The depolarization-evoked, Ca2+-dependent release of exosomes from mouse cortical nerve endings: new insights into synaptic transmission.

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Abstract

Background and purpose Exosomes, nanosized extracellular vesicles, emerged as players in the cell-to-cell communication in the central nervous system (CNS), having a role in the modulation of the synaptic activity. This study aimed at evaluating whether exosomes can be actively released from presynaptic nerve terminals. Experimental Approach Mouse cortical synaptosomes were exposed to a depolarizing stimulus (25 mM KCl medium) and exosomes were isolated from the synaptosomal supernatants. Exosomes were characterized by dynamic light scattering, transmission electron microscopy, western blot and flow cytometry analyses. We also evaluated whether and how removing external Ca2+ ions or activating presynaptic GABAB receptors by exposing synaptosomes to (±)-baclofen (10 μM) affects the 25 mM KCl-evoked release of exosomes. Key Results The structural and biochemical analysis unveiled that synaptosomal supernatants contained vesicles having the size and the shape of exosomes, immunopositive for the exosomal markers TSG101, flotillin-1, CD63 and CD9. The content of these proteins in the exosomal fraction isolated from synaptosomal supernatants increased upon the exposure of nerve terminals to a depolarizing stimulus and occurred in a Ca2+-dependent fashion, mimicking the release of glutamate. (±)-Baclofen significantly reduced glutamate exocytosis but failed to affect the release of exosomes from cortical synaptosomes. Finally, presynaptic exosomes were shown to carry selected NMDA and AMPA receptors subunits. Conclusion and implications Our study unveils the Ca2+-dependent, depolarization-evoked release of exosomes from mouse cortical nerve terminals, which is insensitive to presynaptic release-regulating GABAB receptors. These findings add new insights into the mechanisms of exosomes-mediated communication in CNS.

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The structural and biochemical analysis unveiled that synaptosomal supernatants contained vesicles having the size and the shape of exosomes, immunopositive for the exosomal markers TSG101, flotillin-1, CD63 and CD9. The content of these proteins in the exosomal fraction isolated from synaptosomal supernatants increased upon the exposure of nerve terminals to a depolarizing stimulus and occurred in a Ca^{2+}-dependent fashion, mimicking the release of glutamate. (±)-Baclofen significantly reduced glutamate exocytosis but failed to affect the release of exosomes from cortical synaptosomes. Finally, presynaptic exosomes were shown to carry selected NMDA and AMPA receptors subunits.

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Keywords

Synaptosomes, exosomes, calcium dependency, glutamate release, $GABA_B$ receptor, GluN subunits, GluA subunits.

List of abbreviations

CNS, central nervous system; ESCRT-I, endosomal sorting complex required for transport I; EV, extracellular vesicles; LDH, lactate dehydrogenase; MVB, multivesicular body; PBS, phosphate saline buffer; PSD95, postsynaptic density protein 95; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; t-TBS, Tris-buffered saline-Tween; TEM, transmission electron microscopy; Tris, Tris-(hydroxymethyl)-amino methane; TSG101, tumor susceptibility gene 101 protein; WG, Wingless

Bullet point summary

What is already known

- Exosomes are key players in the cell-to-cell communication in the central nervous system
- Neurons release exosomes, but clear evidence they are released also from axonal terminals is lacking

What this study adds

- Mouse cortical nerve terminals release exosomes in response to a depolarizing stimulus
- The release of exosomes is a Ca^{2+}-dependent event, insensitive to presynaptic release-regulating $GABA_B$receptors

Clinical significance

Our study gives new insights into the knowledge of the exosomal-mediated communication in CNS

Introduction

Extracellular vesicles (EVs) have emerged in the last decades as a novel way of cell-to-cell communication (Raposo and Stoorvogel, 2013). Exosomes are nanosized EVs (30-100 nm in diameter) of endosomal origin, secreted by most cells in the body, known to vehiculate complex cargoes, including proteins, lipids and nucleic acids, whose composition depends on the cell they originate from. Once released in the extracellular fluids, they are taken up by the selected target cells, influencing their functions (Mathieu et al., 2019).

Exosomes mediate intercellular communication also in the central nervous system (CNS) and they are actively released by all the CNS cells, including astrocytes (Verkhratsky et al., 2016), microglia (Paolicelli et al., 2018), oligodendrocytes (Frühbeis et al., 2013) and neurons (Chivet et al., 2012). Data in the literature demonstrated that the exosomal trafficking has important implications in CNS physiology and pathology, from supporting neurogenesis, neuroprotection and brain homeostasis, to favoring the cell-to-cell spreading of "pathogenic proteins" (β amyloid peptides, tau and prions; Holm et al., 2018; Bavisotto et al., 2019). Emerging evidence suggests that exosomes are also involved in the modulation of synaptic transmission

and plasticity. It has been demonstrated that cultured cortical neurons release exosomes, mainly from the soma and the dendrites, and that their secretion is increased by depolarization and strictly dependent on synaptic glutamatergic activity. Interestingly, neuron-derived exosomes carry selected proteins involved in synaptic transmission, such as GluA2/3 AMPA receptor subunits, representing a new possible mechanism for regulating synaptic strength after neuronal activation (Faurè et al., 2006; Lachenal et al., 2011). Furthermore, at the *Drosophila*neuromuscular junction it has been described the trans-synaptic transferring of the Wnt-family signaling protein Wingless (Wg) through exosomes released from the synaptic boutons and containing the Wg-binding protein Evenness Interrupted (Korkut et al., 2009; Koles et al, 2012). In the same model, also synaptotagmin-4 was found to be delivered through exosomes from the presynaptic motor neuron to the muscle fiber, to mediate the activity-dependent synaptic growth (Korkut et.al, 2013).

It has not yet been clarified whether exosomes can be actively released also from presynaptic neuronal terminals of CNS mammalian synapses, although some evidence supports the hypothesis (Xu et al., 2013; Janas et al., 2016; Wang et al., 2017).

Synaptosomes are isolated nerve endings, which carry the structural features and the properties of the *in vivo* neuronal terminals they originate from. They are a widely recognized *in vitro* model for studying selectively the presynaptic mechanisms of neurotransmission, like the release of neurotransmitters and its modulation by receptors (Raiteri, 2001 and 2008; Langer, 2008).

By using this model, we aimed at investigating whether a release of EVs having the morphological and proteomic features of exosomes takes place also at axonal terminals, as already described for other neuronal compartments. Our results are consistent with the depolarization-evoked, Ca^{2+}-dependent release of exosomes-like vesicles from mouse cortical nerve endings and suggest new unexpected aspects of synaptic transmission.

Methods

Animals

Mice (male, strain C57BL/6J) were obtained from Charles River (Calco, Italy) and housed in the animal facility of the Department of Pharmacy (DIFAR), Pharmacology and Toxicology Section (Genoa, Italy), under controlled environmental conditions (ambient temperature = 22°C, humidity = 40%) on a 12-h light/dark cycle with food and water ad libitum. Mice were euthanized by cervical dislocation, followed by decapitation, and the cortices were rapidly removed. The experimental procedures were in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC), the ARRIVE guidelines and recommendations made by the British Journal of Pharmacology (McGrath et al., 2015), and they were approved by the Italian Ministry of Health (DDL 26/2014 and previous legislation; protocol number n° 75F11.N.I.MY). Experiments were performed following the Guidelines for Animal Care and Use of the National Institutes of Health. In line with the 3Rs rules (replacement, refinement and reduction), any effort was made to reduce the number of animals to obtain statistically reliable results.

Preparation of synaptosomes

Mouse cortical purified synaptosomes were prepared as previously described (Olivero et al., 2018). The tissue was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with Tris-(hydroxymethyl)-amino methane (Tris, final concentration 0.01 M) using a glass/Teflon tissue grinder (clearance 0.25 mm). Synaptosomes were isolated through differential centrifugation. The homogenate was first centrifuged at 1,000 g for 5 min to remove nuclei and debris. The supernatant was gently layered on a discontinuous Percoll gradient (6, 10, and 20% v /v in Tris-buffered 0.32 M sucrose) and then centrifuged at 33,500 g for 6 min. The layer between 10 and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation at 19,000 g for 15 min. Synaptosomes were then resuspended in a physiological medium having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.4.

Depolarization and pharmacological treatments

Cortical synaptosomes were divided in identical aliquots and pre-incubated for 5 minutes in a physiological 3 mM KCl medium in a water bath at 37°C under mild shaking. Synaptosomal aliquots were then incubated for 2 minutes in basal condition (3 mM KCl medium, control synaptosomes) or in the presence of a mild depolarizing stimulus (25 mM KCl medium, depolarized synaptosomes). When indicated, synaptosomes were incubated for 2 minutes with a 25 mM KCl medium lacking Ca^{2+} ions (25 mM KCl/Ca^{2+}-free medium) or with a 25 mM KCl medium in the presence of the $GABA_B$ receptor agonist (±)-baclofen (10 μM, added 1 min before the depolarizing stimulus, Tocris Bioscience, Bristol, UK).

Cold medium was added to the reaction tubes to stop the depolarization reaction. The synaptosomal suspensions were then centrifuged at 19,000 g for 10 min and the synaptosomal pellets and supernatants were collected.

The synaptosomal pellets were quantified for the protein content with Pierce BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). and lysed in modified RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, protease inhibitors) for Western Blot analysis, or resuspended in phosphate saline buffer (PBS) for transmission electron microscopy and dynamic light scattering analyses.

Synaptosomal supernatants were centrifuged again at 19,000 g for 30 min to remove any synaptosomal residues and collected for consequent analyses.

Isolation of the exosomes from the synaptosomal supernatants

Identical volumes of the synaptosomal supernatants from the different experimental conditions were incubated with the Total Exosome Isolation Reagent (from cell culture media, Invitrogen, Thermo Fisher Scientific) overnight at 4°C and then centrifuged at 10,000 g for 1 h at 4°C. Pellets, representing the exosomal fractions, were collected and resuspended in modified RIPA lysis buffer for the western blot analysis, or in PBS, when performing flow cytometry, transmission electron microscopy and dynamic light scattering analyses.

Western blot analysis

The exosomal lysates from the different experimental conditions were dissolved in SDS-PAGE sample buffer, boiled for 5 min at 95°C, subjected to 10% SDS-PAGE, and then blotted onto PVDF membranes (Merck, Darmstad, Germany). Membranes were blocked for 1 h at room temperature with Tris-buffered saline-Tween (t-TBS: 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20), containing 5% (w/v) non-fat dried milk, and then incubated overnight at 4°C with the following primary antibodies: rabbit anti-TSG101 (1:500, T5701, Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-flotillin-1 (1:1000, 18634, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-CD63 (1:1000, sc-5275, Santa Cruz Biotechnology, Dallas, Tx, USA) and rabbit anti-CD9 (1:300, ab92726, Abcam, Cambridge, UK). After extensive washes in t-TBS, membranes were incubated for 1 h at room temperature with appropriate horseradish peroxidase-linked secondary antibodies (1:10000, A9044 and A9169, Sigma-Aldrich).

Also the cortical synaptosomal lysates (5 μg/lane) were analysed with immunoblot analysis and membranes were probed with the above-mentioned primary antibodies (concentrations as previously indicated) and with mouse-anti-sintaxin-1a antibody (1:500, GTX18010, GeneTex, Irvine, CA, USA).

In a set of western blot experiments, equal amount of the synaptosomal and exosomal lysates (2 μg/lane) were compared for the content of selected proteins by using the already mentioned primary antibodies, at the concentrations indicated, and the following ones: mouse anti-synaptotagmin-1 (1:500, 105 011, Synaptic System, Goettingen, Germany), rabbit anti-PSD95 (1:1000, NBP1-40474, Novus Biologicals, Centennials, CO, USA), mouse anti-GluN1 (1:500, MAB 1586, Merck), mouse anti-GluN2A (1:500, ab174636, Abcam), mouse anti-GluN2B (1:500, ab93610, Abcam), rabbit anti-GluA2/3 (1:500, ab52896, Abcam).

Immunoblots were visualized with the enhanced chemiluminescence Western blotting detection system Immobilon Forte Western HRP substrate (Merck). Images were acquired by using the Alliance LD6 images capture system (Uvitec, Cambridge, UK) and analysed with UVI-1D software (Uvitec).

In western blot experiments membranes were cut into pieces to probe different regions of the same blot with multiple antibodies.

Transmission electron microscopy

Freshly prepared synaptosomes were washed out in 0.1 M cacodylate buffer and immediately fixed in 0.1 M cacodylate buffer containing 2.5% glutaraldehyde (Electron Microscopy Science, Hatfield, PA, USA), for 1 h at room temperature. The synaptosomes were postfixed in osmium tetroxide for 2 h and 1% uranyl acetate for 1 h. Subsequently, samples were dehydrated through a graded ethanol series and embedded in resin (Poly-Bed; Polysciences, Inc.,Warrington, PA, USA) for 24 h at 60°C. Ultrathin sections (50 nm) were cut and stained with 5% uranyl acetate in 50% ethanol.

Electron microscopic analysis on isolated vesicles preparations was performed as follows. The extracellular vesicles preparations were resuspended in 20 μL PBS (pH 7.4) and fixed by adding an equal volume of 2% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Extracellular vesicles were then adsorbed for 10 minutes to formvar-carbon coated copper grids by floating the grids on 5 μL drops on parafilm. Subsequently, grids with adhered vesicles were rinsed in PBS and negatively stained with 2% uranyl acetate for 5 minutes at room temperature. Stained grids were embedded in 2.5% methylcellulose for improved preservation and air dried before examination (Cortese et al., 2020). Electron micrographs were taken at Hitachi TEM microscope (HT7800 series, Tokyo, Japan) equipped with Megaview 3 digital camera and Radius software (EMSIS, Germany).

Dynamic light scattering and zeta-potential

Particle size, polydispersity and zeta-potential were analyzed using the Zetasizer Nano ZS90 particle sizer at a 90° fixed angle (Malvern Instruments, Worchestershire, UK). Vesicles size was evaluated by dynamic light scattering allowing the analysis of particles within the range of 0.1 nm up to 10 μm, as previously described (Marimpietri et al., 2013). Briefly, exosomes and synaptosomes were suspended in PBS and the measure was performed at a constant temperature of 25°C in UV-transparent cuvettes. The translational diffusion coefficient of the solutions was calculated from the time autocorrelation of the scattered light intensity and the translational diffusion coefficient was extracted from the correlogram using the method of cumulants as applied in the proprietary Malvern software. The diameter of the exosomes was obtained from the application of Stokes–Einstein equation: d(H)=kT/3 pgD where d(H) is the hydrodynamic diameter, k the Boltzmann constant, T the temperature, g the shear viscosity of the solvent and D the diffusion coefficient of the particles.

Flow cytometric analysis

Exosomes were analyzed for the presence of CD9 and TSG101 by flow cytometry after vesicles adsorption onto latex beads as previously reported (Marimpietri at al., 2013). In brief, 1 mL of purified exosomes was incubated with 4 μL of 4 mm diameter aldehyde/sulfate latex beads (Invitrogen, Thermo Fisher Scientific) for 2 h at room temperature; PBS supplemented with 4% fetal bovine serum was then added to each sample, and the incubation was prolonged for 30 min. After washing, exosome-coated beads were incubated for 30 min at 4°C with primary rat anti-mouse CD9 PE-conjugated monoclonal antibody (12-0091-81, Invitrogen, Thermo Fisher Scientific) or with unlabeled mouse anti-TSG101 monoclonal antibody (ab43, Abcam) plus a further incubation for 20 min at 4°C with a goat anti-mouse PE-conjugated secondary antibody (PA5-33249, Thermo Fisher Scientific). As negative control an isotype-matched primary monoclonal antibody was used. All antibodies were used in accordance with manufacturer instructions. Samples were then analyzed by Gallios flow cytometer and Kaluza software (BeckmanCoulter, Brea, CA, USA).

Glutamate assay

The endogenous glutamate content in the synaptosomal supernatants was analysed by high performance liquid chromatography analysis after precolumn derivatization with ophthalaldehyde and separation on a C18 reverse-phase chromatographic column (10x4.6 mm, 3 μm; at 30@C; Chrompack, Middleburg, The Netherlands) coupled with fluorimetric detection (excitation wavelength, 350 nm; emission wavelength, 450

nm). Buffers and the gradient program were previously described (Salamone et al., 2014). Homoserine was used as internal standard. The amount of glutamate was expressed as pmol/mg of synaptosomal proteins.

LDH assay

The LDH activity in the synaptosomal supernatants was evaluated by using the Pierce LDH Citotoxicity Assay Kit (Thermo Fisher Scientific) according to manufacturer's manual. The LDH activity in the supernatants was expressed as percent of the total synaptosomal LDH activity (LDH activity in synaptosomal pellets and in the supernatants).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Data represents the mean ± SEM of n independent values (n indicated in the figure legends). The n values correspond to the number of the animals used within each experiment, except for the data in the figure 4 and in the figure 7, where the cortices from two mice were pooled together to carry out the experiments.

Sigma Plot 10 data analysis and graphing software package was used for data handling/statistics and for graph drawing. Analysis of variance was performed by ANOVA followed by Tukey's multiple-comparisons test; direct comparisons were performed by Student's t test. Post hoc tests were done only if F value was significant. Data were considered significant if $p<0.05$.

Results

Cortical synaptosomes possess proteins that are markers of exosomes

Purified cortical synaptosomes, isolated as described in figure 1A (see also Methods section), were examined at the ultrastructural level by transmission electron microscopy (TEM). Consistent with previous data in the literature (Feligioni et al., 2003; Nisticò et al., 2015), synaptosomes appeared as round-shaped membrane-bound structures having a diameter in the 500-1200 nm range, containing intraterminal mitochondria, small clathrin-coated vesicles and abundant synaptic vesicles. In addition, a subpopulation of synaptosomes were endowed with endosome-like structures, including multivesicular body (MVB)-like organelles, with intraluminal vesicles (Figure 1B).

Cortical synaptosomes were also analysed with dynamic light scattering, which confirmed the size range observed in TEM analysis, with a bell-shape size distribution profile of the synaptosomal particles peaking at 790 ± 67 nm (Figure 1C).

The presence of MVB-like organelles in the purified synaptosomes prompted us to further investigate by western blot analysis the immunopositivity of cortical synaptosomal lysates for proteins widely recognized as exosomal markers: the endosomal sorting complex required for transport I (ESCRT-I) tumor susceptibility gene 101 protein (TSG101), the lipid raft-associated protein flotillin-1, and the tetraspanins CD63 and CD9 (Andreu, Z. & Yànez-Mò, 2014; Zhang et al., 2019). The selective antibodies unveiled a clear immunoreactivity having appropriate mass for all the above-mentioned proteins in the cortical synaptosomal lysates (expected molecular weights: TSG101, 46 kDa; flotillin-1, 49 kDa; CD63, 30-60 kDa for the glycosylated forms of the protein; CD9, 25 kDa; Figure 1D).

Cortical synaptosomes exposed to a depolarizing stimulus release extracellular vesicles having the biochemical and morphological features of exosomes.

It has been demonstrated that exosomes are released in a depolarization-evoked, Ca^{2+}-dependent manner from cortical neurons (Faure et al., 2006; Lachenal et al., 2011). Based on the presence of MVB-like organelles in cortical synaptosomes and on the immunoreactivity for the exosomal markers TSG101, flotillin-1, CD9 and CD63 in the synaptosomal lysates, we asked whether exosomes can be actively released also from axonal terminals.

To test this hypothesis, identical aliquots of cortical synaptosomes were incubated for 2 minutes in basal, non-depolarizing condition (3 mM KCl medium, control synaptosomes) or in the presence of a mild depolarizing stimulus (25 mM KCl medium, depolarized synaptosomes). Identical volumes of the synaptosomal supernatants from both experimental conditions were incubated with the Total Exosome Isolation Reagent to isolate extracellular vesicles (EVs) having the dimension and the features of exosomes (Figure 2A, see also Methods section). The isolated particles, we refer to as EVs fraction, were analysed with western blot for protein composition to verify the presence of the exosomal markers TSG101, flotillin-1, CD63 and CD9. An immunoreactivity for all the above-listed proteins was detected in the EVs fractions isolated from the supernatants of both control and depolarized synaptosomes. Figure 2B displays a representative blot showing bands for the four exosomal markers at the expected molecular weights. Notably, the signal for TSG101, flotillin-1, CD63 and CD9 was more pronounced in the EVs fraction isolated from the supernatants of the depolarized synaptosomes. The quantification confirmed the significant increase in the exosomal proteins content in the EVs fraction from the depolarized synaptosomes when compared to control ones (Figure 2C).

To corroborate these data, cortical synaptosomes incubated in basal condition and in 25 mM KCl medium were also analysed by TEM. According to the biochemical data, the comparative ultrastructural analysis unveiled that depolarized synaptosomes were almost devoid of MVB-like organelles, but still contained endosomal-like structures (Figure 2D).

The EVs isolated from the synaptosomal supernatants were then analysed by dynamic light scattering for their size and zeta potential. The size distribution profile of the EVs isolated from the depolarized synaptosomes did not differ significantly from that of the EVs isolated from control synaptosomes. Both curves showed a bell-shaped profile that reaches the peak at 50.78 ± 2.10 nm for the EVs isolated from the supernatants of control synaptosomes and at 52.54 ± 1.85 nm for the EVs isolated from the supernatants of depolarized synaptosomes (Figure 3A and 3B). The size range for both profiles is consistent with the expected size values for exosomes. Furthermore, the EVs from both control and depolarized conditions showed a comparable zeta potential (Figure 3A), suggesting a good and similar nanoparticle stability in terms of dispersion, aggregation or flocculation. As shown in Figure 3A, the mean count rate of dynamic light scattering (the parameter that represents the average scattering intensity during the measurement) was almost twice (125.41 ± 6.22 kcps) when analysing the EVs isolated from the supernatants of depolarized synaptosomes in comparison to that recorded during the analysis of the EVs from the supernatants of control synaptosomes (66.79 ± 3.12 kcps). Since the mean count rate is directly proportional to the concentration of the EVs in the analysed sample, this finding seems best interpreted by assuming that the depolarizing stimulus increased the EVs content in the synaptosomal supernatants. In a whole, these data indicate that the EVs isolated from the supernatants of control and depolarized synaptosomes differ by a quantitative point of view, but not qualitatively.

Based on this observation, we decided to extend the study focussing on the EVs isolated from the supernatants of the 25 mM KCl-depolarized synaptosomes.

The latex beads cytofluorimetric assay revealed good levels of signal for CD9 and TSG101, two of the most typical exosomal surface markers (Figure 3C).

Furthermore, TEM analysis demonstrated that the EVs released from the synaptosomes after depolarization have a round shape and smooth surface, with a diameter ranging from 40 to 70 nm, which is consistent with exosomal ultrastructure (Figure 3D).

In a whole, all these findings support the conclusion that cortical synaptosomes release EVs having the morphological and biochemical features of exosomes and that this event is significantly enhanced when exposing synaptosomes to a depolarizing stimulus. Based on these observations, the term “exosomes” will be used from here on to indicate the released EVs.

Comparative analysis of exosomal and synaptosomal markers in synaptosomal and exosomal lysates

Western blot analysis was carried out to compare the expression of synaptosomal and exosomal markers in the lysates of the exosomes and of the synaptosomes they are released from. Identical amounts of proteins (2 μg/lane) of both the synaptosomal and the exosomal lysates were first analysed for their contents of the exosomal markers TSG101, flotillin-1, CD63 and CD9. The exosomal lysates were particularly enriched in TSG101, CD63 and CD9 proteins when compared to the synaptosomal ones, while flotillin-1 was strongly detected also in synaptosomes. We then analysed the presence of the following synaptosomal markers: synaptotagmin-1, a synaptic vesicles membrane protein; syntaxin-1a, which is located in presynaptic plasma membranes; postsynaptic density protein 95 (PSD-95), a membrane protein having a postsynaptic localization (Bonanno et al., 2005; Bonfiglio et al., 2019; Franchini et al., 2019). Synaptosomal lysates showed a clear immunoreactivity for all the three proteins (synaptotagmin-1, 60 kDa; syntaxin-1a, 36 kDa; PSD-95, 95 kDa), while the exosomal lysates were immunopositive for syntaxin-1a, although to a lower extent than the synaptosomal lysates, but lack the synaptotagmin-1 and PSD-95 signals (Figure 4). These observations unveiled a qualitative difference in the protein composition of the synaptosomal and the exosomal preparations.

The release of exosomes from cortical synaptosomes is a Ca^{2+}-dependent event.

It's known that the exposure of synaptosomes to 25 mM KCl medium causes a membrane depolarization that in turn allows a Ca^{2+}-dependent exocytosis of neurotransmitter. We asked whether the influx of Ca^{2+} ions into synaptosomes elicited by the high-KCl-induced depolarization could also play a role in the release of exosomes from cortical synaptosomes. To test the hypothesis, identical aliquots of cortical synaptosomes were exposed to the depolarizing 25 mM KCl medium containing physiological amount of the divalent cation (1.2 mM Ca^{2+}-containing medium) or lacking Ca^{2+} ions (Ca^{2+}free medium).

As shown in the representative western blot in the figure 5A, a significant reduction in TSG101, flotillin-1, CD63 and CD9 proteins content was observed in the exosomal fraction isolated from the supernatants of synaptosomes exposed to the 25 mM KCl /Ca^{2+} free medium when compared to synaptosomes depolarized with the 25 mM KCl / 1.2 mM Ca^{2+}-containing medium (Figure 5B).

It's worth noting that the significant increase of the endogenous glutamate content in the supernatants from synaptosomes exposed to the 25 mM KCl /1.2 mM Ca^{2+}-containing medium was significantly reduced when calcium ions were omitted in the depolarizing stimulus (Figure 5C).

To exclude that a non-specific synaptosomal leakage might account for the results so far described, we verified the viability of the synaptosomes in the different experimental conditions by measuring the endogenous lactate dehydrogenase (LDH) activity in the supernatants. The LDH activity was unmodified in the supernatants of control and depolarized synaptosomes and also the removal of external Ca^{2+} ions from the 25 mM KCl medium did not affect this parameter (Figure 5D).

The release of exosomes from cortical synaptosomes is not modulated by presynaptic $GABA_B$ heteroreceptors.

Glutamate exocytosis from cortical synaptosomes is an active process controlled by auto- and heteroreceptors presynaptically located in nerve terminals. In particular, presynaptic $GABA_B$heteroreceptors exist in cortical glutamatergic nerve endings: they are inhibitory G-coupled receptors, whose activation reduces the adenylyl cyclase activity and the Ca^{2+} conductance, inhibiting glutamate exocytosis from the nerve endings (Bonanno and Raiteri., 1993; Grilli et al., 2004; Vergassola et al., 2019; Pittaluga 2019). Accordingly, the glutamate content in the supernatants of synaptosomes exposed to 25 mM KCl medium in the presence of the $GABA_B$receptor agonist (±)-baclofen (10 μM) was significantly lower than that in the supernatants of synaptosomes exposed to the depolarizing stimulus alone (Figure 6A). Differently, the amount of TSG101, flotillin-1, CD63 and CD9 in the exosomal fraction isolated from the synaptosomes exposed to the depolarizing stimulus in the presence of (±)-baclofen (10 μM) did not significantly differ from that in the exosomal fraction isolated from synaptosomes exposed to the depolarizing stimulus alone (Figure 6B and 6C).

Exosomes released by cortical synaptosomes vehiculate selected glutamate receptor subunit

proteins.

Exosomes are known to deliver selected cargoes, including cellular proteins. We asked whether the exosomes released from the depolarized cortical synaptosomes can vehiculate receptor proteins. In particular, we focused on NMDA and AMPA receptors proteins because synaptosomes are endowed with these receptors (Pittaluga et al., 1997; Nisticò et al., 2015), which are known to modulate the release of neurotransmitters and to undergo both constitutive and induced in-out synaptosomal plasma membrane trafficking (Pittaluga et al., 2006; Summa et al., 2011).

NMDA receptors are tetramers composed of the GluN1, GluN2 and GluN3 subunits. Data in literature suggest that presynaptic release regulating NMDA autoreceptors mainly consist of GluN1, GluN2A and GluN2B subunits assembly (Olivero et al., 2019). Accordingly, western blot analysis confirmed the presence of GluN1, GluN2A and GluN2B proteins in the cortical synaptosomal lysates (GluN1, 100 kDa; GluN2A and GluN2B, 166 kDa). However, we found a different level of the expression of these proteins in the exosomal lysates. In particular, GluN1 and GluN2A subunits were undetectable in the exosomal lysates, while a clear immunoreactivity was detected for the GluN2B subunit, more pronounced when compared to synaptosomal lysates (Figure 7A).

As far as the AMPA receptors are concerned, we focussed on GluA2/3 subunits, since evidence was provided that these proteins are carried by cortical neuronal exosomes (Faurè et al., 2006; Lachenal et al., 2011). An immunoreactivity for the GluA2/3 subunits was found, although to a different level, in both the synaptosomal and the exosomal lysates. To note, in the exosomal lysates the antibody unveiled exclusively the presence of bands compatible with a multimeric associations of the receptor subunits (about 250 kDa), while in the synaptosomal lysates also a band corresponding to the monomeric form (100 kDa, figure 7B).

Discussion

In recent years, exosomes have emerged as non-conventional messengers in inter-cellular communication in the CNS and their role in the modulation of synaptic activity in physiological and pathological conditions gained interest (Smalheiser, 2007; Saedi et al., 2019; Schiera et al., 2020).

The possibility that exosomes can be released from the nerve endings was first proposed by studies on the *Drosophila* neuromuscular junction (Koles et al. 2012; Korkut et al., 2013).

Exosomes are produced within the endosomal compartment in cells. The presence of the endosomes in the presynaptic component of central synapses is still matter of discussion: evidence suggest that at this level they are dynamic organelles having a role in synaptic vesicles endocytosis (Leenders et al., 2002; Jähne et al., 2015). Furthermore, although MVBs, the late endosomal organelles exosomes originate and are released from, are less present in central axonal terminals than in other neuronal compartments (i.e. soma and dendrites; Von Bartheld and Altick, 2011; Men et al., 2019), some studies supported their presynaptic location and the possibility that they can fuse with nerve terminals plasma membranes (Janas et al., 2016 and references therein; Zappulli et al., 2016; Zhang and Yang, 2018). Despite all these observations, clear evidence demonstrating the release of exosomes from presynaptic structures in central synapses is lacking.

Owing to address this question, we focussed on synaptosomes, which are re-sealed pinched off nerve terminals that retain the functional properties of the presynaptic component they originate from (Pittaluga et al., 2019) and that we propose as an appropriate model to highlight the presence and the release of exosomes from nerve terminals.

In this study, we demonstrated that mouse cortical synaptosomal lysates are immunopositive for endosomes-associated proteins, commonly considered as exosomal markers (i.e. TSG101, flotillin-1, CD63 and CD9), consistent with the presence of exosomes in isolated nerve endings. In line with the hypothesis, the TEM ultrastructural analysis unveiled the presence of MVBs-like organelles containing intraluminal vesicles in cortical synaptosomes.

Secondly, we demonstrated that mouse cortical synaptosomes release EVs with structural and biochemical

features of exosomes in response to a depolarizing stimulus. In fact:

i) The supernatants of cortical synaptosomes exposed to a mild depolarizing stimulus (25 mM KCl medium) were enriched with EVs that at both dynamic light scattering and TEM analyses display size and shape consistent with those of exosomes.

ii) The size and the zeta-potential of the EVs isolated from the supernatants of synaptosomes in both basal and depolarized conditions were largely conserved. However, the significant increase in the mean count rate of dynamic light scattering when analysing EVs from depolarized terminals suggest an accumulation of EVs at the outer side of synaptosomes upon the application of the releasing stimulus.

iii) The EVs fraction isolated from the supernatants of depolarized synaptosomes were more reactive for TSG101, flotillin-1, CD63 and CD9 when compared to the EVs fraction collected in basal condition.

On the other hand, MVB-like organelles were rarely, if ever, detected in depolarized synaptosomes when compared to control synaptosomes: this could be best interpreted by assuming a depletion of this organelles in nerve terminals because of their stimulus-induced fusion with membranes to release exosomes.

The comparative analysis of specific synaptosomal and exosomal proteins in synaptosomal and exosomal lysates unveiled a different proteomic profile in the two preparations.

The exosomal lysates were enriched in the exosomal proteins TSG101, CD9 and CD63, confirming the purity of the exosomal preparation. Differently, flotillin-1 was also highly expressed in the synaptosomal lysates, consistent with the fact that it concentrates within lipids rafts, cholesterol-enriched microdomains present also in neuronal plasma membranes, where it plays a role in the synapse formation (Swanwick et al., 2015). Synaptotagmin-1, a v-SNARE protein located in vesicular membranes, was largely expressed in synaptosomal lysates, but almost absent in the exosomal fraction, that would exclude a contamination of synaptic vesicles in the exosomal preparation, as well as PSD95, a specific marker for the postsynaptic compartment. Differently, syntaxin-1a, a t-SNARE membrane protein mediating vesicular exocytosis, which has been demonstrated to have a role in permitting the anchoring of MVBs to plasma membranes and the release of exosomes from*Drosophila* neuromuscular junction (Koles et al., 2012), was also detected in the exosomal lysates.

Synaptosomes represent the simplest model to study the Ca^{2+}-dependent exocytosis of transmitters from synaptic vesicles (Raiteri and Raiteri, 2000; Pittaluga, 2019). The finding that the enrichment of exosomal markers in the exosomal fraction isolated from the supernatants of depolarized synaptosomes was almost undetectable when nerve terminals were stimulated with the 25 mM KCl medium lacking Ca^{2+} ions suggests that the release of exosomes is a Ca^{2+}-dependent event, as already described in the literature (Savina et al., 2003; Krämer-Albers et al., 2007; Lachenal et al., 2011). To correlate the efficiency of exosomal outflow and the efficiency of transmitter exocytosis., we analysed the supernatants for their endogenous glutamate content. Cortical synaptosomes are a heterogenous population in which glutamatergic ones prevail, therefore the study of glutamate exocytosis would allow to monitor the release activity in most of the synaptosomal population (Grilli et al., 2004). As expected, we detected a significant increase of the glutamate content in the supernatants of synaptosomes exposed to the depolarizing stimulus that was significantly reduced by omitting external Ca^{2+} ions. In a whole, these findings support the conclusion that transmitter exocytosis and exosomes release are two Ca^{2+}-dependent events that occur concomitantly at nerve endings exposed to a depolarizing stimulus.

The transmitter exocytosis is presynaptically controlled by auto- and heteroreceptors (Raiteri, 2001; Langer, 2008; Pittaluga, 2019). Interestingly, the depolarization-evoked Ca^{2+}-dependent secretion of exosomes from cultured cortical neurons was shown to be controlled by $GABA_A$ligands, allowing the conclusion that the release of exosomes from these cells can be mediated by synaptic receptors activity (Lachenal et a., 2011). It is known that glutamatergic synaptosomes possess inhibitory $GABA_B$ heteroreceptors controlling glutamate exocytosis (Bonanno and Raiteri, 1993). Our study demonstrated that presynaptic $GABA_B$ heteroreceptors do not control the secretion of exosomes from cortical nerve endings but inhibit efficiently glutamate exocytosis. In fact, the immunoreactivities of the exosomal markers in the exosomal fraction isolated from

the supernatant of synaptosomes exposed to the depolarizing stimulus in the presence of (±)-baclofen were comparable to those detected in the absence of the ligand, while the content of glutamate in the supernatant was significantly reduced. In a whole, these observations suggest that, although transmitter exocytosis and exosomes sorting are both dependent on a common triggering circumstance involving the influx of Ca^{2+}ions within terminals, the two events may involve different intraterminal pathways, as suggested by the different sensitivity to the receptor-mediated presynaptic mechanisms of control of the synaptic activity. Further studies are required to correctly address this issue.

Finally, our study demonstrate that exosomes released from cortical nerve endings carry selected AMPA and NMDA receptors subunits. Presynaptic exosomes were found to possess GluA2/3 proteins, as already reported for exosomes derived from cultured neurons (Fauré et al. 2006; Lachenal, 2011). Quite interestingly, the GluA subunits preferentially adopted a multimeric association in exosomes, best consistent with the transport of receptor assemblies. This could reflect a preferential localization of AMPA receptors clusters in exosomes within lipid rafts (Hering et al., 2003; Smallheiser 2007). Moreover, exosomes were endowed with the GluN2B subunit, which preferentially adopted the monomeric form, while GluN1 and GluN2A subunits were absent. Although preliminary, these data could suggest the trafficking of glutamatergic ionotropic receptor subunits through exosomes within the glutamatergic synapse as well as to adjacent cells. Since both GluA2/3 and GluN2B subunits control the kinetic properties of the respective receptors, these observations could describe a new mechanism of control of the glutamate receptors assembly and functions that might be relevant to dictate the efficiency of synaptic connections in CNS during development but also in pathological conditions.

Taking into consideration that, as far as the synaptic active zone is concerned, it has been demonstrated that exosomes released postsynaptically by cortical neurons migrate to the presynaptic side (Chivet et al., 2014), it seems conceivable to conclude that these particles would assure a bidirectional control of synaptic strength at synaptic contacts. We cannot however exclude the possibility that synaptosomal exosomes also target bystander cells, allowing the transfer of selected receptor proteins extrasynaptically, in a cell-cell interaction involving a new form of volume diffusion. Further studies are needed to address these points.

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Figure legends

Figure 1

Structural analysis of purified synaptosomes isolated from the mouse cortex. A) Schematic representation of the discontinuous Percoll gradient isolation procedure of the purified synaptosomes from the mouse cortical homogenate. For further details, see Methods section. B) Ultrastructural analysis of synaptosomes by transmission electron microscopy (TEM). The figure shows an isolated synaptosome, containing multiple vesicles, a mitochondrion and a multivesicular body-like organelle. Scale bar: 200 nm. The image is representative of the TEM analysis of n=5 different synaptosomal preparations. C) Size distribution of synaptosomes assessed by the Zetasizer Nano ZS90 particle sizer. The curve is representative of the analysis of n=5 different synaptosomal preparations. D) Western blot analysis of the exosomal markers TSG101, flotillin-1, CD63 and CD9 in cortical synaptosomal lysates (5 μg/lane). Sintaxin-1 was used as a synaptosomal marker. The image is representative of the analysis of n=6 synaptosomal lysates.

Figure 2

Cortical synaptosomes release extracellular vesicles immunopositive for the exosomal markers TSG101, flotillin-1, CD63 and CD9 and their secretion is increased by depolarization. A) Schematic representation of the isolation procedure of the exosomes from the supernatants of the synaptosomes incubated for 2 min in 3 mM KCl medium (control synaptosomes) or in 25 mM KCl medium (depolarized synaptosomes). For further details, see Methods section. B) Western blot analysis of TSG101, flotillin-1, CD63 and CD9 proteins in the exosomal fractions isolated from the supernatants of control (lane 3 mM KCl) and depolarized synaptosomes (lane 25 mM KCl). The image is representative of the results of n=5 (TSG101), n=6 (CD63) and n=11 (flotillin-1 and CD9) western blot analyses. C) Quantification of the change in TSG101, flotillin-1, CD63 and CD9 proteins in the exosomal fraction from the supernatants of depolarized synaptosomes (25 mM KCl, black bars) when compared to control synaptosomes (3 mM KCl, empty bars). Results are expressed as percent of control (3 mM KCl). Data represent the means ± SEM. $p<0.05$ versus respective control. D) Ultrastructural analysis of control and depolarized cortical synaptosomes by TEM. The figure shows a representative image of synaptosomes incubated for 2 min in 3 mM KCl medium (a) and in 25 mM KCl medium (b). Scale bar: 200 nm. The image is representative of n=5 different synaptosomal preparations.

Figure 3

Characterization of the exosomes released from cortical synaptosomes by light dynamic scattering, flow cytometry and transmission electron microscopy (TEM). A) Size (nM), zeta-potential (mV) and mean count rate (kcps) values detected by Zetasizer Nano ZS90 particle sizer during the analysis of the exosomes isolated from the supernatants of control (3 mM KCl) and depolarized synaptosomes (25 mM KCl). Data represent the means ± SEM of n=5 (control synaptosomes) and n=7 (depolarized synaptosomes) analyses. $p<0.05$ versus the mean count rate value of 3 mM KCl. B) Size distribution of the exosomes assessed by the zetasizer nano ZS90 particle sizer. The curve is representative of the analysis of n=8 exosomal preparations. C) Flow cytometric analysis of TSG101 and CD9 expression in the exosomes purified from the supernatants

of depolarized synaptosomes and coated to latex beads. Images are representative of the analysis of n=5 exosomal preparations. D) Negative-stained exosomes isolated from the supernatants of depolarized synaptosomes observed by TEM. The left image shows the round and smooth morphology of isolated exosomes, the right picture represents an enlargement (squared box) of the original image. The electron micrographs are representative of n=5 exosomal preparations. Scale bar right: 140 nm; scale bar left: 100 nm.

Figure 4

Comparative western blot analysis of selected proteins in the cortical synaptosomal and exosomal lysates. Identical amounts (2 μg proteins/lane) of the cortical synaptosomal lysate (lane Syn) and of the exosomal lysate (lane Exo) were loaded on 10% SDS-PAGE and analysed for the contents of the exosomal markers TSG101, CD63, CD9 and flotillin-1, and of the synaptosomal markers synaptotagmin-1, sintaxin-1a and PSD-95. The image is representative of the western blot analysis on n=5 synaptosomal and exosomal preparations.

Figure 5

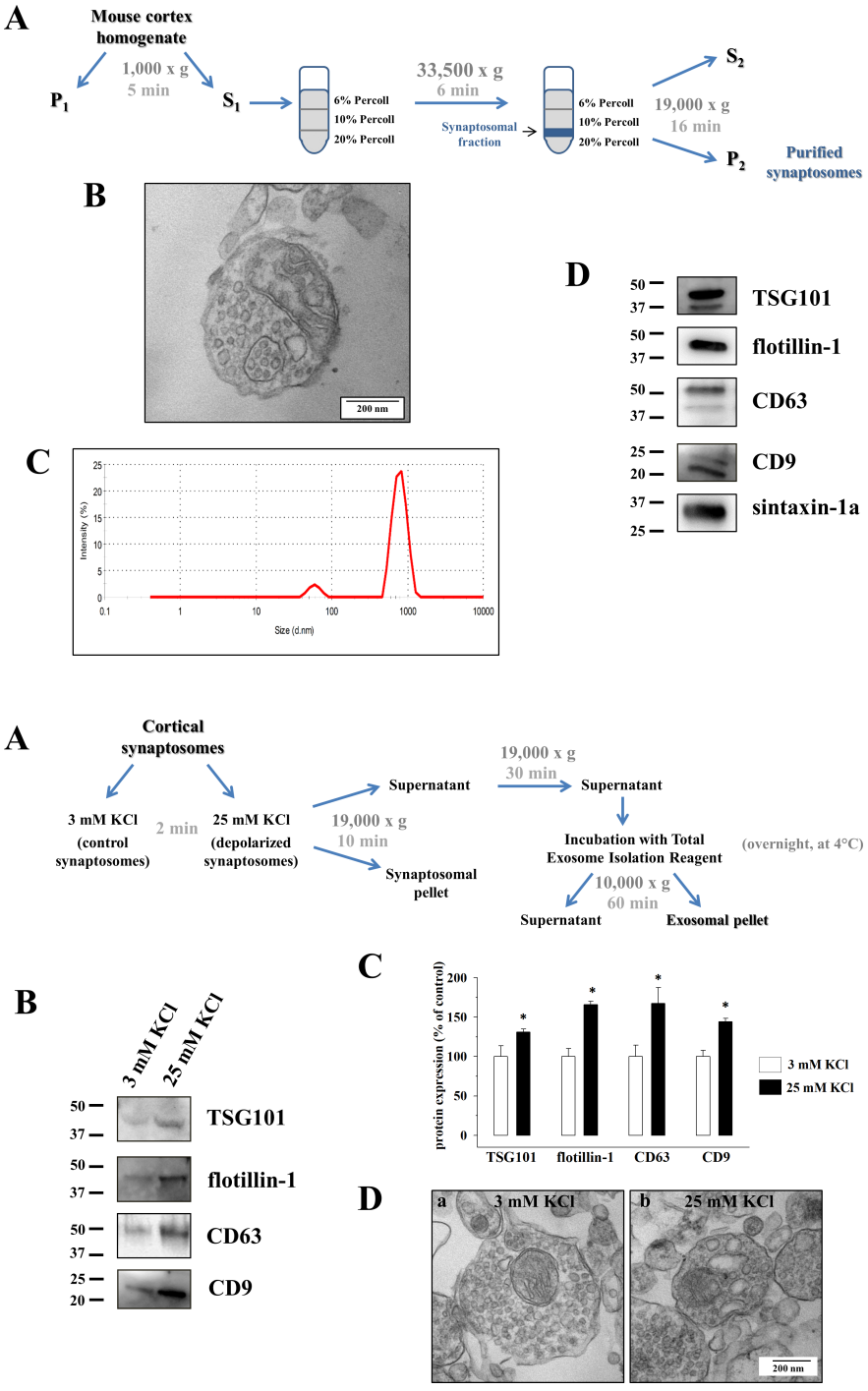
The release of exosomes from cortical synaptosomes is a Ca^{2+} dependent event. Synaptosomes were incubated for 2 min in a 25 mM KCl medium containing 1.2 mM Ca^{2+}ions or lacking Ca^{2+} ions (Ca^{2+} free medium). A) Western blot experiments were carried out to analyse TSG101, flotillin-1, CD63 and CD9 proteins content in the exosomal fractions isolated from the supernatants of the synaptosomes in the two experimental conditions (respectively, lane 25 mM KCl and lane 25 mM KCl / Ca^{2+} free). The image is representative of the results of n=5 (TSG101 and CD63), n=6 (CD9) and n=8 (flotillin-1) western blot analyses. B) Quantification of the change in TSG101, flotillin-1, CD63 and CD9 proteins in the exosomal fraction from the supernatants of the synaptosomes exposed to 25 mM KCl/Ca^{2+} free medium (grey bars) when compared to 25 mM KCl /1.2 mM Ca^{2+} medium (25 mM KCl, black bars). Results are expressed as percent of control (25 mM KCl). Data represent the means ± SEM. $p<0.05$ versus respective control. C) Endogenous glutamate content in the supernatants of the synaptosomes incubated in 3 mM KCl medium (empty bar), 25 mM KCl /1.2 mM Ca^{2+}-containing medium (25 mM KCl, black bar) and 25 mM KCl / Ca^{2+}free medium (grey bar). The endogenous glutamate content is expressed as pmol/mg synaptosomal proteins. Data represent the means ± SEM of the analysis of n=11 samples. * $p<0.05$ versus 3 mM KCl; # $p<0.05$ versus 25 mM KCl. D) Lactate dehydrogenase (LDH) activity in the supernatants of the synaptosomes incubated in 3 mM KCl medium (empty bar), 25 mM KCl /1.2 mM Ca^{2+}-containing medium (25 mM KCl, black bar) and 25 mM KCl / Ca^{2+}free medium (grey bar). The LDH activity in the supernatants is expressed as percent of total LDH activity (LDH activity in the synaptosomal pellets and supernatants). Data represent the means ± SEM of the analysis of n= 5 samples.

Figure 6

The release of exosomes from cortical synaptosomes is not modified by presynaptic $GABA_B$ receptors activation. Synaptosomes were incubated for 2 min in a 25 mM KCl medium in the absence or in the presence of the $GABA_B$ receptor agonist (±)-baclofen (10 μM). A) Endogenous glutamate content in the supernatants of the synaptosomes incubated in 3 mM KCl medium (empty bar), 25 mM KCl medium (black bar) and 25 mM KCl / (±)-baclofen (10 μM) medium (rising right hatched grey bar). The endogenous glutamate content is expressed as pmol /mg synaptosomal proteins. Data represent the means ± SEM of the analysis of n=12 samples. * $p<0.05$ versus control;# $p<0.05$ versus 25 mM KCl. B) Western blot experiments were carried out to analyse TSG101, flotillin-1, CD63 and CD9 proteins content in the exosomal fractions isolated from the supernatants of synaptosomes stimulated with 25 mM KCl alone (lane 25 mM KCl) or in the presence of (±)-baclofen (10 μM) (lane 25 mM KCl / (±)-baclofen (10 μM)). The image is representative of the results of n=5 (TSG101), n=6 (CD63), n=9 (flotillin-1) and n=10 (CD9) western blot analyses. C) Quantification of the change in TSG101, flotillin-1, CD63 and CD9 proteins in the exosomal fraction from the supernatants of the synaptosomes exposed to 25 mM/ (±)-baclofen (10 μM) medium (rising right hatched grey bars) when compared to 25 mM KCl medium (black bars). Results are expressed as percent of control (25 mM KCl).

Figure 7

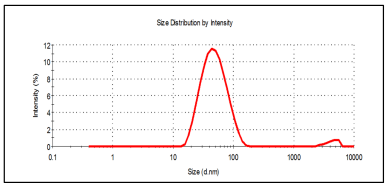
Exosomes released by cortical neurons carry selected glutamate receptors subunit proteins. Identical amounts (2 µg proteins/lane) of the lysates of cortical synaptosomes (lane Syn) and exosomes (lane Exo) were analysed for their content in GluN1, GluN2A and GluN2B NMDA receptor subunits and GluA2/3 AMPA receptor subunits. The image is representative of the western blot analysis on n=5 different synaptosomal and exosomal lysates.



A

	3 mM KCl	25 mM KCl
Size (nm)	50.78 ± 2.10	52.54 ± 1.85
Zeta potential (mV)	-18.03 ± 1.49	-21.46 ± 1.94
Mean count rate (kcps)	66.79 ± 3.12	125.41 ± 6.22 *

B



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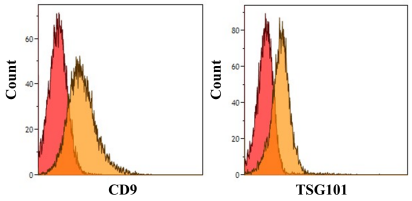

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