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Abstract The effects of cocaine (150 nM, 300 nM and 150 µM) on human glioblastoma cell cultures were studied on tunneling nanotube formation (1h cocaine treatment) and extracellular vesicle release (1h, 3h and 8h cocaine treatment). Cocaine significantly increased the number of tunneling nanotubes only at the lowest concentration used. The release of extracellular vesicles (mainly exosomes) into the medium was stimulated by cocaine at each concentration used with a maximum effect at the highest concentration tested (150 µM). Moreover, cocaine (150 nM) significantly increased the number of vesicles with 61-80 nm diameter while at concentrations of 300 nM and 150 µM, the smaller vesicles (30-40 nm diameter) were significantly increased with a reduction of the larger vesicles (41-60 nm diameter). A time dependence in the release of extracellular vesicles was observed. In view of the proposed role of these novel intercellular communication modes in the glial-neuronal plasticity, it seems possible that they can participate in the processes leading to cocaine addiction. The molecular target/s involved in these cocaine effects could be specific molecular components of plasma membrane Lipid Rafts and/or cocaine-induced modifications in cytoplasmic lipid composition.

Key words Cocaine; Glioblastoma cell culture; Tunneling nanotubes; Exosomes; Lipid Rafts

Introduction

Cocaine, a natural alkaloid from the leaves of the coca plant, is among the most widely abused drugs in modern society. Cocaine binds to Dopamine Transporter (DAT) with an affinity around 600 nM (Woodward et al.,1995) and inhibits the reuptake of dopamine (DA), resulting in marked increases in DA neurotransmission (Di Chiara and Imperato, 1988; Koob and Bloom, 1988; Kalivas and Volkow, 2005).Through the acute increase of cocaine induced ventral and dorsal striatal DA transmission, reward and its expectancy as well as the salience of the stimulus are experienced (Zink et al., 2003). The enhanced DA signaling in the mesolimbic system is proposed to be the main mechanism leading to cocaine addiction (Ritz et al., 1987; Mortensen and Amara, 2003; Torres et al., 2003). However, recent findings showed that cocaine may have actions independent of DAT (Genedani et al., 2010; Ferraro et al., 2012). Hence, a direct allosteric action of cocaine on the D2 receptors has been indicated as well as an action of cocaine on the remodeling of Lipid Rafts (LRs) (Genedani et al., 2010; Ferraro et al., 2012).

The existence of new specialized structures for intercellular communication, such as tunneling nanotubes (TNTs) (Rustom et al., 2004; Baluška et al., 2006; Goncharova and Tarakanov, 2008) and extracellular vesicles (EVs) (in particular exosomes) (for a review, see Cocucci et al., 2009; Simons and Raposo, 2009; Raposo and Stoorvogel, 2013) has recently been indicated. TNTs are transient transcellular channels with a diameter of 50-200 nm, a length up to several cell diameters with variable lifetimes ranging from less than 60 minutes up to several hours (Gurke et al., 2008). Thus, they connect two cells in a transient way and form a ‘private’ channel [i.e. a new type of Wiring Transmission (WT); Agnati et al. 2010] which allows exchange of molecules, proteins and whole organelles between cells (Rustom et al., 2004; Baluška et al., 2006; Goncharova and Tarakanov, 2008). Mitochondrial DNA (mtDNA) as well as RNAs can migrate along TNTs from one cell to neighboring cells either inside or along their surface (Rustom et al., 2004; Koyanagi et al., 2005; Onfelt et al., 2006; Belting and Wittrup, 2008; Agnati et al., 2009).

A set of different messages [e.g. specific proteins, RNAs (mRNA, ncRNA), mtDNA] can also be sent from cell to cell via extracellular vesicles (acting as protective containers), dispatched into the extracellular space and/or the CSF followed by diffusion until the proper targets are reached (Simons and Raposo, 2009). This type of intercellular communication belongs to the Volume Transmission (VT) mode of communication (no virtual continuous channel) and in this case a “safe” transfer of the message occurs (Agnati et al., 2010). Different types of extracellular vesicles are described (Cocucci et al., 2009; Raposo and Stoorvogel, 2013) even if special attention so far is paid only to exosomes that are released after fusion with the membrane of multivesicular bodies, which contain intra-lumenal vesicles 30–100 nm in diameter.

The view that exosomes as well as shedding vesicles (vesicles 100-1000 nm in diameter) are safe vesicular carriers for targeted intercellular communication (Simons and Raposo, 2009; Raposo and Stoorvogel, 2013) is largely supported by experimental findings. As a matter of fact, it was shown that intrinsic features of exosomes and of shedding vesicles likely allow their specific interaction with the appropriate target cells. They fulfill their task by moving along energy gradients from the source-cell to the target-cell(s) (Mallegol et al., 2007; Belting and Wittrup, 2008; Lakkaraju and Rodriguez-Boulan, 2008; Schorey and Bhatnagar, 2008; Simons and Raposo, 2009). Lakkaraju and Rodriguez-Boulan image exosomes to be itinerant workers who travel away from the source of production and roam from cell to cell to disseminate important information (Lakkaraju and Rodriguez-Boulan, 2008). Thus, we called this type of VT, the 'Roamer Type of VT' (Agnati et al., 2010). Therefore, in view of these peculiar features, both TNTs and EVs can play a pivotal role in the integrative actions of the Complex Cellular Networks of the CNS (Agnati and Fuxe, 2000).
These findings as well as data on the involvement of LRs both in TNT formation and in EV release (de Gassart et al., 2003; Wubbolts et al., 2003; Fifadara et al., 2010) prompted us to investigate the effects of cocaine on TNT formation and EV release in human glioblastoma cell cultures. Possible involvement of planar LRs was also studied by β-methyl-cyclodextrin (BMC), which induces a depletion of membrane cholesterol, and cholera toxin B (CTX-B) which alters LRs composition by specific binding to ganglioside 1 (GM1). Lipid Rafts are membrane micro-domains enriched in sphingolipids (e.g gangliosides in particular GM1) and cholesterol. Literature data suggest a role of LR in TNT formation (Fifadara et al., 2010; Kabaso et al., 2011, 2012).

Materials and methods

Cell culture and reagents

Human U87MG glioblastoma cells were cultured routinely at 37 °C with 5% CO_2 in Eagle's minimal essential medium (E-MEM; Lonza BioWhittaker, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 1.5 g/L sodium bicarbonate. All cell culture reagents, β-methyl-cyclodextrin and cholera toxin subunit B were obtained from Sigma-Aldrich (Aldrich, St. Louis, MO, USA). Cocaine hydrochloride was purchased from Macfarlan Smith Ltd (Edinburgh, UK).

TNT evaluation

For TNT studies, cells ($2x10^5$) were plated on 12 mm diameter glass based dishes (Iwaki Cell Biology, Thermo Fisher Scientific Inc, Waltham, MA, USA) and allowed to grow for 24 h. Then, cells were treated for 1 hour at 37 °C with cocaine hydrocloride (150, 300 nM and 150 µM). Respective control cells were exposed for the same period to serum free medium. To evaluate a possible effect of cocaine on LRs we used β-methyl-cyclodextrin (BMC) to deplete cholesterol from the cells or cholera toxin subunit B (CTX-B) to coat plasma membrane ganglioside -1 (GM1). Thus, cells were pretreated for 30 min at 37 °C with 6.6 mg mL^{-1} BMC or 5 µg mL^{-1} CTX-B dissolved in serum-free medium. Finally, live cells were observed at the confocal microscope for TNT evaluation.

Extracellular vesicle isolation

For extracellular vesicle preparation U87MG cells ($5x10^6$) were plated on 25 cm^2 flasks (Iwaki Cell Biology, Thermo Fisher Scientific Inc, Waltham, MA , USA) and allowed to grow for 72 h. Then, cells were treated for 1, 3 or 8 hour at 37 °C with cocaine hydrocloride (150, 300 nM and 150 µM). Respective control cells were exposed for the same period to serum free medium. For each treatment 33 mL of conditioned medium were collected and processed for exosome isolation. Extracellular vesicles were purified by differential centrifugation at 4 °C, starting with a centrifugation at 300 ×g (10 min) and followed by centrifugations at 12,000 ×g for (20 min), and 100,000 ×g (120 min). The resulting EV pellets were washed with phosphate buffered saline (PBS) and then collected again by ultracentrifugation at 100,000×g (120 min) and resuspended in 500 µL PBS.

Live confocal microscopy

After the different treatments, live cells were washed and observed by means of a Leica DMIRE2 confocal microscope equipped with a 63x oil objective using transmitted light. During every experimental section, we observed 3 randomly selected different fields of the dish. For each field we acquired ten consecutive images (237x237 µm). Then, for each field we counted the number of TNTs between cells and the number of cells present in the same field. Data were expressed as the ratio between TNT number and cell number.

Atomic Force Microscopy (AFM)

Purified EVs were further diluted up to 1:60 ratio with PBS and evaluated for number and size by AFM analysis. In detail, 10 µL of the obtained suspension were adsorbed to freshly cleaved mica sheets for 30 min at room temperature, rinsed with deionized water, and air dried. A Nanoscope IIIa Multimode AFM (Veeco) in tapping mode and silicon probes (K ≈ 50 N m^{-1}) were used. Constant force was maintained for imaging all samples. Topographic (height) and amplitude images were recorded simultaneously at 512x512 pixels at a scan rate of 2.03 Hz. Height image processing was performed using Gwyddion 2.5 software.

Statistical Analysis

Data were expressed as means ± s.e.m. and analyzed for significance by analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. The statistical analysis was carried out using the SPSS 13.0 software (SPSS Inc., Chicago, USA).

Results

Cocaine and TNT formation

As shown in Fig. 1, cocaine at the concentration of 150 nM significantly increases the TNT formation. Higher concentrations of cocaine (300 nM or 150 µM) have no effect.

LR compostion and cocaine-induced TNT formation

Exposure of human U87MG glioblastoma cells to BMC (6.6 mg mL^{-1}) for 30 min to deplete membrane cholesterol reduces, although not significantly, TNT formation (Fig. 2).
A significant reduction is observed after pre-treating cells with CTX-B (5 µg mL^{-1}; 30 min) which acts on plasma membrane GM1 (Fig. 2). In these conditions the TNT number remains above the control value also in the presence of cocaine (150 nM) (Fig. 2).

AFM analysis of EVs

Topographic and amplitude images obtained by AFM reveal that the vast majority of the samples consisted of small, 30-100 nm in diameter, vesicles (Fig. 3A). The size of all analyzed vesicles matches the size of exosomes. A few large particles were also present, which may be exosome

aggregates, difficult to avoid after ultracentrifugation (Thery et al., 2006), or larger vesicles. AFM experiments show that exosome content in conditioned medium is inversely proportional to the time of the collection of conditioned medium (1, 3 and 8 h) (Fig. 3B) suggesting a possible intracellular recycling of the released vesicles.

Cocaine and exosome release

In view of above, we chose 1 h exposure to investigate the influence of cocaine. As shown in Fig. 4, cocaine treatment increases the number of exosomes with a maximum effect at the highest concentration used (150 µM). Topographic images are used for EV quantification.
The size distribution shows that cocaine at the lowest concentration (150 nM) significantly increases the number of exosomes with 61-80 nm in diameter while, at the higher concentrations (300 nM and 150 µM), the smaller exosomes (30-40 nm in diameter) are significantly increased with a significant reduction of the larger exosomes (41-60 nm in diameter) (Fig. 5).

Time-dependent EV release

The time dependency in the release of EVs is evident in cocaine-treated cells (150 µM). The highest increase was observed after 1 h of treatment (increase of + 254% vs. control). A reduced increase was observed after 3 and 8 h exposure to cocaine (increase of about +50% vs. control) (Fig. 6).

Discussion

In previous work it was suggested that cocaine can have actions independent of DAT in vitro and in vivo (Genedani et al., 2010; Ferraro et al., 2012). The present in vitro results on glioblastoma cells highlight other possible DAT-independent actions of cocaine, which may have a relevant role also in vivo. As a matter of fact, the current paper demonstrates that cocaine, at least in vitro, influences novel intercellular communication modes in glioblastoma cells. Thus, it increases TNT formation as well as exosome release. Both of these actions are already evident at a low concentration of cocaine (150 nM) which does not block DAT (Woodward et al., 1995). In particular, TNTs are significantly increased only at this concentration while the release of exosomes is influenced by cocaine at every concentration used with a maximal effect at the highest concentration (150 µM).
As previously reported, TNTs allow transfer of a set of signals from cell to cell at a short distance (WT). While accumulating evidence from recent studies indicates their importance for a variety of cellular functions, we still have a limited molecular understanding of TNTs regarding their structure

and biogenesis. A role of cholesterol-sphingomyelin membrane nanodomains in the stability of TNTs has been suggested both showing an enrichment of these nanodomains along TNTs and a reduction in TNT density depleting cholesterol from the plasma membrane (Lokar et al., 2012). Our results, obtained in cells pre-exposed to β-methyl-cyclodextrin, give further support to these data. As regards TNT biogenesis, M-Sec, in cooperation with RalA, involved in controlling actin cytoskeletal remodeling (Moskalenko et al., 2002; Sugihara et al., 2002), and the exocyst complex seem to have a pivotal role (Hase et al., 2009; Kimura et al., 21012)moreover, the involvement of yet unknown factor(s) cannot be excluded.

Several reports indicate the ability of cocaine to interact and modify membrane components. A non-specific weak binding of cocaine to lipids has been reported (Bailey, 1998) but this does not seem to have a relevant role on cocaine-induced TNTs formation and/or vesicle release. On the contrary, a greater relevance could be played by the action of cocaine on specific membrane microdomains, in particular LRs. Our previous data reported the ability of cocaine to increase planar LRs (Genedani et al., 2010). Moreover, cocaine rapidly concentrates into cells (Nassogne et al., 2004) and activates sigma-1 receptors which, in turn, increases plasma membrane LRs (Hayashi and Su, 2005). These data are also confirmed by “in vivo” studies showing that exposure to cocaine can induce marked changes in ganglioside composition in different areas of the brain (Leskawa et al., 1994). Cocaine influence on LR expression could account, at least in part, for the increased TNT formation that we observed at the lowest concentration used (150 nM). At the higher concentrations (300 nM and 150 μM), cocaine does not modify TNTs and this could depend from major modifications of plasma membrane components; for example, we previously observed that the exposition of CHO cells to high concentration (1.5 mM) of cocaine induced caveolin-1 fragmentation (Genedani et al. 2010).

Exosomes represent a ‘Roamer Type of VT’ able to transfer a number of signals to specific recipient cells both at short and long distance from donor cells. They are extracellularly released as a consequence of multivesicular endosome (MVE) fusion with the plasma membrane (Raposo and Stoorvogel, 2013). The generation of MVEs involves the lateral segregation of cargo at the delimiting membrane of an endosome and inward budding and pinching of vesicles into the endosomal lumen. The endosomal sorting complex responsible for transport (ESCRT) seems to be involved (Raiborg and Stenmark, 2009; Hurley, 2010) but it does not completely account for exosome biogenesis. Similarly, the mechanism for generation of exosomes from the plasma membrane is largely undefined through cytoskeleton (actin and micro- tubules), associated molecular motors (kinesins and myosins), molecular switches (small GTPases), and the fusion machinery (SNAREs and tethering factors; Cai et al., 2007) are required. Moreover, it has been shown that GM1 and cholesterol enriched LRs, also able to recruit secretory proteins such as SNAREs, are critically involved in docking and exocytosis of secretory vesicles. In fact,

immobilized CTX-B facilitates vesicle docking and trafficking in a cholesterol dependent manner (Soo et al., 2010). The cocaine-induced modifications of LRs (Hayashi and Su, 2005; Genedani et al., 2010), with consequent possible influence on MVE generation, docking and exocytosis of secretory vesicles, could have a main role on the observed stimulatory effect of cocaine on the release of exosomes.

However, our data on the different size distribution of exosomes released by different concentrations of cocaine suggest that additional mechanisms could be involved. Interestingly, two different populations of MVEs have been identified and that primarily involved in exosome secretion seems to be the cholesterol-rich MVE population (Möbius et al., 2002) suggesting a relationship between intracellular lipid components and exosome release. Several literature data show that cocaine modifies cytoplasmic lipid composition both by direct action on specific enzymes and by interaction with sigma-1 receptors. In cultured fibroblasts cocaine accumulates into lysosomes (at 30 min its concentration is 100-fold higher than the concentration introduced in the culture medium) and may induce a typical lysosomal lipid overload. Particularly, cocaine produces a 20% increase of total cell phospholipid content including phosphatidylcholine, phosphatidylserine and particularly phosphatidylethanolamine, as well as sphingomyelin (Nassogne et al., 2004). On the other hand, sigma-1 receptors, primary localized on the endoplasmatic reticulum (ER) and specific targets of cocaine, play important roles in the compartmentalization of lipids into the ER lipid storage sites and in the export of lipids to peripheries of cells (Hayashi and Su,2005). Recently, the phospholipids phosphatidylserine and Bis(Monoacylglycero) Phosphate, and the neutral lipid ceramide has been shown to trigger an exosome biogenesis pathway independent of the ESCRT machinery (Trajkovic et al., 2008). Moreover, the lipid composition of exosomes indicates a major sorting of lipid molecules from the parental cells with enrichment in sphingomyelin and saturated molecular species of phosphatidylcholine and phosphatidylethanolamine (Laulagnier et al., 2004; Subra et al., 2010; Llorente et al., 2013). Overall, these data suggest a possible relationship between cocaine-induced accumulation of intracellular lipids and exosome biogenesis. This might also account for the differences in diameter of exosomes released by cocaine at low and high concentrations. Cocaine at the lowest concentration used (150 nM) increases the number of exosomes with 61-80 nm in diameter while at the higher concentrations (300 nM and 150 µM), the smaller exosomes (30-40 nm in diameter) are significantly increased with a significant reduction of the larger exosomes (41-60 nm in diameter) mainly at the concentration of 300 nM. The cocaine-induced increase of total cell phospholipid content as well as sphingomyelin depends on the concentration of the drug; thus, at the lowest concentration the quantity of intracellular lipids is low allowing mainly the biogenesis of bigger exosomes at higher concentrations the accumulation of lipids is higher thus facilitating the biogenesis of smaller exosomes. As far as the functional meaning, in view of the belonging of this

type of intercellular communication to the VT mode of communication (Agnati et al., 2010) it can be proposed that at low cocaine concentrations exert spatially limited effects, that is only on the neighboring cells. On the other hand, the cocaine-induced release of smaller exosomes at the higher concentrations suggests long-distance effects thus more widespread actions of this drug of abuse.

In summary, the current data show for the first time an action of cocaine on TNT formation and exosome release in glioblastoma cell cultures. These novel cocaine actions found in glioblastoma cells, which appear to be DAT independent, at least at the two lowest concentrations used which are substantially below the Ki value (600 nM) for DAT inhibition by cocaine (Woodward et al., 1995), will be explored for their development also in neuronal cells including in vivo studies. It seems possible that they can participate in processes leading to cocaine addiction. The molecular targets mediating these cocaine effects can be specific components of membrane planar LRs modulating in an unknown way the release of exosomes and the formation of TNTs and/or cocaine-induced modifications in cytoplasmic lipid composition.

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Legends to the figures

Fig. 1 Influence of 1 h exposition to cocaine (150 nM, 300 nM and 150 µM) on Tunneling Nanotube (TNT) formation between human glioblastoma U87MG cells. To visualize TNTs, live cells were observed by means of a Leica DMIRE2 confocal microscope. The panel A shows an example of TNTs in the presence of cocaine (150 nM) and the respective control cells. For a quantitative analysis, live cells were observed by means of the confocal microscope equipped with a 63x oil objective using transmitted light. TNTs and cells have been counted after acquisition of ten consecutive (237x237 µm) images and data were expressed as the ratio between TNT number and cell number. Percent data vs. the respective control are reported in panel B as means ± s.e.m. of at least 9 different experiments. *p< 0.05 vs. control; ANOVA with Bonferroni correction for multiple comparisons.

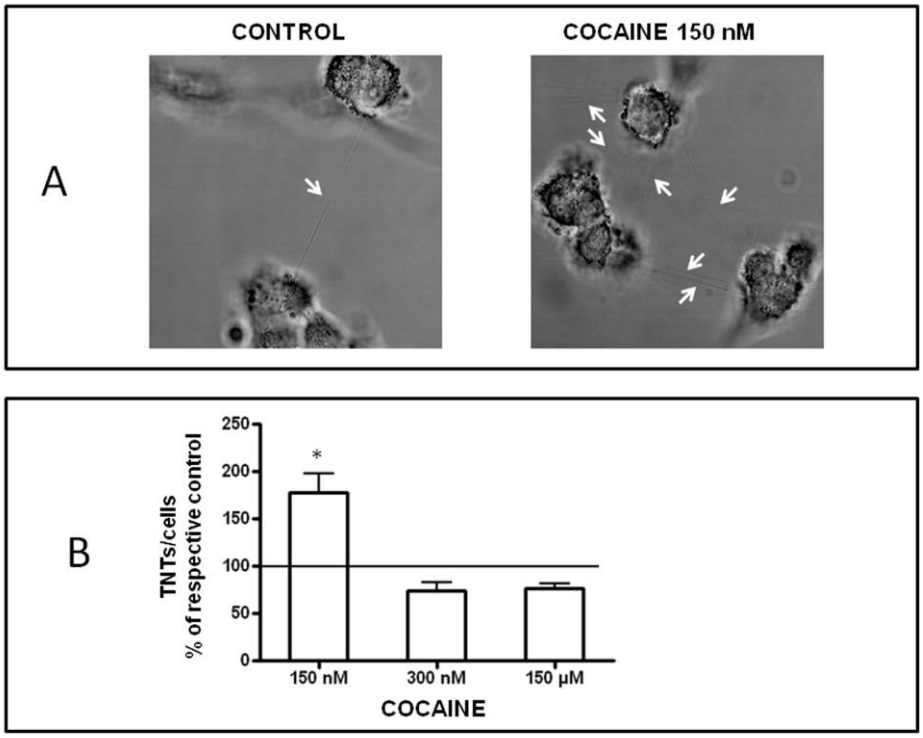
Fig. 2 Influence of β-methyl-cyclodextrin (BMC; 6.6 mg mL^{-1}) or cholera toxin subunit B (CTX-B; 5 µg mL^{-1}) alone or in association with cocaine (150 nM), on Tunneling Nanotube (TNT) formation between human glioblastoma U87MG cells. Cells were pretreated for 30 min with BMC or CTX-B and successively exposed to cocaine (150 nM) for 1 h. Control cells were exposed for the same periods to serum-free medium. To visualize TNTs, live cells were observed by means of a Leica DMIRE2 confocal microscope. For a quantitative analysis, live cells were observed by means of the confocal microscope equipped with a 63x oil objective using transmitted light. TNTs and cells have been counted after acquisition of ten consecutive (237x237 µm) images and data were expressed as the ratio between TNT number and cell number. Percent data vs. the respective control are reported as means ± s.e.m. of at least 9 different experiments. **p< 0.02; *p<0.05 vs. control; ANOVA with Bonferroni correction for multiple comparisons.

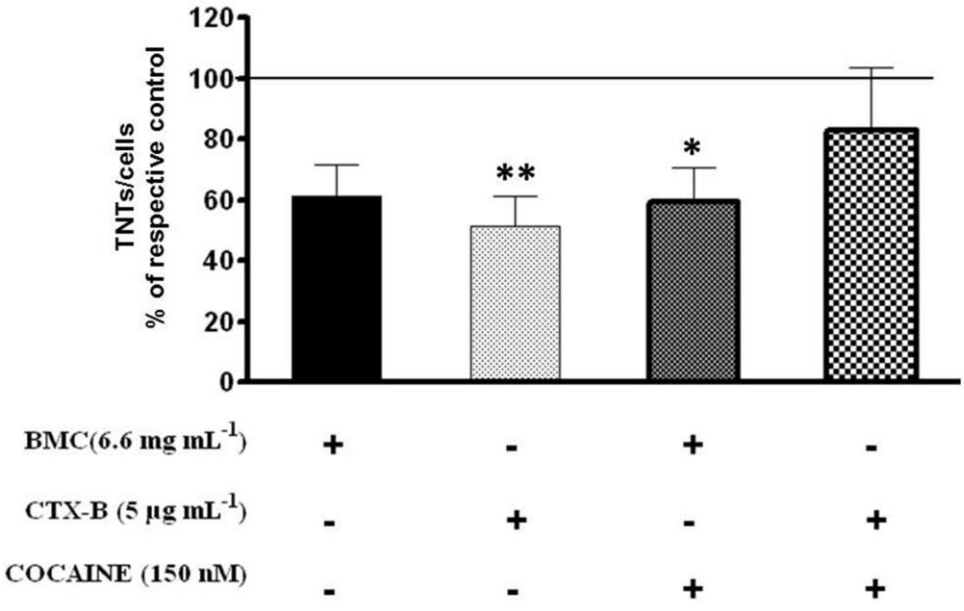
Fig. 3 Extracellular vesicle (EV) release from not-treated human glioblastoma U87MG cells. EVs were collected from cellular conditioned media by serial centrifugations and then resuspended in 500 µL PBS. This exosomal suspension was further diluted 1:60 in PBS and 10 µL were adsorbed to freshly cleaved mica sheets for 30 min at room temperature, rinsed with deionized water and air dried. A Nanoscope IIIa Multimode AFM (Veeco) in tapping mode was used to acquire topographic images (1x1 µm) successively elaborated with Gwyddion software for a quantitative comparison. Panel A shows size distribution of EVs. Data are expressed as percentage of EV frequency/μm^2 with a specific diameter range (nm) and reported as means ± s.e.m. of 14 different experiments. The release of EVs at different times (1, 3 and 8 h) is reported in panel B. Data are means ± s.e.m. of 14 different experiments.

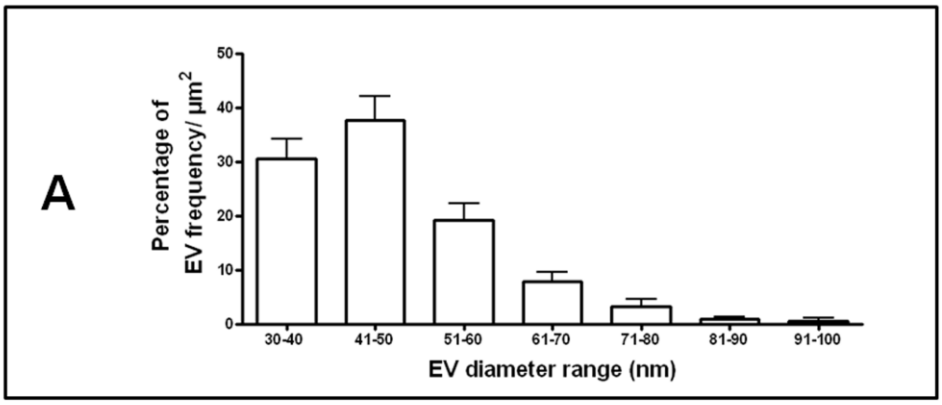
Fig. 4 Influence of 1 h exposition to cocaine (150 nM, 300 nM and 150 µM) on extracellular vesicle (EV) release from human glioblastoma U87MG cells. Control cells were exposed for 1 h to serum-free medium. EVs were collected from cellular conditioned media by serial centrifugations and then resuspended in 500 µL PBS. This exosomal suspension was further diluted 1:60 in PBS and 10 µL were adsorbed to freshly cleaved mica sheets for 30 min at room temperature, rinsed with deionized water and air dried. A Nanoscope IIIa Multimode AFM (Veeco) in tapping mode was used to acquire topographic images (1x1 µm) successively elaborated with Gwyddion software for a quantitative comparison. Examples of AFM images (1x1 µm) are reported (control, cocaine 150 nM, 300 nM and 150 µM) in the upper part of the figure. Each panel shows AFM amplitude image at the left and topographic (z = 0-10 nm) image at the right. Data are reported as means ± s.e.m. of at least 9 different experiments. $^{\S}p<0.0001$, $^{**}p<0.02$ vs. control; ANOVA with Bonferroni correction for multiple comparisons.

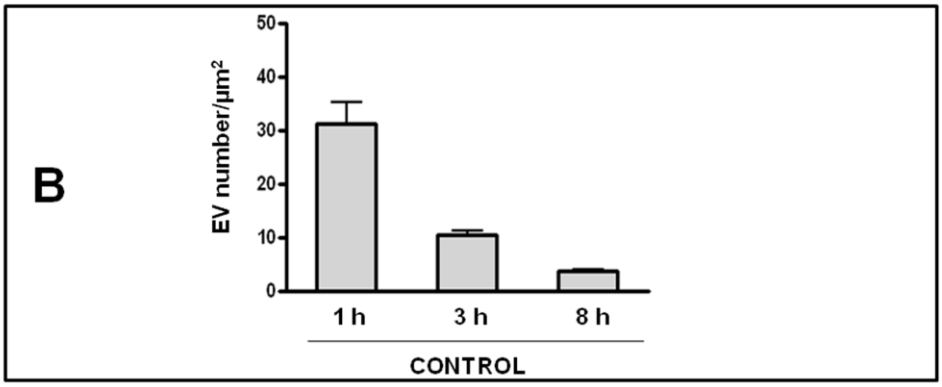
Fig. 5 Comparison of EV size distribution, according to the diameter (nm), among exosomes released from human gliobastoma U87MG cells exposed to different cocaine concentrations (150 nM, 300 nM and 150 µM) or to serum-free medium (control) for 1 h. A Nanoscope IIIa Multimode AFM (Veeco) in tapping mode was used to acquire (1x1 µm) topographic images successively elaborated with Gwyddion software for a quantitative comparison. Data are expressed as percentage of EV frequency/μm^2 with a specific diameter range (nm) and reported as means ± s.e.m. of at least 9 different experiments. $^{**}p<0.02$, $^{\S}p<0.0001$ vs. control; ANOVA with Bonferroni correction for multiple comparisons.

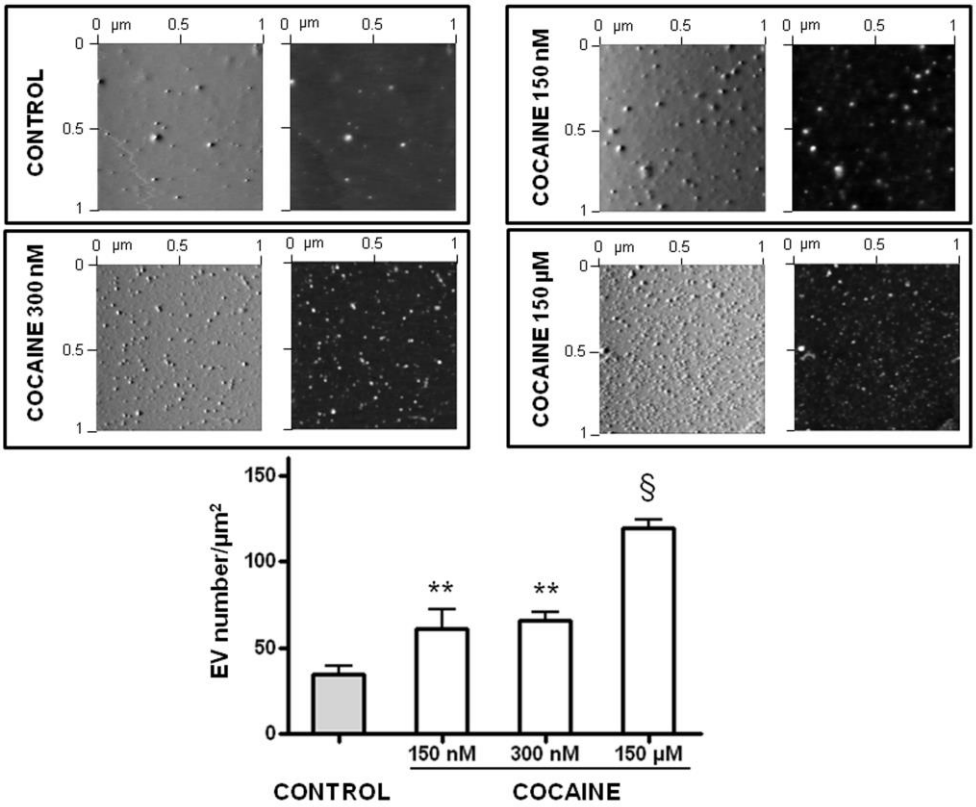
Fig. 6 Influence of cocaine (150 µM) on extracellular vesicle (EV) release from human glioblastoma U87MG cells. Cells were treated with cocaine or serum-free medium (control) for 3 different periods (1, 3 and 8 hours); the respective conditioned media were collected and serial centrifuged in order to isolate EVs. Each EV pellet was resuspended in 500 µL PBS, further diluted 1:60 and 10 µL were adsorbed to freshly cleaved mica sheets for 30 min at room temperature, rinsed with deionized water and air dried. A Nanoscope IIIa Multimode AFM (Veeco) in tapping mode was used to acquire (1x1 µm) topographic images successively elaborated with Gwyddion software for a quantitative comparison. Data were expressed as the percentage of released EVs from treated cells in respect to the relative control and reported as means ± s.e.m. of at least 9 different experiments. In the upper part of the figure examples of AFM amplitude images are reported at the three different times of exposure to cocaine. $^{\S}p<0.0001$ vs. control; ANOVA with Bonferroni correction for multiple comparisons.

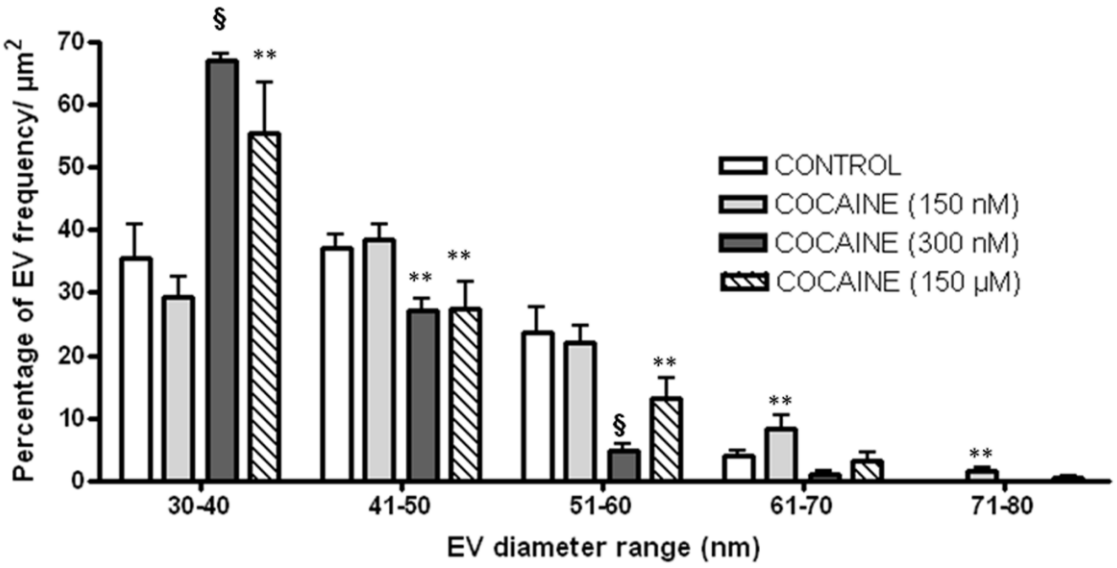

A
CONTROL
COCAINE 150 nM
B
TNTs/cells
% of respective control
250
200
150
100
50
0
*
150 nM
300 nM
150 µM
COCAINE

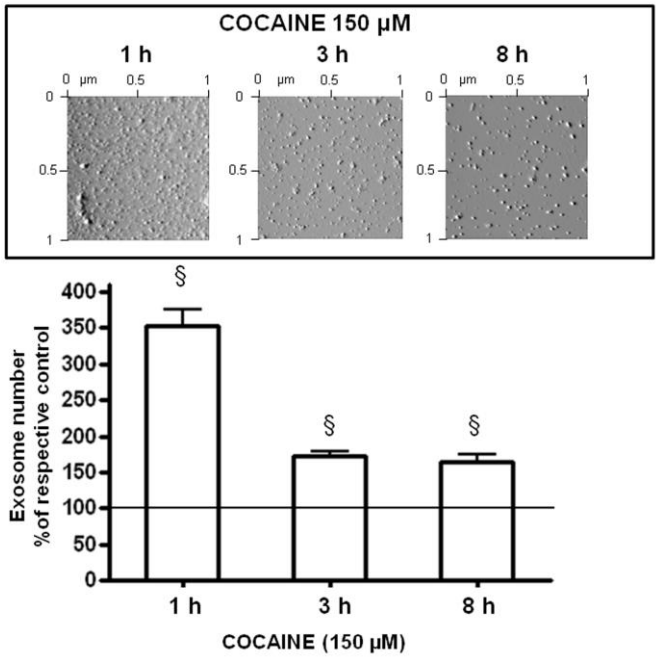

TNTs/cells
% of respective control
120
100
80
60
40
20
0
**
*
BMC(6.6 mg mL-1)
+ - + -
CTX-B (5 µg mL-1)
- + - +
COCAINE (150 nM)
- - + +


A
Percentage of EV frequency/ µm²
50
40
30
20
10
0
30-40
41-50
51-60
61-70
71-80
81-90
91-100
EV diameter range (nm)


B
EV number/µm²
50
40
30
20
10
0
1 h
3 h
8 h
CONTROL


CONTROL
COCAINE 150 nM
COCAINE 300 nM
COCAINE 150 µM
0 µm
0.5
1
EV number/µm²
150
100
50
0
**
**
§
150 nM
300 nM
150 µM
CONTROL
COCAINE


Percentage of EV frequency/ µm²
70
60
50
40
30
20
10
0
§
**
**
**
**
§
**
**
CONTROL
COCAINE (150 nM)
COCAINE (300 nM)
COCAINE (150 µM)
30-40
41-50
51-60
61-70
71-80
EV diameter range (nm)


COCAINE 150 µM
1 h
3 h
8 h
0 µm 0.5 1
0
0.5
1
Exosome number
%of respective control
400
350
300
250
200
150
100
50
0
§
§
§
1 h
3 h
8 h
COCAINE (150 µM)