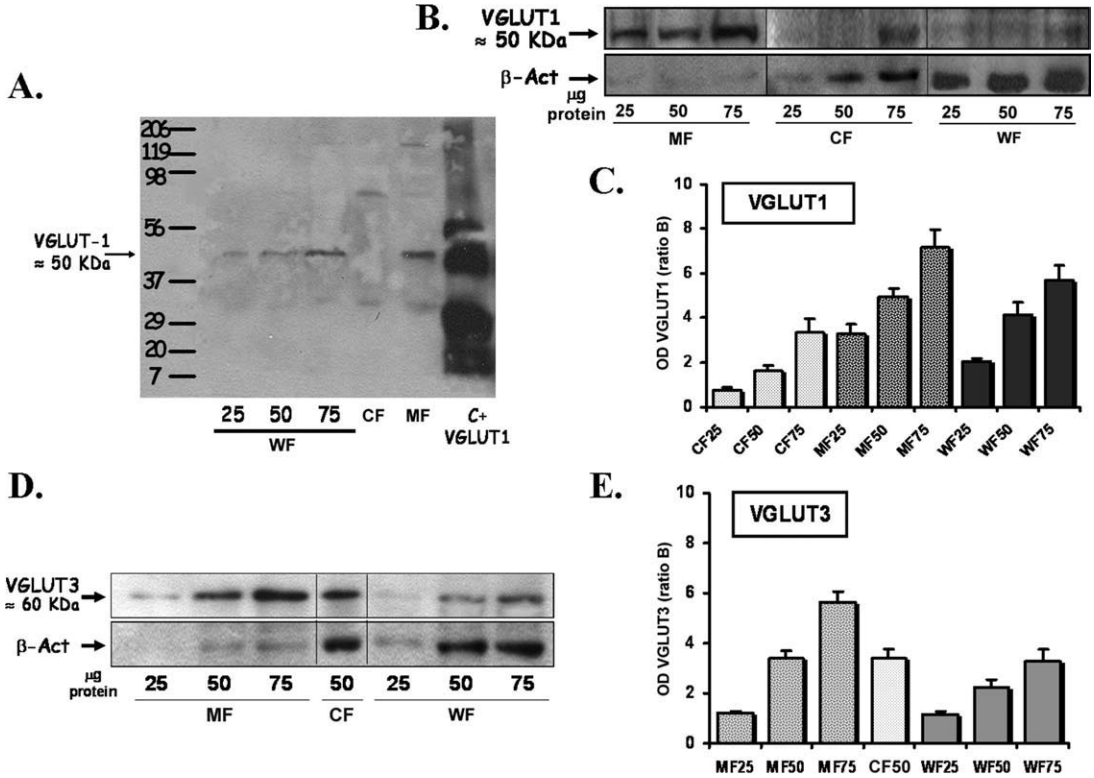

Fig. 5. VGLUT1 and VGLUT3 expression in subcellular fractions of bovine chromaffin cells determined by Western blot analysis. **A,B,D**: Representative images of Western blot experiments for VGLUT1 (A,B) and VGLUT3 (D) in whole fraction (WF), membrane fraction (MF), and cytosolic fraction (CF) of chromaffin cells at indicated amounts of total protein. Estimated molecular weights are indicated. **C,E**: Quantitative analysis of VGLUT1 (C) and VGLUT3 (E) expression in subcellular fractions of chromaffin cells. The data represent the ratios of densitometric values normalized against the ECL backgrounds obtained for each Western blot and are mean ± SEM values obtained from three experiments with different cell batches each performed ion duplicate. β-Actin signals were obtained in order to assess the purity of different fractions and the quantity of proteins in each assay.

55.82% ± 2.60% of cells express VGLUT1 and 26.43% ± 3.53% of cells express VGLUT3. However, only 1.62% ± 0.41% of cells were immunolabelled for VGLUT2 (Fig. 4B). These results confirm that chromaffin cells express VGLUT1 more abundantly, followed by VGLUT3, but that these cells do not seem to express VGLUT2. Similarly, we observed that while almost the whole population of chromaffin cells express EAAT3 (95.42% ± 1.80%; Fig. 4D; a proportion very similar to that observed for synaptophysin; 99.2% ± 0.83%); only 64.7% ± 3.4% of cells were immunolabelled for EAAT2, and only 33.7% ± 5.1% of cells were immunolabelled for EAAT1 (Fig. 4C). Thus, these results seem to confirm those of microscopic studies, described above, showing that the plasma membrane glutamate transporter expressed more abundantly in chromaffin cells is EAAT3, followed by EAAT2 and EAAT1.

Subcellular Localization of Glutamate Transporters Expressed in Chromaffin Cells Through Western Blot Techniques

The expression of VGLUT1 and -3 and EAAT3 and -2, shown above led us to perform a subcellular distribution study of these transporters in whole homogenates and cytosolic and membrane fractions of chromaffin cells by Western blot to determine the approximate molecular weight and have additional evidence on the localization of these transporters within these cells. Western blot analysis revealed the appearance of a band of approximately of 50 kDa mw, corresponding to VGLUT1, in whole homogenate fractions of chromaffin cells, the intensity of which was directly proportional to the protein amount in the sample (Fig. 5A). This signal was about three times stronger in membrane fractions than in cytosolic fractions ($P < 0.001$; ANOVA test) and was always proportional to the quantity of protein used for the assays (Fig. 5B,C), thus indicating that VGLUT1 is expressed mainly in particulate fractions of chromaffin cells.

We also found a band with an mw of about 60 kDa for VGLUT3 (Fig. 5D). In contrast to VGLUT1, the intensity of signals obtained for VGLUT3 expression was very similar in membrane and in cytosolic fractions, indicating a similar distribution of this vesicular transporter between the two subcellular compartments (Fig. 5E).

With regard to the EAATs expression, our results showed bands of expression for the three transporters at 62, 69, and 63 kDa mw for EAAT1, EAAT2, and

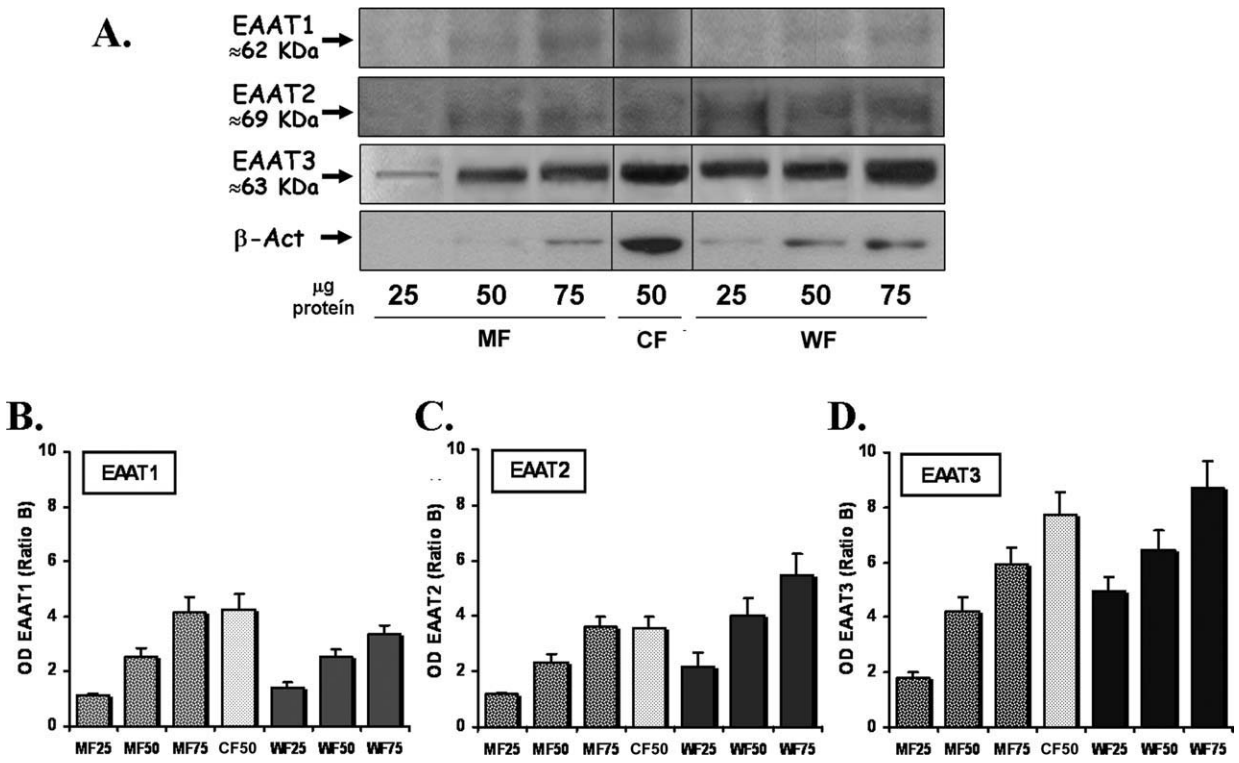

Fig. 6. EAAT1-3 expression in subcellular fractions of bovine chromaffin cells determined by Western blot analysis. **A**: Representative images of Western blot experiments for EAAT1, EAAT2, and EAAT3 in whole fraction (WF), membrane fraction (MF), and cytosolic fraction (CF) of chromaffin cells at indicated amounts of total protein. Estimated molecular weights for each glutamate transporter are indicated. **B–D**: Quantitative analysis of EAAT1 (B), EAAT2 (C), and EAAT3 (D) expressions in indicated subcellular fractions of chromaffin cells. The data represent the ratios of densitometric values normalized against the backgrounds obtained for each Western blot and are mean ± SEM values obtained from three experiments with different cell batches each performed in duplicate. β-Actin signals were obtained in order to assess the purity of different fractions and the quantity of proteins in each assay.

EAAT3, respectively (Fig. 6A). However, EAAT3 expression was significantly higher than that of EAAT2 and EAAT1 ($P < 0.001$; ANOVA test). Moreover, unlike the case with VGLUTs, cytosolic signals for the three EAATs were always higher than those found for membrane fractions (Fig. 6B–D), indicating the existence of some traffic of these transporters to plasma membrane through the cytosol. Band intensities were also directly proportional to the protein amount in the samples.

Chromaffin Cells Express VGLUT1 and VGLUT3 and EAAT3 and EAAT2 mRNAs, Which Show Different Regulation by Cytokines and Glucocorticoids

To delve more deeply into the study of glutamate transporter expression in chromaffin cells and its specific regulation, PCR (semiquantitative for Fig. 7A,B and quantitative for Fig. 7D,E) experiments were set for VGLUT1–3, EAAT1–3, and G3PDH genes. Semiquantitative RT-PCR experiments confirmed the presence of specific mRNAs for VGLUT1 and -3 and not for VGLUT2 (Fig. 7A) or for EAAT1–3 (Fig. 7B). To confirm the validity of the primers used, the same experiments were performed in whole homogenates of rat cerebral cortex (Cx) and hippocampus (Hc). Positive signals were obtained for all tested primers, thus confirming their validity (Fig. 7C). Figure 7D,E presents data on the expression of mRNA for EAAT2 and -3 (Fig. 7D) and for VGLUT1 and -3 (Fig. 7E) mRNA expressions obtained by quantitative real-time PCR techniques (RT-qPCR) after chromaffin cell incubation with TNFα, IFNγ, and LPS and specific combinations of them or dexamethasone. These data show that all the cytokines assayed and LPS were able to increase mRNA EAAT3 and VGLUT1 expression, although these treatments do not significantly affect VGLUT3 or EAAT2 mRNA expressions (Fig. 7D,E). Moreover, treatment with dexamethasone was able to increase mRNA EAAT3 and VGLUT1 expression 2 and 3.5 times, respectively, whereas no statistically significant effects were found in the case of EAAT2 or VGLUT3. These results were confirmed by Western blot experiments (Fig. 8). Thus, the treatment of chromaffin cells with the same cytokines for 24 hr revealed the increase in expression of EAAT3 and VGLUT1 at the protein level, but not those of EAAT2 or VGLUT3, except for some cytokine combinations. In this case, the specific combi-

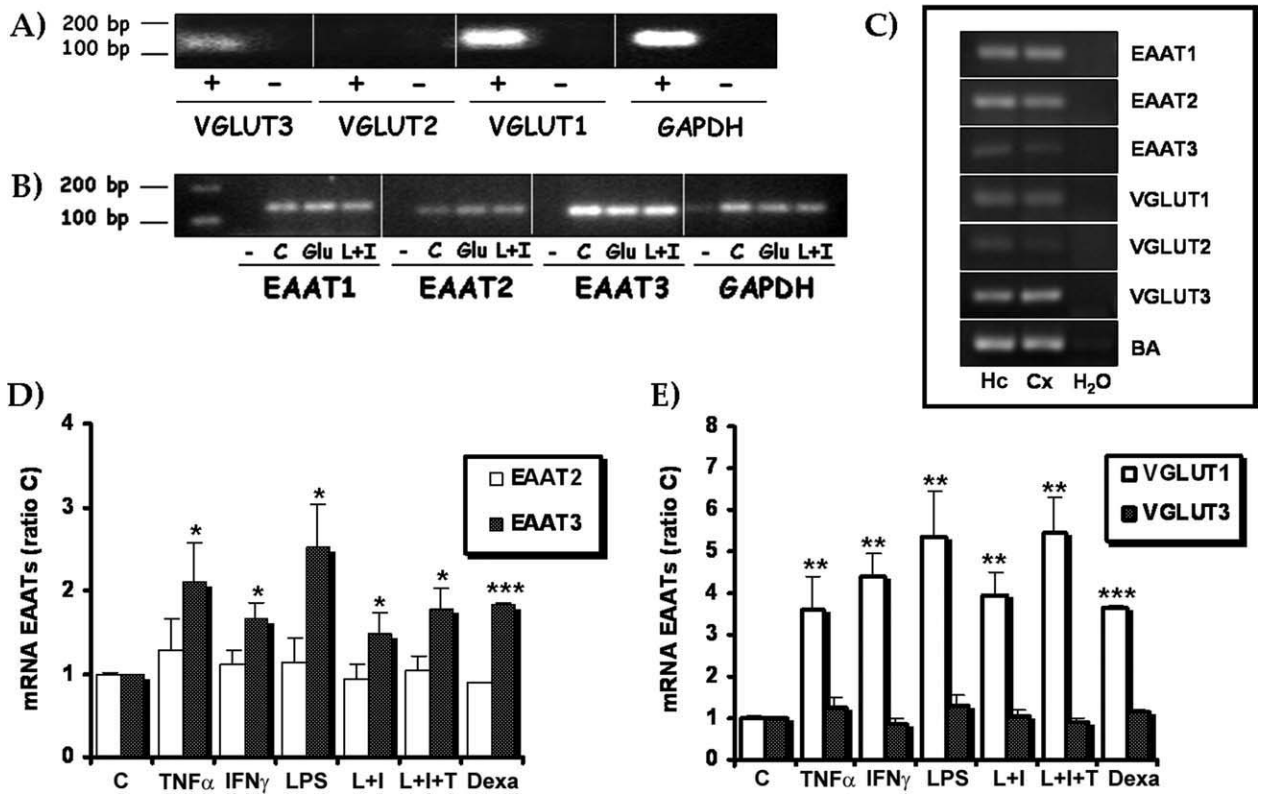

Fig. 7. mRNA expression of different EAATs and VGLUTs in bovine chromaffin cells and regulation of EAATs by cytokines and corticosteroids. **A**: Expression of VGLUTs mRNA in chromaffin cells assayed by conventional RT-PCR. mRNA was extracted with the RNeasy Qiagen Kit and subjected to reverse transcription (RT) and subsequent amplification with an appropriate set of primers for VGLUTs or for GAPDH, used as a housekeeping gene. PCR products RT^- (–) or RT^+ (+) were run on an agarose gel and images collected as described in Materials and Methods. **B**: Regulation of EAAT mRNAs expression in chromaffin cells assayed by conventional RT-PCR. Cells were incubated for 6 hr in the absence (controls; C) or in the presence of 10 μM LPS plus 10 nM IFNγ (L + I) or 1 mM glutamate (Glu). mRNA was extracted and subjected to RT and subsequent amplification with appropriate set of primers for EAATs, and PCR products were run on an agarose gel as described in Materials and Methods. GAPDH was used as a housekeeping gene. **C**: Conventional RT-PCR for mRNA from rat brain cortex (Cx) and hippocampus (Hc) tissues, as positive controls of primers used. **D,E**: For real-time RT-PCR, bovine chromaffin cells were incubated for 6 hr with 10 μM LPS, 10 nM IFNγ, 10 nM TNFα or their specific combinations (L + I or L + I + T) or with 100 nM dexamethasone (Dexa). mRNA was extracted and subjected to reverse transcription and subsequent quantitative amplification with appropriate sets of primers for EAAT2 and -3 (D) or VGLUT1 and -3 (E) and GAPDH as a control, as described in Materials and Methods. Data are expressed as ratios over their controls and are mean ± SEM values obtained from three experiments each performed in triplicate. Statistics compare the effect of cytokines or dexamethasone with their specific controls (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; one-way ANOVA test).

nations of cytokines potentiate the individual expression separately obtained with each one, an effect that was not apparent at the mRNA expression level, which suggests the existence of additional posttranscriptional or translational regulation. Thus, taken together, these results confirm the expression of specific EAATs and VGLUTs subtypes in chromaffin cells with different regulation by cytokines and glucocorticoids at both mRNA and protein levels.

DISCUSSION

In recent years, evidence has emerged for a role of glutamate as an extracellular signal mediator in the autocrine and/or paracrine system (intestine, testes, placenta, kidney, bone), in addition to its excitatory amino acid neurotransmitter role in the CNS (Skerry and Genever 2001; Hinoi et al., 2004). Recent evidence also points out a role for glutamate as a regulatory molecule in neuroendocrine hypothalamic-hypophysary systems (Hrabovszky and Liposits, 2008) and in endocrine tissues such as pineal gland (Danbolt, 2001) and pancreas where glutamate has a regulatory role in glucagon and insulin secretion (for review see Hinoi et al, 2004). By analogy with neurotransmission in the central glutamatergic synapses, the expression of GLTs could be essential for the termination of glutamate signals by reducing the extracellular concentration in mechanisms underlying possible signal transduction mediated by this amino acid in the adrenal gland. VGLUTs' discovery has provided a reli-

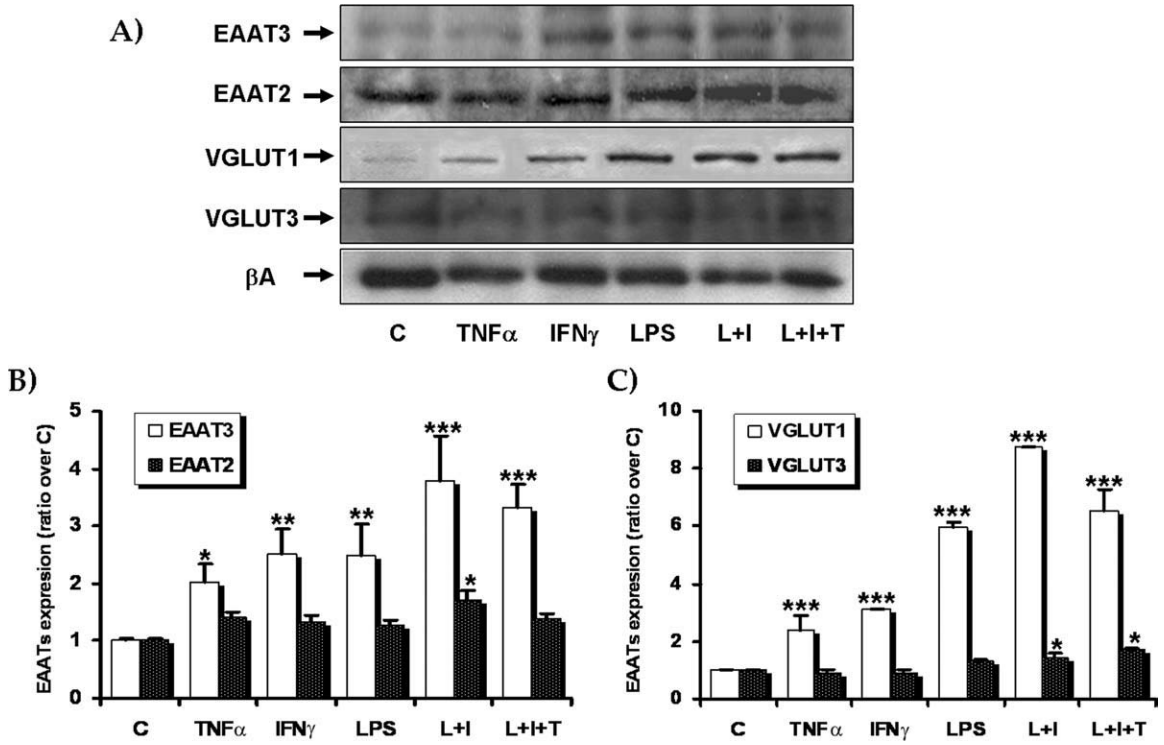

Fig. 8. Regulation of EAATs and VGLUTs expression by cytokines in bovine chromaffin cells, determined by Western blot analysis. Bovine chromaffin cells were incubated for 24 hr with 10 μM LPS, 10 nM IFNγ, 10 nM TNFα, or their specific combinations (L + I or L + I + T), and Western blots were carried out as described in Material and Methods. **A**: Representative images of Western blot experiments for EAAT2, EAAT3, VGLUT1, and VGLUT3 in whole homogenates of chromaffin cells. **B,C**: Quantitative analysis of EAAT2 and -3 (B) or VGLUT1 and -3 (C) expression in chromaffin cells. Data represent the ratios of densitometric values against controls, normalized with β-actin, and are mean ± SEM values obtained from three experiments with different cell batches each performed in duplicate. Statistics compare the effect of cytokines with specific controls for each glutamate transporter (*$P < 0.05$, **$P < 0{,}01$, ***$P < 0.001$; one-way ANOVA test).

able marker of glutamate secretory cells. Because of this, we proposed the study of these transporters in bovine chromaffin cells by several experimental approaches. This paper discusses, in the light of new results on the expression of glutamate transporters in chromaffin cells, the possible role of glutamate in chromaffin cells, which would support the newly proposed nonsynaptic role of glutamate in neuroendocrine communication.

The present study demonstrates, for the first time, that catecholamine-secreting adrenal chromaffin cells exhibit VGLUT1 and -3 and EAAT2 and -3 immunoreactivities and show specific expression of these glutamate transporters at both mRNA and protein levels. The synthesis of VGLUTs and EAATs exhibits robust up-regulation in response to certain endocrine challenges, such as the cytokines TNFα, IFNγ, and LPS, as well as by glucocorticoids such as dexamethasone, indicating that altered glutamatergic signalling may represent an important adaptive mechanism.

Previous results from our group show that ionotropic and metabotropic glutamate receptor agonists can elicit catecholamine secretion and increase intracellular calcium levels in adrenal medulla at endocrine release sites (González et al., 1998). Structural constituents of adrenal medulla, chromaffin cells contain elements of glutamatergic transmission, including glutamate receptors and enzymes of the glutamate/glutamine cycle (Schwendt and Jezová, 2001; Sarriá et al., 2006).

Our previous results also indicate that chromaffin cells release different amounts of glutamate when stimulated by depolarizing agents that act by different mechanisms. In comparison with cortical neurones, the magnitude of glutamate secretion is lower, but it is similar in both the relative percentages of release in any case and the order of potency obtained for different secretagogues in both cell types: KCl > 4-AP > veratridine (Romero et al., 2003). All these catecholamine secretagogues induced glutamate secretion by two mechanisms: 1) a Ca^{2+}-dependent, exocytotic mechanism and 2) a Ca^{2+}-independent mechanism mediated by reversion of the electrogenic glutamate transporter.

Our previous results on the subcellular location of glutamate indicate that 90% of the cell-releasable glutamate pool is located in the cytoplasm and that only 10% is contained in the secretory granules (chromaffin granules and synaptic-like microvesicles; Romero et al., 2003). This glutamate distribution is similar to that of GABA (81% cytosolic and 19% granular; Oset-Gasque

et al., 1990). However, the compartmentation of cellular catecholamines is very different, insofar as 97% of total catecholamines are mainly located in chromaffin granules.

These previous results suggested the presence in chromaffin cells of specific glutamate transporters, whose identity has been revealed in the present work. Thus, by using multitechnical approaches (confocal microscopy, flow cytometric analysis, Western blot, and qRT-PCR techniques), the presence of specific plasma membrane (EAATs) and vesicular glutamate (VGLUTs) transporters has been investigated. We found a specific expression of both EAATs and VGLUTs. With respect to EAATs expression, immunocytochemical flow cytometry analysis showed a very high expression of the neuron-specific transporter EAAT3 (about 95% of cells), followed by EAAT2 (about 65% of cell) and EAAT1 (about 30% of cells). These results were confirmed by Western blot techniques, showing estimated molecular weights of about 69, 63, and 62 kDa for EAAT2, EAAT3, and EAAT1, respectively, which are similar to the weights described for brain (Sánchez-Mendoza et al., 2010) and for dorsal root ganglia and sciatic cells (Carozzi et al., 2008) and by qPCR techniques. However, the low expression obtained for EAAT1 at the protein level, along with the fact that specific expression of EAAT1 was not systematically found in all experiments, urges us to think that this transporter might be more important in the endothelial cells, as it is in astrocytes or endothelial cells at the brain level (Teichberg et al., 2009). The low EAAT1 expression in chromaffin cells could be in agreement with results from Lee et al. (2001) showing that the expression of GLAST (EAAT1) is transiently increased at 3 weeks of age in rat adrenal glands and with the fact that, while GLT1 (EAAT2) is highly expressed throughout the CNS, the expression of GLAST is low and restricted to a few brain regions, such as the Bergmann glia in cerebellum (Nedergaard et al., 2002).

The most important plasmalemmal glutamate transporter found in chromaffin cells is EAAT3, which is what we expected, given the neural origin of these cells. Interestingly, this transporter shows an important regulation by cytokines, such as IFNγ and TNFα, and by LPS and corticosteroids, such as dexamethasone, which is very relevant given the high quantity of these steroid hormones, which come from adrenal cortex, to which these cells are subjected. Thus, this specific regulation of EAAT3, different from that of EAAT2, suggests that the expression of both transporters is not redundant in adrenal medulla, because these transporters could be subjected to different regulation, as demonstrated in the brain. Thgus, it has been shown that, although ischemic preconditioning ''in vivo'' increases the expression of EAAT2 and EAAT3 glutamate transporters, the up-regulation of the latter is, at least partially, mediated by the TNFα-converting enzyme/TNFα/TNFR1 pathway (Pradillo et al., 2006), whereas EAAT2 expression is up-regulated by PPARγ agonists (Romera et al., 2007).

In chromaffin cells, EAATs share their subcellular location between membrane and cytosolic fractions. This is in accordance with previous studies in neurons suggesting that the expression of EAAT3 is located in both neuronal cytoplasm and plasma membrane (Pradillo et al., 2006), although EAAT3 is localized mainly at the plasma membrane level when its up-regulation by ischemic preconditioning takes places (Pradillo et al., 2006). Moreover, a large portion of total EAAT2 (and a minor portion of total EAAT1, EAAT3, and EAAT4) was associated with cholesterol-rich lipid raft microdomains of the plasma membrane, and the association with these cholesterol-rich microdomains is important for its localization and function (Butchbach et al., 2004).

The presence of EAATs in adrenal chromaffin cells seems to support previous data from our group indicating that both neurotransmitters (glutamate and catecholamines) are released to a different extent when chromaffin cells are stimulated by different secretagogues (Romero et al., 2003). The presence of a large pool of cytosolic glutamate in chromaffin cells could explain the greater involvement of EAATs in glutamate release in comparison with catecholamine release. The expression of EAATs along with the mainly cytosolic location of glutamate in chromaffin cells confers upon these glutamate transporters a special relevance in both the glutamate secretory process and the potential excitotoxic effect of glutamate in these cells (Vicente et al., 2006).

Although our previous results demonstrated that vesicular glutamate makes up only 10% of the cell-releasable glutamate pool, this is a large enough pool to account for the measured Ca^{2+}-dependent release (1–7% of total glutamate in 5 min; Romero et al., 2003). Thus, pool size/depletion is not limiting to consider Ca^{2+}-dependent glutamate release as exocytotic secretion. Results presented in this study clearly demonstrate the specific expression of VGLUT1 and -3 in about 55% and 25%, respectively, of the whole population of chromaffin cells. However, we did not find specific expression of VGLUT2, which is also what we expected, given the segregation and complementary expression patterns of both vesicular transporters in glutamatergic neurons from brain (Fremeau et al., 2004). Unlike EAATs, VGLUTs are localized mainly in the membrane fraction. Their estimated molecular weights of about 50 kDa for VGLUT1 and 60 kDa for VGLUT3 are very similar to those found in the brain (Sánchez-Mendoza et al., 2010). RT-qPCR techniques confirm the expression of these glutamate transporters at the mRNA level and show a different regulation between them by cytokines and glucocorticoids. The strong regulation by cytokines and dexamethasone found for VGLUT1 indicates that, as in the case of EAAT3, VGLUT1 could have an important role in the regulation of glutamate release and catecholamine release in the adrenal medulla. In fact, the expression of VGLUT1 mRNA in about 50% of corticotrophs but only in 7.7% of luteinizing hormone gonadotrophs in anterior and intermediate lobes of pituitary has recently been demonstrated (Kocsis

et al., 2010). Thus, the regulation of VGLUT1 in chromaffin cells by glucocorticoids seems to support a putative functional role of this transporter in the response to glutamate in chromaffin cells. The fact that EAAT3 and VGLUT1 undergo a similar regulation by cytokines and glucocorticoids suggests the existence of a functional relationship between the two transporters in control of extracellular glutamate levels in the adrenal medulla. Our recent data showing the up-regulation of nNOS by glucocorticoids and iNOS by cytokines in chromaffin cells (Pérez-Rodríguez et al., 2009), together with the fact that NO increases catecholamine (Oset-Gasque et al., 1994) and glutamate secretion (unpublished results), seem to support this conclusion. Moreover, these results support previous evidence showing the presence of VGLUT1 [known first as differentiation-associated Na^+-dependent inorganic phosphate cotransporter (DNPI), as an isoform of brain specific BNPI], in neuroendocrine cells, such as α cells in Langerhans islets and pynealocytes (Hayashi et al., 2001), adding important further evidence for a role of L-glutamate as an intracellular signaling molecule in endocrine peripheral organs with an important role in the regulation of hormone secretion. In addition, glutamate in adrenal medulla could serve as a precursor for GABA synthesis.

Therefore, taken together, the interesting results presented here, together with our previous results on glutamate release from adrenal medulla (Romero et al., 2003) and its role in apoptosis induced by NO in chromaffin cells (Pérez-Rodríguez et al., 2009), support 1) the participation of these glutamate transporters in the process of glutamate transport and release in chromaffin cells and in the regulation of their neurosecretory function in adrenal medulla and 2) the validity of chromaffin cells as a model for the study of neurosecretion, neurotransmission, and neurodegeneration mechanisms.

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