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Research Article

Microvesicle and tunneling nanotube mediated intercellular transfer of g-protein coupled receptors in cell cultures

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ABSTRACT

Recent evidence shows that cells exchange collections of signals via microvesicles (MVs) and tunneling nano-tubes (TNTs). In this paper we have investigated whether in cell cultures GPCRs can be transferred by means of MVs and TNTs from a source cell to target cells.

Western blot, transmission electron microscopy and gene expression analyses demonstrate that A_{2A} and D_2 receptors are present in released MVs. In order to further demonstrate the involvement of MVs in cell-to-cell communication we created two populations of cells (HEK293T and COS-7) transiently transfected with D_2R-CFP or A_{2A}R-YFP. These two types of cells were co-cultured, and FRET analysis demonstrated simultaneously positive cells to the D_2R-CFP and A_{2A}R-YFP. Fluorescence microscopy analysis also showed that GPCRs can move from one cell to another also by means of TNTs.

Finally, recipient cells pre-incubated for 24 h with A_{2A}R positive MVs were treated with the adenosine A_{2A} receptor agonist CGS-21680. The significant increase in cAMP accumulation clearly demonstrated that A_{2A}Rs were functionally competent in target cells.

These findings demonstrate that A_{2A} receptors capable of recognizing and decoding extracellular signals can be safely transferred via MVs from source to target cells.

Introduction

In multi-cellular organisms, intercellular communication is of fundamental importance in all physiological and pathological processes, in fact biological functions are performed by complex and organized assemblies of cells whose integrated actions are based on inter-cellular communication processes. Usually, intercellular communication is achieved via either electrical signals or the release of specific signals (soluble molecules) by a source cell in the surrounding environment and the recognition/decoding of these messages by their corresponding cognate receptors in the target cell.

It has been recently demonstrated that cells can exchange collections of signals using more complex structures such as Tunneling Nano-Tubes (TNTs) and microvesicles (MVs) [1,2].

TNTs are structures involved in the intercellular communication, which have been discovered by means of in vitro studies [3]. TNTs

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have a diameter of 50–200 nm and a length up to several cell diameters. These transcellular channels could lead to the formation of syncytial cellular networks [3–6]. They have been identified in a variety of cultured cell systems, including immune (for example T cells and macrophages), kidney (HEK293T), neuronal (PC12) and human glioblastoma (U87MG) cells [7]. Several in vitro studies demonstrated that these structures make possible the exchange of molecules, proteins, and whole organelles between cells [8,9].

Despite MVs have long been regarded as cellular debris, in the last years this view is rapidly changing toward a reconsideration for an important role of exocytic vesicles in cellular communication [10–12]. Two modes of microvesicles release have been characterized: the exocytosis of internal luminal vesicles formed in the multivesicular bodies (MVBs) and the direct budding of small vesicles from the plasma membrane. Exosomes represent a specific subclass of such membrane vesicles that are released by cells upon fusion of MVBs with the plasma membrane. In contrast to the fate of the proteins trafficked for degradation to the lysosomal system, secreted exosomes are biologically active entities that are important for a variety of pathways, particularly in the immune system [13–16]. Exosomes can be taken up by other cells and modulate the activity of recipient cells in vitro [17–19] and in vivo [15,20,21]. Exosomes are characterized by a diameter ranging from 30 to 100 nm and bear specific protein markers such as endosomal–lysosomal sorting proteins (Alix, Tsg101), tetraspanins (CD63, CD9, CD81) and heat-shock proteins (Hsp70 and Hsp90) [22–25].

On the contrary, shedding vesicles originate from the outward blebbing of the cellular plasma membrane [10]. These secreted vesicles may range in sizes between 100 and 1000 nm in diameter and show a prominent exposure of phosphatidylserine residues on their outer surface. As far as cargo proteins are concerned, shedding vesicles are characterized by metallo-proteinases and integrins [26]. In the present paper the term microvesicles will be used to collectively indicate both exosomes as well as shedding vesicles.

MVs have a high content in protein and about 350–400 different proteins have been characterized by means of proteomic analyses [27,28]. Among these, MVs contain a collection of peripheral membrane proteins such as MHC I and II, integrins, transferrin receptors and tetraspanins [29] which can activate downstream signaling pathways in target cells, triggering for instance calcium signaling [30], MAPK activation [31], or NKG2D signaling [32].

MVs can also convey important lipid molecules such as sphingomyelins, cholesterol, phosphatidylserine, lyso-phosphatidylcholine, prostaglandins, etc., depending on the donor cell type. Furthermore, it has been shown that lipid rafts play a crucial role in MVs generation, as a matter of fact, raft-domains are incorporated in reticulocytes and B lymphocytes exosomes [33]. Many studies show that tetraspanins are highly enriched in exosomes and lipid composition analyses of B cell exosomes have highlighted high levels of cholesterol, sphingomyelin and glycolipid GM3 [24,34]. All these molecules have been found in a raft like microdomains and can actively interact with peripheral G-protein coupled receptors (GPCRs) [35–37].

These findings have recently been extended by the detection of functional mRNA and microRNA species in certain types of MVs [2,38]. These data open the possibility that cells can alter the expression of genes in neighboring and distant cells by transferring genetic information [39]; after entering the target cell, the MV RNA can reprogram the cell phenotype and confer acquisition of specific features of the donor cell [40,41].

Therefore MVs behave as 'safe containers' of signal units allowing a 'private' intercellular transfer of information [11].

As discussed by Smalheiser [22] as well as by our group [11,42] exosomal transfer of proteins and RNAs may be a fundamental mode of communication in multi-cellular organisms, in particular within the Central Nervous System (CNS).

Microvesicles are important players in intercellular signaling processes in view of their ability of transferring signalosome components to target cells [43]. However, investigations on functional implications of this mode of communication in the CNS are still in its infancy.

Since GPCRs are key molecules in decoding neurotransmitters information and previous studies have demonstrated that MVs carry some G proteins [44], we have investigated whether MVs may transport functionally competent (GPCRs) from a donor to a target cell. Thus, we studied the ability of target cells to recognize and decode signals by means of receptors that they did not previously express.

Materials and methods

Plasmid constructs

The constructs presented here were made using standard molecular biology techniques employing PCR and fragment replacement strategies. The cDNA encoding dog adenosine A_{2A} and human D_2 receptor without its stop codons were subcloned in pEYFP-N1 (A_{2A}R-YFP) and pECFP-N1 (D_2R-CFP) (Clontech), respectively. The cDNA encoding the N-terminal tagged hemagglutinin dog adenosine A_{2A}R (HA-A_{2A}R) was kindly provided by Dr. Mark E. Olah, Duke University Medical Center, North Carolina, USA.

Cell culture, transfection and microvesicle isolation

U87MG, COS-7 or HEK293T cells (American Type Culture Collection, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (FBS) at 37 °C and in an atmosphere of 5% CO_2. COS-7 or HEK293T cells were transiently transfected using TransIT-LT1 Transfection Reagent (Mirus Bio LLC). For microvesicles isolation, FBS was previously centrifuged overnight at 4 °C and 110,000 *g* using a SW28 rotor in a Beckman ultracentrifuge; the supernatant was carefully removed with a pipette, passed through a 0.22 μm filter and then added to culture medium. Conditioned medium from 5×10^7 cells was collected after 24 h. Microvesicles were purified by differential centrifugation at 4 °C, starting with a centrifugation at 1000 ×*g* (15 min) and followed by centrifugations at 12,000 ×*g* for (20 min), 18,000–20,000 ×*g* (20 min) and 100,000 ×*g* (70 min). The resulting microvesicles pellets were washed in 13 ml phosphate buffered saline (PBS), filtered through a 0.22 μm filter and then collected again by ultracentrifugation at 1100,000 ×*g* (70 min) and resuspended in PBS.

Western blotting analysis

For SDS-PAGE, samples containing 30 μg of protein were mixed with Laemmli sample buffer (1:1 ratio [45]) and loaded onto 12% SDS-PAGE gels. Subsequently, proteins were transferred to a nitrocellulose

membrane (GE Healthcare). Primary used antibodies were: Tsg101 (1:2000 dilution, clone 4A10 Abcam), Calnexin (1:2000 dilution, ab13504 Abcam), HA (1:5000 dilution, ab9110 Abcam), Rab5 (1:500 dilution, R4654 Sigma-Aldrich). Primary antibodies were incubated overnight at 4 °C, followed by washing and the application of secondary HRP-conjugated antibody (Pierce). Immune complexes were visualized using the Supersignal Dura reagent (Pierce).

Transmission electron microscopy (TEM) and immunogold labeling

Microvesicles isolated by differential centrifugation from conditioned media of transiently transfected HEK293T cells were loaded on a Formvar/carbon-coated grid (Agar Scientific Ltd.) and fixed with 2% paraformaldehyde in PBS for 5 min. The exosome-loaded grids were then washed three times with PBS and then three times with PBS containing 1% BSA before incubation with primary antibody for 2 h (1/50 in normal serum 2% + 0.01% Triton X-100 in PBS).

The antibodies used were mouse anti HA (Santa Cruz Biotechnology, Milano) for $A_{2A}R$ and rabbit anti D_2R (kindly provided by Dr. Watson). The grids were then washed twice in PBS and twice in BSA buffer and labeled with 10 nm of colloidal gold goat anti-mouse and 15 nm of colloidal gold goat anti-rabbit at 1/50 in 2% normal serum PBS for 1 h. Grids were washed three times in PBS, twice in double-distilled water. Grids were stained with 1% uranyl acetate for 3 min and viewed on a JEM-2010 computer-controlled high-contrast 200 kV transmission electron microscope associated with a Telecamera Slow Scan CCD Gatan 694. 3000× images were collected.

mRNA detection from released microvesicles

Total RNA was isolated from purified microvesicles using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Thereafter, the presence of A_{2A} and D_2 receptor mRNAs from purified microvesicles was evaluated by RT-PCR. Total RNA treated with DNaseI (about 100 ng, Ambion) was reverse transcribed using Sensiscript RT Kit (Qiagen) and random primers (Promega), according to the manufacturer's instructions.

PCR specific amplification was carried out using the following primers: $A_{2A}R$ receptor (Forward: 5′-CTTGGGTTCTGAGGAAGCAG-3′ and Reverse: 5′-TGCTCTGTGGAGACAAGGTG-3′); D_2R (Forward: 5′-CTGCAGACCACCACCAACTA-3′ and Reverse: 5′-CGTCCAGAGTGACGAAGATGT-3′). RT-PCR amplifications were conducted using HotStarTaq Master Mix Kit (Qiagen) according to the manufacturer's instructions, with 300 nM primers and 2 μl of cDNA in a 20 μl final reaction volume. Thermocycling was conducted using a PTC-200 (MJ Research) initiated by a 10 min incubation at 95 °C, followed by 40 cycles (95 °C for 5 s; 60 °C for 5 s; 72 °C for 10 s). The specificity of the amplification products obtained was confirmed by examining sample separation in a 3% agarose gel.

Microvesicle visualization by confocal microscopy

Microscopic observations were made by means of a Leica TCS SP2 (Leica Microsystems, Mannheim, Germany) confocal multiband scanning laser equipment with AOBS system adapted to an inverted Leica DM IRE2 microscope interfaced with an argon-kripton laser setted at a power of 8 mW, 488 nm. To minimize the noise and to keep a low photobleaching rate, it was selected an acquisition time of 1 s per scan and averaged 8 scans to produce each 1024×1024 pixel image. All images were recorded with a HCX PL APO 63× objective, zoom 2–8.

FRET-based acceptor photo-bleaching experiments analyzed by confocal microscopy

HEK293T cells were transiently transfected with the plasmid cDNA encoding the D_2 receptor-CFP (D_2R-CFP) or A_{2A} receptor-YFP ($A_{2A}R$-YFP). Twenty-four hours after transfection, the two different cell populations were co-cultured for 24 h, fixed and analyzed by confocal laser scanning microscopy. The same protocol was performed using COS-7 cells. Moreover, a co-culture of COS-7 cells transiently transfected with the $A_{2A}R$-YFP or D_2R-CFP plasmids was set up maintaining the two cell populations physically separated by means of a 0.4 μm pore size membrane. Twenty four hours after cell interaction, the upper well, containing the cells transfected with $A_{2A}R$-YFP was removed and the cells at the bottom of the plate were fixed and analyzed.

In some set of experiments, two inhibitors of MVs release, 2.5 mM methyl-β-cyclodextrin (MβCD) and 10 μM GW4869 (specific inhibitor of neutral sphingomyelinase) were added to the co-cultures for 24 h.

Confocal laser scanning microscopy was performed using a Leica Multiband TCS SP2 microscope (Leica Microsystems, Mannheim, Germany) equipped with an acoustic-optical beam splitter (AOBS) and a 100-milliwatt argon laser for excitation from 458 nm to 514 nm. CFP was excited with the 458 nm laser, YFP was excited with the 514 nm laser, and images were acquired in the following sequence:

(I) A pre-photo-bleach YFP (acceptor) image was acquired by scanning while exciting with the 514 nm laser line. (II) A pre-photo-bleach CFP (donor) image was acquired by scanning while exciting with 458 nm laser line. (III) A region of interest was selected and the acceptor (YFP) was subsequently photo-bleached by scanning repeatedly with the 514 nm laser line until fluorescence signals were reduced of 70%. (IV) A post photo-bleach image for CFP was acquired by scanning with the 458 nm laser line. (V) A post-photo-bleach image for YFP was acquired by scanning with the 514 nm laser.

Hardware parameters listed in the table reported in Fig. 3 were identical in all experiments. FRET efficiency was calculated automatically by the LCS 2.61.1537 Leica software FRET Acceptor Bleaching application using the reported formula: $FRETEff = \frac{Dpost - Dpre}{Dpost}$; for all $Dpost > Dpre$; where *Dpost* is the fluorescence intensity of the Donor after acceptor photo bleaching and *Dpre* the fluorescence intensity of the Donor before acceptor photo bleaching. An average FRET efficiency of 9 cells was calculated for cells expressing CFP and YFP and cells expressing CFP or YFP alone as negative controls.

cAMP assay

cAMP was measured in COS-7 and HEK293T cells exposed for 24 h to purified HA-A_{2A} MVs or MVs harvested from untransfected cells (control cells) using the Direct cAMP ELISA kit (Stressgen) according to the manufacturer's instructions. Cells were trypsinized and washed twice before cAMP assay.

In a set of experiments, cells incubated with MVs positive for HA-A_{2A} receptor in the presence of cycloheximide (20 μg/ml) for 24 h were used.

CGS-21680 at the concentration of 200 nM for 2 h was used as the specific agonist of A_{2A} receptors.

Statistical analysis

Data were analyzed for significance by analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons and Student's *t*-test. The statistical analysis was carried out using the SPSS 13.0 software (SPSS Inc., Chicago, USA).

Results

Microvesicles are carriers of the A_{2A} and D_2 GPCRs

In the present paper, we investigated whether MVs can transport functionally competent GPCRs from a donor to a target cell. Firstly, we tested the ability of GPCRs to segregate in released MVs; for this purpose HEK293T and COS-7 cells were transfected with the plasmid HA-A_{2A} which encodes for the fusion protein hemagglutinin A_{2A} receptor. Twenty-four hours after transfection the MVs released into a culture medium were harvested by a serial centrifugation procedure and subsequently characterized by western blotting using antibodies against well-defined markers such as Tsg101 and Rab5A [46]. By contrast, Calnexin was not detected in the preparations demonstrating the presence of MVs in the purified samples without contaminations by endoplasmic reticulum proteins. In these MVs, western blot analysis showed also the presence of the GPCR A_{2A}R corroborating the hypothesis of an intercellular transfer of GPCRs (Fig. 1A). In order to further demonstrate that MVs can be the vehicle of signal units, like the heterodimer A_{2A} and D_2 receptors, HEK293T and COS-7 cells were co-transfected with the plasmids HA-A_{2A} and D_2R. Using TEM analysis combined with immunogold labeling it was possible to directly visualize the presence of HA-A_{2A} (10 nm diameter size particles) and D_2 receptors (15 nm diameter size particle) in the same MV (Fig. 1B). Furthermore, mRNAs coding for A_{2A} and D_2 receptors were identified in the MVs released by co-transfected HEK293T and COS-7 cells, and more interesting we could detect the presence of the A_{2A} mRNA in MVs produced by

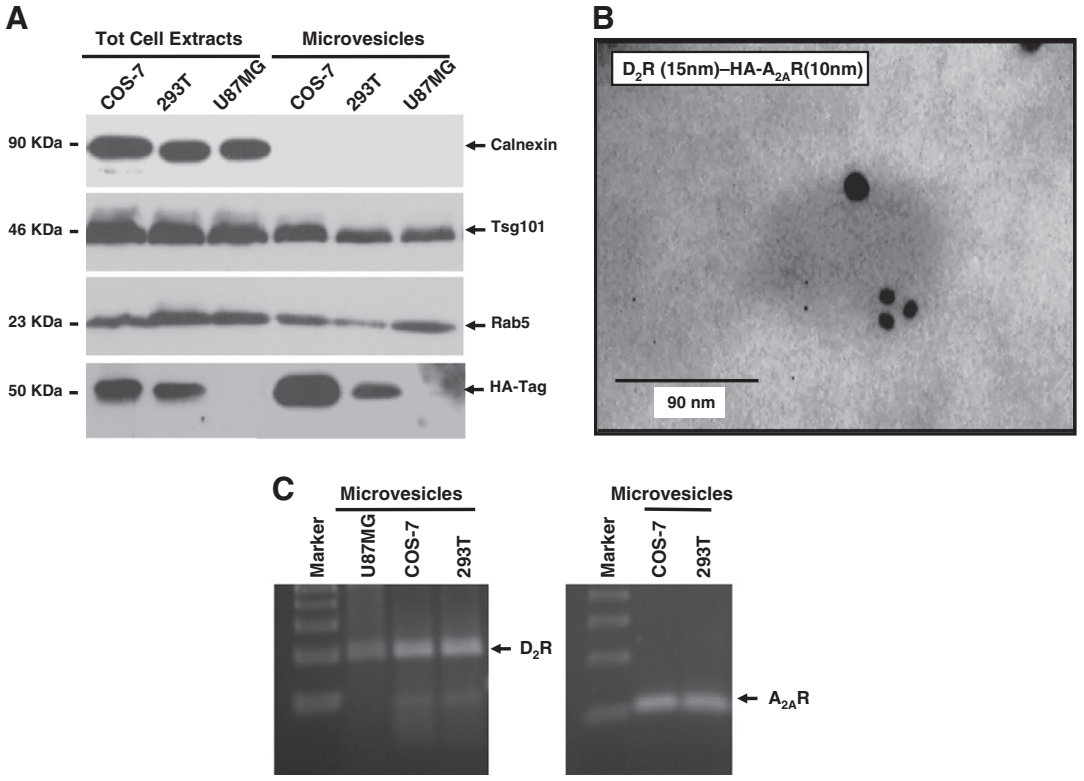


Fig. 1 – Transfer of GPCRs via microvesicles. A) Western Blot analysis of microvesicles isolated from 24 h conditioned medium of HEK293T and COS-7 transfected with A_{2A} receptor and glioblastoma (U87MG) cells. HEK293T and COS-7 cells were transfected with the plasmidic DNA coding for hemagglutinin (HA)-tagged dog adenosine A_{2A} receptor (HA-A_{2A}). Microvesicles and whole cellular lysate proteins from HEK293T and COS-7 transfected with HA-A_{2A} receptor and U87MG cells were separated on SDS-PAGE and electroblotted to nitrocellulose membrane. Blots were probed with antibodies against Calnexin (a marker of endoplasmic reticulum), Tsg101 and Rab5A (markers of exosomes) and HA epitope (HA-A_{2A} receptor). Molecular mass of each marker is shown in the left. B) TEM image of microvesicles released from HEK293T cells co-transfected with HA-A_{2A} and D_2 receptors. Microvesicles were isolated from HEK293T cell co-transfected with HA-A_{2A} and D_2 receptors by 110,000 *g* centrifugation of culture media and, after purification, loaded on a Formvar/carbon-coated grid. Grids were processed for D_2-A_{2A} immunogold staining (15 and 10 nm respectively), stained with 1% uranyl acetate and viewed on a JEM-2010 computer-controlled high-contrast 200 kV TEM associated with a Slow Scan CCD camera (Gatan 694; 3000× images were collected). C) Electrophoretic separation on agarose gel of the amplification products of the A_{2A} and D_2 receptor cDNA from MVs released by Glioblastoma cells, and HEK293T or COS-7 cells co-transfected with A_{2A} and D_2 receptors.

Glioblastoma cells (Fig. 1C). Altogether, these data demonstrate that not only some GPCRs can be accumulated in released MVs as proteins but also mRNAs coding for those GPCRs can be present in detectable amounts.

Transfer of the GPCRs among cultured cells

The evidence that GPCRs are present in MVs is a necessary but not sufficient condition to demonstrate that these proteins can be transferred to neighboring cells. As a matter of fact, MVs could be simply a mechanism through which cells discard GPCRs. In order to demonstrate the involvement of MVs in cell-to-cell communication and GPCRs transfer to target cells, the interaction of labeled MVs obtained from transfected cells with Glioblastoma cells has been evaluated. As reported in Fig. 2, fluorescence confocal microscopy analysis showed that labeled MVs bind to the glioblastoma cell surface.

Obviously, a key question to be answered was whether these GPCRs were incorporated in the plasma membrane of the recipient cells and if they were functionally competent.

The first question was investigated by means of the fluorescence resonance energy transfer (FRET) technology, a widely used methodology to study protein–protein interactions, in particular, GPCRs hetero-dimer formation [47].

We created two populations of cells transiently transfected with a plasmid cDNA encoding the D_2R-CFP or with A_{2A}R-YFP, respectively. The day after transfection, the two cell populations were put in co-culture and allowed to interact. Cells will be positive to the FRET analysis only if the two fluorophores closely colocalize (below a distance of about 10 nm). In the case of FRET signaling exchange of D_2R-CFP and/or A_{2A}R-YFP between the two populations of cells has occurred and, in addition, the transferred GPCRs are colocalized, likely forming a hetero-dimer.

Fig. 3 shows the results obtained after one day of co-culture of D_2R-CFP transfected HEK293T cells with A_{2A}R-YFP transfected HEK293T cells. In cells positive for the two fluorophores the FRET signals have been evaluated in two regions (ROI 1 and ROI 2), which showed a FRET efficiency of about 18% and 20%, respectively. These data clearly demonstrated the simultaneously presence of the D_2R-CFP and A_{2A}R-YFP in cells that had the genetic information to code only one of these GPCRs. This evidence gives a strong support to our hypothesis about the transfer of GPCRs among cells.

GPCRs are exchanged by means of MVs and TNTs

The experiments based on FRET assay shed light on the transfer of membrane receptors among cells but these did not deal with the carriers responsible for the transport, even if our data on GPCR presence in released MVs strongly suggested their involvement in this process. However, other types of intercellular communication could be involved as well, in particular TNTs. As a matter of fact, fluorescence confocal microscopy analysis showed that COS-7 cells can make TNTs connecting A_{2A}R-YFP labeled cells with D_2R-CFP positive cells. Notably, the TNT showed a marked stain for YFP demonstrating that A_{2A}R can migrate along this tubular structure and more interestingly we found FRET positive signals in the region of contact between the two cells (Fig. 4; ROI 2 areas). This finding clearly showed that GPCRs can move from one cell to another by means of TNTs. Thus, the involvement of MVs in the shuttling of GPCRs remained to be demonstrated. In order to clarify this issue, we set up an alternative experimental model in which the day after transfection the two cell populations (one transfected with A_{2A}R-YFP and the others with D_2R-CFP) were grown in the same well but the physical contact among cells was prevented by a 0.4 μm pore size membrane (Fig. 5A). Although, in this experimental system the formation of TNTs between A_{2A}R-YFP and D_2R-CFP labeled cells was blocked, we were still able to find cells showing FRET signals (Fig. 5B–F). These results strongly support the notion that GPCRs can be intercellularly transferred via MVs.

Furthermore, two known inhibitors of MVs release were tested for their effects on GPCR exchange in the presence of cellular separation by means of membrane. These were methyl-β-cyclodextrin

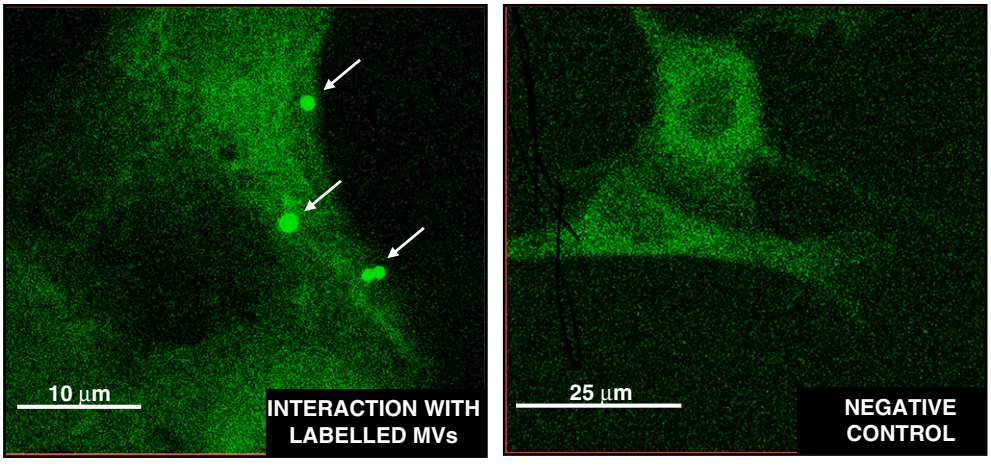
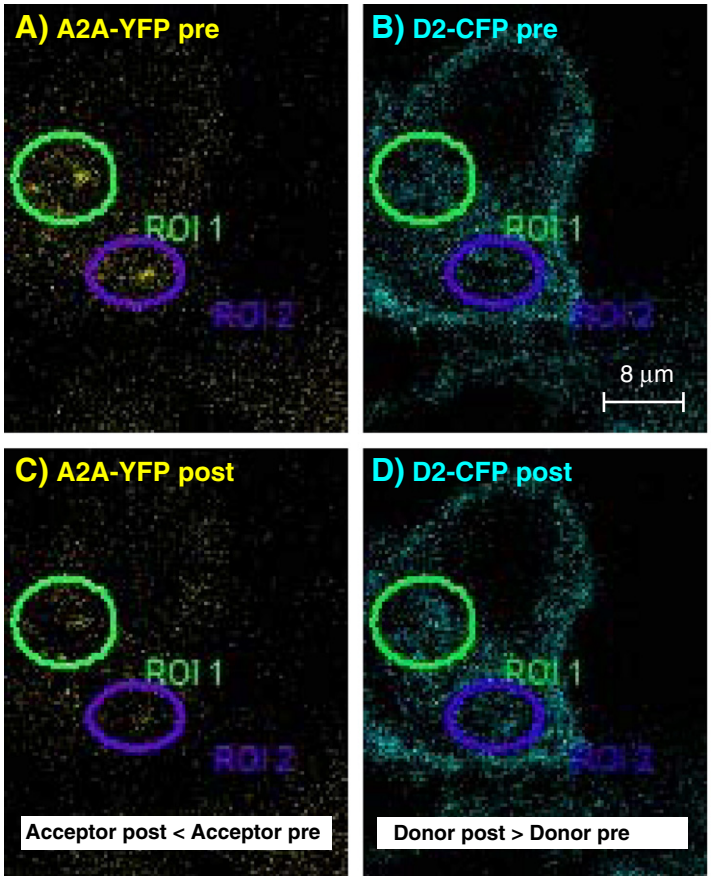
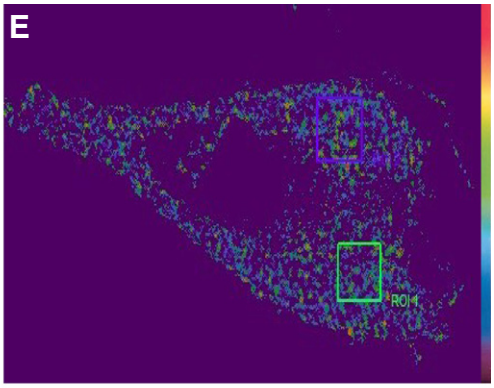


Fig. 2 – Confocal microscopic analysis of Glioblastoma cells incubated with MVs prepared from HEK293T cells. Microvesicles were labeled by the over-expression of the fusion protein Rab5a-GFP in HEK293T cells. Rab5a has been chosen because it was detected both in endosomes and in exosomes. The released MVs were harvested from 24 h culture medium of transfected cells, purified by serial centrifugations, and incubated with Glioblastoma cells. Left panel: Glioblastoma cells allowed to interact with GFP labeled MVs for 6 h. Right panel: control Glioblastoma cells. The arrows indicate the labeled MVs interacting with the surface of Glioblastoma cells.





$$FRET_{Eff=}\frac{D_{post}-D_{pre}}{D_{post}} \quad \text{for all } Dpost > Dpre$$

D2-CFP : DONOR (458 nm)

A2A-YFP : ACCEPTOR (514 nm)

	Donor- acceptor labelled cell		Donor labelled cell used as negative control	
ROI	**ROI_1**	**ROI_2**	**ROI_1**	**ROI_2**
Donor pre	34.57	76.06	14.44	12.47
Donor post	45.19	93.05	14.75	12.67
Acceptor pre	22.64	19.26	8.72	7.22
Acceptor post	13.19	10.19	4.28	4.34
FRETeff (%)	23.50	18.66	2.08	1.56

Fig. 3 – FRET acceptor photo-bleaching analysis of HEK293T co-cultures. HEK293T cells were transiently transfected either with the cDNA encoding the D_2 receptor-CFP (D_2R-CFP) or A_{2A} receptor-YFP (A_{2A}R-YFP). Twenty-four hours after transfection, the two different cell populations were co-cultured for 24 h, fixed and analyzed by confocal laser scanning microscopy. A) pre-photo-bleach YFP (acceptor) image was acquired by scanning while exciting with the 514 nm laser line; B) pre-photo-bleach CFP (donor) image was acquired by scanning while exciting with 458 nm laser line; C) Two regions of interest (ROI 1 and ROI 2) were selected and the acceptor (YFP) was subsequently photo-bleached by scanning repeatedly with the 514 nm laser line until fluorescence signals were reduced of 70%. This resulted in a decrease of fluorescence signals corresponding to the acceptor (YFP). D) post photo-bleach image for CFP was acquired by scanning with the 458 nm laser line after bleaching of the acceptor (YFP). In this case donor (CFP) fluorescence signals increase due to the lack of fluorescence energy absorption by the acceptor (YFP). E) Representation of FRET efficiency in cells labeled only with D_2-CFP, used as negative control. FRET efficiency was calculated automatically by the LCS 2.61.1537. Leica software Fret Acceptor Bleaching application using the reported formula:

$$FRETEff = \frac{Dpost-Dpre}{Dpost} \text{ forall} Dpost > Dpre.$$

FRET signals demonstrate the exchange of GPCRs between the cells, since after co-culture A_{2A} and D_2 receptors are present as a heterodimer in the same cell.

(MβCD; 2.5 mM), used to disrupt lipid rafts by removing cholesterol from membranes [48], and GW4869 (10 µM) a specific inhibitor of neutral sphingomyelinase [49]. After inhibitor treatments, we were not able to detect cells positive for the two fluorophores, consequently, the FRET signals were similar to negative controls (Fig. 6A). These results demonstrated that cells can transfer GPCRs using MVs as carriers in addition to TNTs.

Transferred GPCRs are functionally competent

Finally, it has been investigated whether these receptors, in particular A_{2A} receptors, were functionally competent after the delivery to the acceptor cells. Thus, we harvested the MVs released from COS-7 or HEK293T cells transfected with the fusion protein hemagglutinin A_{2A} receptor, then the purified MVs were incubated with untransfected cells. Co-culture of recipient cells with MVs harvested from untransfected cells was used as negative control. After 24 h from interaction, these cells were stimulated for 2 h using 200 nM CGS-21680, a potent adenosine A_{2A} receptor agonist, and the amount of cAMP was evaluated. Fig. 6B shows that CGS treatment causes a significant higher accumulation of cAMP in cells incubated with MVs carrying HA-A_{2A} receptor (as demonstrated in Fig. 1) than in control cells.

Furthermore we investigated if HA-A_{2A} receptors expressed within the recipient cells might be translated from the corresponding mRNA rather than directly derived from proteins transported by

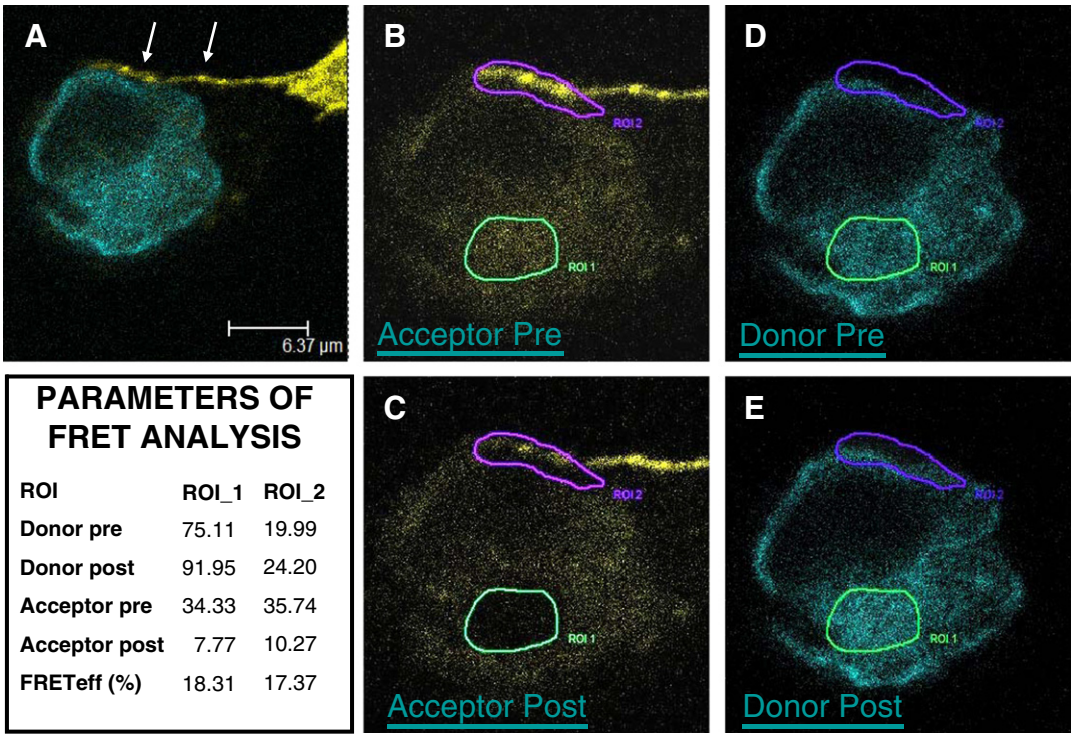


Fig. 4 – FRET analysis of co-cultures of the COS-7 cells transfected with A_{2A} R-YFP or D_2R-CFP. COS-7 cells were transiently transfected either with the A_{2A}R-YFP or with the D_2R-CFP plasmids. The day after transfection, the two cell populations were co-cultured and allowed to interact for 24 h. Confocal laser scanning microscopy analysis was conducted as reported in Fig. 3. A) In the panel A it is shown a TNT that lengthens from a A_{2A}R-YFP stained cell to a D_2R-CFP positive cell (indicated by arrows). B, C, D, E) FRET acceptor photo-bleaching analysis showed a FRET efficiency of about 17% in the region of contact (ROI_2 area; for more details on the FRET analysis see the legend of Fig. 3). These data clearly show that GPCRs can move from one cell to another by means of TNT structures.

the MVs. To approach this point, recipient cells were incubated with HA-A_{2A} MVs for 24 h in the presence of 20 µg/ml cycloheximide (a potent protein synthesis inhibitor). Thereafter, cells were treated with CGS 200 nM for 2 h. cAMP assays highlighted that, when mRNA translation was inhibited, the recipient cells showed a reduced response to CGS stimulation suggesting that fusion protein HA-A_{2A} receptor could be principally transferred in the form of mRNA that could be translated in the recipient cells (Fig. 6C).

Discussion

As pointed out in the Introduction and discussed by Smalheiser [22] as well as by our group [11,42] the release of MVs containing proteins and RNAs may be a fundamental mode of communication in multi-cellular organisms, in particular within CNS.

As a matter of fact MVs quantitatively and qualitatively complement classical methods of intercellular communication such as direct secretion of signaling molecules at synaptic and extra-synaptic level, physical interaction of membrane proteins and involvement of gap junctions. In view of its peculiar features, the MVs mediated transfer of a set of signals has been called the "Roamer Type of Volume Transmission" since the MVs are like the roamer's bag filled up with important materials [11,50]. Several papers have demonstrated that MVs can transfer proteins, different RNA and DNA from cell to cell [31,38,51]. In line with this, it has been reported that astrocytes [52], microglial cells [53], oligodendrocytes [54], and developing neurons [23,55] release exosomes.

Recently, Lachenal and Collaborators [56] have demonstrated that fully differentiated cortical and hippocampal neurons in cultures release exosomes, suggesting that CNS cells could release this class of microvesicles also in vivo. In agreement with such a view exosomes have been isolated from the cerebral spinal fluid [57]. Furthermore, Lachenal and Collaborators provided evidence that this exosomal secretion by neurons is regulated by the calcium influx and by glutamatergic activity [56] pointing to a possible modulator role of exosomes in synaptic function. Thus, Lachenal and Collaborators suggested that the presence of GluR2 subunits in neuronal exosomes may be part of a mechanism for the local elimination of receptors at synapses undergoing plastic changes [56]. However, we could also speculate that MV release is not simply an elimination of molecules at a synaptic level where they are in excess, but it might be thought as a process of redistribution of receptors occurring, for instance, among synapses belonging to the same synaptic cluster [58–60]. This could represent an important and economically advantageous mechanism for the proper concerted tuning of the efficacies of various synapses at a synaptic cluster level.

The present data demonstrating that GPCRs are transferred from cell to cell via MVs and/or TNTs give an indirect support for such a view. Thus, biochemical systems capable of recognizing and decoding extracellular signals such as neurotransmitters and

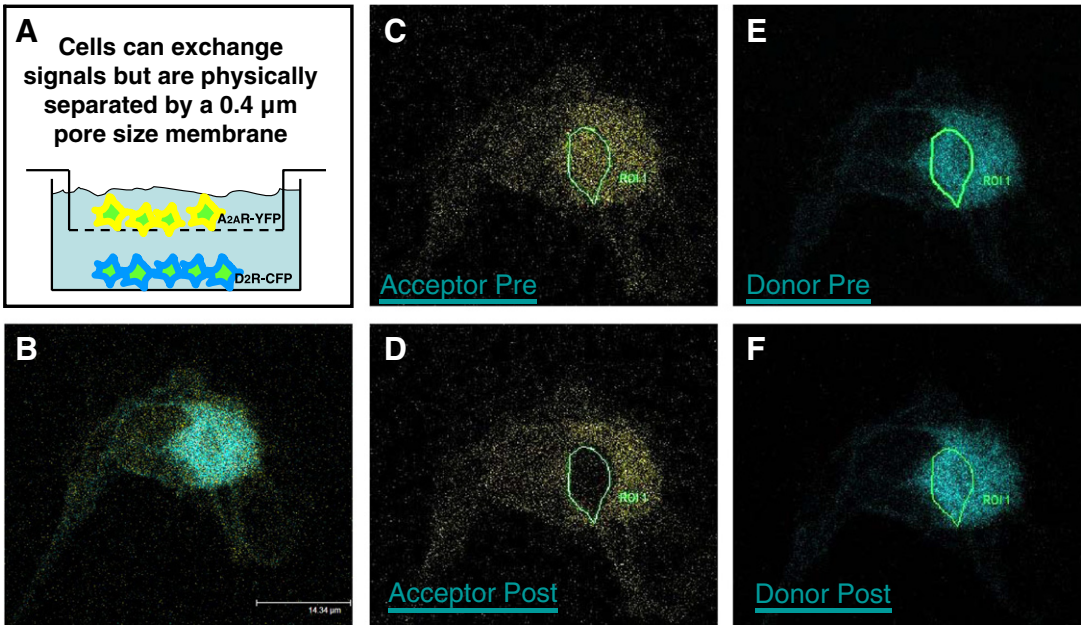


Fig. 5 – Transfer of GPCRs in co-cultures of COS-7 cells separated by a membrane. COS-7 cells were transiently transfected either with the A_{2A}R-YFP or with the D_2R-CFP plasmids. The day after transfection, the two cell populations were put into a co-culture and allowed to interact for another 24 h but, in this experiment set up, the two cells populations were maintained physically separated by means of a 0.4 µm pore size membrane. A) Schematic representation of the interaction model used. At the bottom of the well there are the COS-7 cells transfected with the D_2R-CFP plasmid, while the cells seed above the 0.4 µm pore size membranes were transfected with the A_{2A}R-YFP plasmid. Twenty-four hours after cell interaction, the upper well, containing the cells transfected with A_{2A}R-YFP was removed and the cells at the bottom of the plate were fixed and analyzed. B) The analysis of the obtained cells shows an extensive labeling for D_2R-CFP with few signals of A_{2A}R-YFP. C, D, E and F) FRET acceptor photo-bleaching analysis showed a FRET efficiency of about 17% (ROI_1 area of analysis; for more details on the FRET analysis see the legend of Fig. 3). These data demonstrate that GPCRs can be exchanged among cells even in the absence of physical interactions supporting the hypothesis that MVs could be the carriers for this type of cellular communication.

hormones can be safely transferred via MVs and/or TNTs from a source cell to target cells. Data were also provided demonstrating that the transferred mRNAs coding for A_{2A} receptor and/or the corresponding proteins are functionally competent.

This evidence that GPCRs could be transported via MVs to recipient cells mainly in the form of mRNA adds a further level of plasticity. In fact, it can be surmised that a recipient cell, after receiving the MV cargo, can actively regulate the translation process of exogen mRNA hence acquiring the ability to respond to a defined neurotransmitter.

Thus, thanks to this process of intercellular communication, target cells not only could acquire the capability to transiently recognize and decode signals by means of receptors that they don't express but also, as pointed out above, modulate the density of their receptors in such a way that the concerted tuning of synaptic efficacies in a restricted brain volume can take place.

This aspect can be further discussed. Fang, indeed, proposed [61] that proteins which exhibit higher order oligomerization (e.g., Receptor Mosaics, see Agnati et al. [62]) and which are associated with the cholesterol-rich domains of the plasma membrane (lipid rafts) are preferentially stored in exosomes. This view is indirectly supported by our data showing that not simply A_{2A}R or D_2R is transferred via MVs but possibly also the A_{2A}R-D_2R heteromer. It could be even surmised that higher-order oligomers, supposed to play an important role in learning and memory, might be transferred among cells [63,64].

These data and speculations are in line with the Smalheiser proposal that exosomes may play many different roles at synaptic level during development as well as in the mature brain [22]. All the roles mentioned by Smalheiser are directly or indirectly related to the synaptic efficacy and hence may play a fundamental role in learning and memory processes [22].

It should also be considered exosomes as an important glia-neuronal communication mode [52,65] hence allowing powerful even if not rapid signal transfer in complex cellular networks of the CNS via volume transmission [66].

It can be also surmised that cells (e.g., astrocytes) may release MVs containing GPCRs that transiently modify the decoding capability of some neurons, inducing in the network they belong to a different elaboration of the information. This feature enlarges the functional meaning of the concept of 'polymorphic networks' that is of the plastic reconfiguration of a neural network to produce different outputs [66–68]. Thus, from a single neuronal network a large number of different functional circuits can be created by changing via the Roamer Type of volume transmission the integrative capabilities of some neurons that can become transiently capable of decoding some, otherwise undetectable, signals.

Future basic aspects in the investigations of the MVs-mediated volume transmission are to clarify the specificity of the mechanism according to which the Roamer Type of VT works that is the mechanisms involved in the selective targeting and uptake of certain MVs into specific recipient cells.

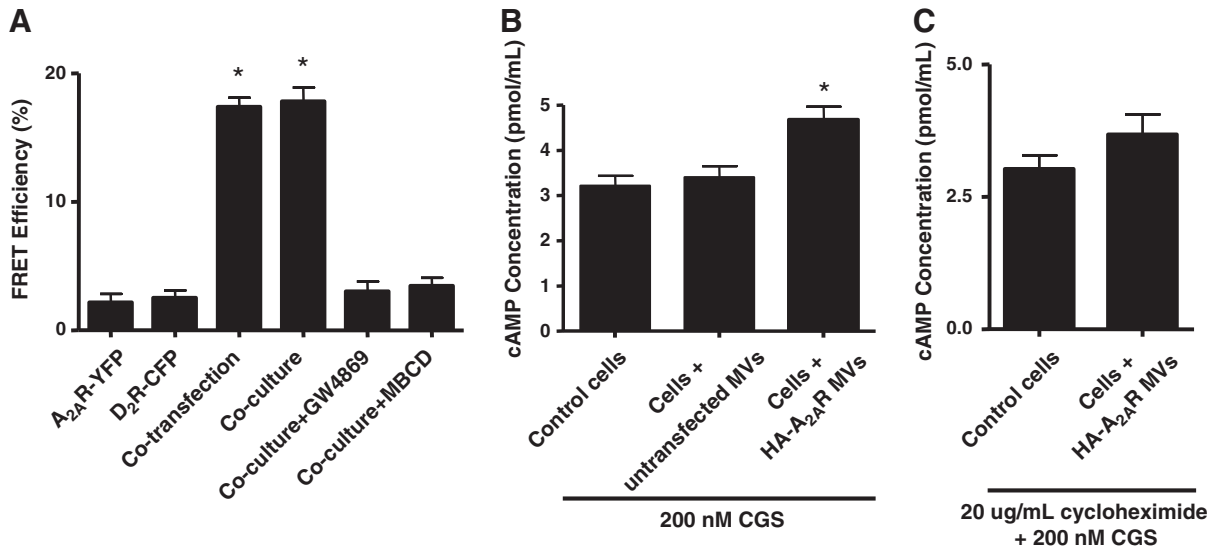


Fig. 6 – Analysis of GPCR exchange by means of MVs. A) FRET analysis of COS-7 co-culture treated with MVs release inhibitor. The day after transfection, A_{2A}R-YFP or D_2R-CFP transfected cells were grown in co-culture but separated by a 0.4 μm pore size membrane, and at the same time these cells were treated with the two inhibitors of MVs release, 2.5 mM methyl-β-cyclodextrin (MβCD) and 10 μM GW4869 (specific inhibitor of neutral sphingomyelinase) for 24 h. After this incubation period confocal laser scanning microscopy analysis failed to find positive cells for either A_{2A}R-YFP or D_2R-CFP, consequently, the FRET analysis was negative. Each bar is the mean ± SEM of three independent experiments. The asterisk marks significant results ($P<0.05$). B) cAMP assay of recipient cells (COS-7 and HEK293T) after incubation (24 h) with microvesicles (MVs) containing A_{2A} receptor-hemagglutinin (HA-A_{2A}). cAMP accumulation was evaluated after treatment with CGS (200 nM for 2 h) in four different experimental conditions:

Control cells: COS-7 and HEK293T cells;
COS-7 and HEK293T cells incubated with MVs harvested from untransfected cells;
COS-7 and HEK293T cells incubated with MVs containing A_{2A} receptor-hemagglutinin;
C) cAMP assay of COS-7 and HEK293T cells treated with 20 μg/ml cycloheximide and incubated with A2A receptor-hemagglutinin MVs. After 24 h cAMP production was stimulated with 200 nM CGS for 2 h. Each bar is the mean ± SEM of three independent experiments. $^*P<0.05$.

Acknowledgments

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