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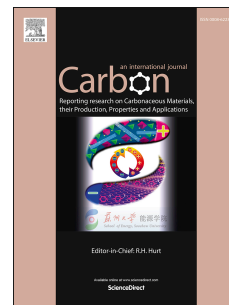
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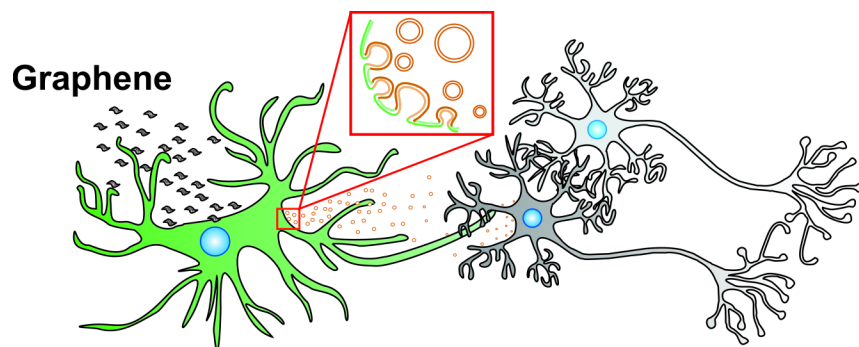
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M.M. performed cell biology, electrophysiology, and immunofluorescence experiments and analysis; M.M. and P.P. designed and performed AFM experiments; M.P. performed IR and UVR experiments and analysis. CM and GDM performed biology and WB experiments; N.L. and K.K. contributed to the synthesis and characterization of thin graphene oxide of biological grade. BB performed SEM micrographs of GO. L.B. and L.C. conceived the study; L.B. conceived the experimental design and contributed to the analysis of data; L.B. wrote the manuscript.



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Abstract

Microvesicles (MVs) generated and released by astrocytes, the brain prevalent cells, crucially contribute to intercellular communication, representing key vectorized systems able to spread and actively transfer signaling molecules from astrocytes to neurons, ultimately modulating target cell functions. The increasing clinical relevance of these signaling systems requires a deeper understanding of MV features, currently limited by both their nanoscale dimensions and the low rate of their constituent release. Hence, to investigate the features of such glial signals, nanotechnology-based approaches and the applications of unconventional, cost-effective tools in generating MVs are needed. Here, small graphene oxide (s-GO) nanoflakes are used to boost

22 MVs shedding from astrocytes in cultures and s-GO generated MVs are compared with those
23 generated by a natural stimulant, namely ATP, by atomic force microscopy, light scattering,
24 attenuated total reflection–fourier transform infra-red and ultraviolet resonance Raman
25 spectroscopy. We also report the ability of both types of MVs, upon acute and transient exposure
26 of patch clamped cultured neurons, to modulate basal synaptic transmission, inducing a stable
27 increase in synaptic activity accompanied by changes in neuronal plasma membrane elastic
28 features.

29

30 **Keywords:** graphene oxide, extracellular vesicles, atomic force microscopy and spectroscopy,
31 FTIR-ATR and UVRR spectroscopy, synaptic activity, cortical neuronal cultures

32

33 **1. Introduction**

34 In biology, newly described forms of intercellular communication comprise the release of
35 vesicles, named extracellular vesicles, from virtually all cell types, including resident glial cells
36 of the central nervous system (CNS), such as astrocytes and microglia.[1,2] In particular, the
37 shedding of membrane vesicles is a recognized form of cross talk in the multidimensional
38 signaling between astrocytes, (i.e. the majority of cells in the mammalian CNS), and neurons in
39 physiology, but also in neurodegenerative and neuroinflammatory diseases as well as in brain
40 tumors. Extracellular vesicle signaling molecules, either stored within their cargo or embedded in
41 their plasma membrane, modulate relevant processes in the development, physiology and
42 pathology of CNS target cells.[3–6] The signaling system based on release of extracellular
43 vesicles comprises shedding microvesicles (MVs) and exosomes, characterized by different size,
44 membrane composition, cargo and origin.[7,8]

45 MVs are nanovesicles able to interact specifically with cells at local or distant sites.[9] In
46 maintaining CNS functions, glial cells intensely communicate with neurons, also *via* the release
47 of MVs, which represents a highly versatile tool to functionally impact the CNS.[10–12] MVs
48 are considered a “vectorized” signaling system able to bind their target cells to transmit specific
49 information. The reported spreading ability of MVs has suggested their potential exploitation as
50 biomarkers or as engineered therapeutic carriers.[13] A comprehensive correlation between
51 conditions used to release and harvest MVs from the same cell type, i.e. astrocytes, and their
52 signaling ability, will impact our understanding of MVs physiology and the design of MV-based
53 biomedical applications in the CNS.[14,15] Particular attention has to be conveyed to devise
54 novel, cost-effective ways in generating MVs, in particular enhancing constitutive release.

55 Here, we concentrate on graphene oxide (GO), the most common derivative of graphene,
56 whose properties can be tailored to adapt to new physical and biological applications.[16,17] GO
57 flakes have been successfully designed for drug delivery applications in biomedicine.[18] In the
58 CNS, small GO nano-flakes (s-GO) were shown to induce constitutive MV release from cultured
59 astrocytes and to potentiate evoked MV release induced upon exposure to bzATP.[19] s-GO
60 flakes, due to their physical features at the nanoscale, were reported to interfere with cellular
61 membrane dynamics.[19,20] In addition, *via* adhesion to the plasma membrane, s-GO may alter
62 the mechanical features of the lipid bilayer[21] triggering genuine biological responses, such as
63 MVs signaling. Thus s-GOs may represent a tool to exploit mechanical signaling at the
64 nanoscale to activate membrane release of MVs. Drug delivery applications where vesicle
65 release from genetically engineered cells is required, may take advantage of the mechanical
66 modulation of vesicle release brought about by graphene-based nanomaterials, representing a
67 safer and cheaper alternative to pharmacological tools. Prolonged exposure to biomolecules able

68 to induce MVs release, such as ATP, could in fact negatively affect cell physiology by
69 promoting astrogliosis and inducing microglia-mediated neuroinflammatory responses.[22]

70 We use the ability of s-GO to substantially increase the production of MVs from astrocytes to
71 provide, for the first time, a robust and comparative vesicle characterization by means of ultra-
72 microscopy, attenuated total reflection–fourier transform infra-red (FTIR-ATR) and UV
73 Resonant Raman (UVRR) spectroscopy. We additionally explore by single cell patch-clamp
74 recordings the impact of acute, local and transient delivery of MVs on neuronal basal synaptic
75 activity and by atomic force microscopy (AFM) the accompanying changes in neuronal plasma
76 membrane elastic features.

77 **2. Material and Methods**

78 *2.1 Graphene oxide nanosheets synthesis*

79 GO was manufactured under endotoxin-free conditions through our modified Hummers' method
80 as previously described.[19] The complete characterization of the material used is shown and
81 summarised in the Supplementary experimental section and Supplementary Figure S1 and Table
82 S1.

83 *2.2 Cell Cultures*

84 All experiments were performed in accordance with the EU guidelines (Directive 2010/63/EU)
85 and Italian law (decree 26/14) and were approved by the local authority veterinary service and by
86 our institution (SISSA-ISAS) ethical committee. All efforts were made to minimize animal
87 suffering and to reduce the number of animals used. Animal use was approved by the Italian
88 Ministry of Health, in agreement with the EU Recommendation 2007/526/EC.

89 Primary glial cultures were obtained from cortices isolated from neonatal rats (Wistar) at
90 postnatal day 2-3 (P2–P3), as previously described [19,23]. Dissociated cells were plated into

91 plastic 150 cm² flasks and incubated at 37 °C; 5 % CO₂ in culture medium composed of DMEM
92 (Invitrogen), supplemented with 10 % fetal bovine serum (FBS; Thermo Fisher), 100 IU/ mL
93 penicillin, and 10 mg/mL streptomycin.

94 Cortical neurons were isolated from neonatal rat cortices (Wistar) at postnatal day 0-1
95 (P0–P1). Dissociated cells were then plated on poly-L-ornithine (Sigma) coated coverslips
96 (Kindler, EU) at a concentration of 150000 cells in a volume of 200 µL and incubated at 37 °C; 5
97 % CO₂ in a culture medium composed of Neurobasal-A (Thermo Fischer) containing 2% B27
98 (Gibco), 10 mM Glutamax and 0.5 µM Gentamycin (Gibco) for 8-10 *days in vitro* (DIV) before
99 performing electrophysiological experiments.

100 2.3 MV Isolation

101 MV shedding and isolation was performed as previously described.[19] One pool of MVs
102 were collected from 21-24 DIV glial cultures previously treated with graphene oxide nanoflakes
103 (s-GO) (10 µg/mL[19]), added to culture medium once and left for 6 days. At the end of 6-days
104 exposure, the medium was removed and substituted with physiological saline solution, with the
105 following composition: 152 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES
106 and 10 mM Glucose (pH adjusted to 7.4), at 37 °C and 5 % CO₂ for 60 min prior to MVs
107 collection and purification. The MVs pool was isolated from cultures treated (30 min) with
108 benzoyl-ATP (bzATP; 100 µM) diluted in physiological saline solution. The 6-days exposure
109 timepoint was chosen on the basis of previous western blot experiments, and confirmed by our
110 current experiments testing MVs release after 3 days of s-GO exposure (supplementary Figure
111 S2).[19] Negative controls were incubated with physiological solution without the presence of
112 bzATP or s-GO. After the incubation period, cell medium was collected and centrifuged for 15
113 min at a speed of 300 × g in order to remove cell debris. Supernatant was then collected and

114 MVs were pelleted by centrifugation at $20000 \times g$ for 2 hours. For Ca^{2+} deprivation experiments,
115 prior to supernatant collection and MVs pellet centrifugation, cultures were pre-incubated for 45
116 min in a saline solution identical to the physiological saline solution except for 0 mM $CaCl_2$, 3
117 mM $MgCl_2$ and 1mM EGTA to allow the depletion of intracellular calcium storage. Upon this
118 pre-treatment, we harvest the MVs from controls, s-GO treated and bzATP (30 min in Ca^{2+}
119 deprived solution; supplementary Figure S3).

120 *2.4 Western blot analysis*

121 MVs were prepared as previously reported, briefly they were re-suspended in lysis buffer (50
122 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % NP40, 0.1 % SDS), sonicated for 30 s, and then
123 boiled at 95 °C for 5 min.[19] Samples were run on a 10 % polyacrylamide gel and blotted onto
124 nitrocellulose membranes (Millipore, Italy). Membranes were then blocked in PBS-Tween-20
125 (0.1 %) plus 5 % nonfat dry milk and incubated with the primary antibody antitubulin-1 (dilution
126 1:1000) for 16 h at 4 °C. Membranes were then washed with PBS-Tween and incubated with
127 peroxidase-conjugated anti-mouse secondary antibody (dilution 1:1000). Detection of
128 immunolabeled ECL-exposed protein bands was measured with UVI-1D software over three
129 independent experiments.

130 *2.5 Immunofluorescence and confocal microscopy*

131 Primary glial and cortical neurons cultures were fixed in 4 % formaldehyde (PFA, prepared
132 from fresh paraformaldehyde) in PBS for 20 min at room temperature (RT) and then washed in
133 PBS. Free aldehyde groups were quenched in 0.1 M glycine solution for 5 min. The samples
134 were permeabilized in 5 % fetal bovine serum (FBS), 0.3 % Triton-X 100 in phosphate buffer
135 solution (PBS) for 30 min at RT. Samples were then incubated with primary antibodies (mouse
136 monoclonal anti-GFAP, Invitrogen, 1:500 dilution; rabbit polyclonal anti- β -tubulin III, Sigma-

137 Aldrich, 1:500 dilution) diluted in PBS with 5 % FBS at 4 °C for 1 hours. Samples were then
138 incubated with secondary antibodies (Alexa 488 goat anti-mouse, Invitrogen, 1:500 dilution;
139 Alexa 594 goat anti-rabbit, Invitrogen, 1:500 dilution), and DAPI (Invitrogen, dilution 1:200) to
140 stain the nuclei, for 45 min at RT and finally mounted on 1 mm thick glass coverslips using
141 Fluoromount mounting medium (Sigma-Andrich). Images were acquired using a Nikon C2
142 Confocal, equipped with Ar/Kr, He/Ne and UV lasers with a 40 × or 60 × (1.4 NA) oil-objective
143 (using oil mounting medium, 1.515 refractive index) to acquire glial cultures images and cortical
144 neurons images respectively. 200 × 200 μm fields were acquired for cortical neurons images and
145 300 × 300 μm fields were acquired for glial cells images. Confocal sections were acquired every
146 0.25 μm for both the cultures.

147 *2.6 Glial cell viability assay*

148 Primary rat astrocytes (21-24 DIV) were exposed to s-GO 10 μg/mL or to equivalent volumes
149 of the vehicle for 6 days. Cells were stained with propidium iodide (PI, 1 μg/ml; 15 min) for cell
150 death quantification and subsequently fixed in PFA and labelled for DAPI for nuclei
151 visualization and GFAP for visualizing astrocytes. The red (PI positive) fluorescent nuclei
152 indicating dead cells were quantified at 40 × (1.4 NA) magnification using a Nikon C2 Confocal
153 microscope, equipped with Ar/Kr, He/Ne and UV lasers, with random sampling of 10 fields per
154 sample (n = 3 coverslips/sample, from 3 independent culture preparations). The average
155 percentage of dead cells was calculated counting visual fields selected.

156 *2.7 FM1-43 staining*

157 Glial cells were incubated with the fluorescent styryl dye FM1-43 (2 μM) for 2 min in order to
158 completely stain plasma membrane, then extensively washed with PBS and exposed for 30 min
159 to bzATP (100 μM) or to standard saline solution.[19] Samples were placed in a recording

160 chamber mounted on an inverted microscope (Nikon Eclipse Ti-U) and observed with a $40 \times$
161 objective (0.6 NA, PlanFluor, Nikon). Images (512×512 px) were acquired for 10 min with an
162 exposure time of 150 ms (6.6 Hz) by a Hamamatsu Orca-Flash 4.0 digital camera controlled by
163 an integrating imaging software package (HCImage, Hamamatsu). Recorded images were
164 analyzed offline with the Clampfit software (pClamp suite, 10.2 version; Axon Instruments).
165 Image time stacks were analyzed in selected regions of interest (ROI) to measure the
166 variations in FM1-43 fluorescence intensity over time. Natural sample bleaching over time,
167 due to prolonged light exposure, has the same time-course and intensity in all the three
168 groups, as previously described[19].

169 *2.8 Atomic Force Microscopy Analysis*

170 AFM characterization was performed as previously described.[19] Briefly, the pellet of MVs
171 was re-suspended in PBS solution after isolation from cell cultures and a $15 \mu\text{L}$ drop of sample
172 solution was placed and left to adsorb (30 min) onto a freshly peeled mica substrate. Vesicles
173 were then fixed with 1% formaldehyde for 1 h (RT) in order to prevent their collapse during
174 AFM acquisition. MVs were then washed with PBS and dried under a gentle stream of nitrogen.
175 AFM analysis was performed in air at RT, using the semicontact mode of a commercial
176 instrument (Solver Pro, NT-MDT, RU). Silicon tips (NSC36/CR-AU, MikroMash, USA) with a
177 typical force constant of 0.6 nN/nm and a resonance frequency of about 65 kHz were employed.
178 Topographic height and phase images were recorded at 512×512 pixels at a scan rate of 0.5 Hz.
179 Image processing was performed using Gwyddion freeware AFM analysis software, version
180 2.40. Diameter and height of each vesicle were evaluated from cross-line profiles, and results
181 were statistically analyzed using Prism (Graphpad software).

182 *2.9 Neuronal Stiffness*

183 9-10 DIV cortical neurons were exposed to MVs obtained by glial cultures treated with bzATP
184 or s-GO and neuronal rigidity was assessed with AFM, 24 hours after MVs exposure. Force
185 spectroscopy measurements were performed with a commercial Smena AFM (NT-MDT, RU)
186 mounted on an inverted microscope (Nikon Eclipse Ti-U). AFM cantilever deflection was
187 measured when pushed against cortical neurons plated on a glass coverslip. Deflection values
188 were subsequently converted into a force versus indentation curve based on cantilever spring
189 constant and its displacement. Neuronal rigidity was evaluated in 50 randomly chosen neurons
190 for each condition (from 3 independent experiments), acquiring three force spectroscopy curves
191 in the center of each cell soma. The AFM tip was positioned by using an inverted microscope in
192 bright field mode.

193 AFM micro-cantilevers with an elastic constant of about 0.03 nN/nm and a resonance
194 frequency of about 10 kHz (CSG01 tipless cantilevers from NT-MDT, RU) were used. A
195 borosilicate glass microsphere of about 18 μm in diameter (18.2 ± 1.0 μm from Duke Standards,
196 CA, USA) was manually glued at the end of each cantilevers using a UV curable glue (Norland
197 Optical Adhesive 61 from Norland Products Inc., NJ, USA). Force spectroscopy measurements
198 were performed at a constant indentation speed of 1 mm/s with a maximum value of indentation
199 deepness set at 500 nm. Elastic modulus values (E), expressed in kPa, were determined by fitting
200 obtained force-indentation curves with a Hertzian model for the tip, using AtomicJ (v. 1.7.3)
201 analysis software.[24]

202 *2.10 Nanoparticle Tracking analysis (NTA)*

203 Measurement and analysis of MVs size distribution by NTA was performed on a NanoSight
204 LM10 system (Malvern) using approximately 500 μL of MVs of both conditions (bzATP-
205 derived and s-GO-derived) diluted 1:20 in MilliQ H_2O . Individual videos of 60 seconds

206 (recorded at 25 FPS; 3 videos per group) for each sample were acquired at RT using the
207 maximum camera gain, a detector threshold equal to 8 and analyzed by the NanoSight particle
208 tracking software to calculate size and vesicle concentration.

209 *2.11 FTIR-ATR Spectroscopy and UV Resonant Raman (UVRR) Measurements*

210 MVs were isolated from 21-24 DIV glial cultures by centrifugation as described above. MVs
211 pellet was successively washed with NaCl solution (150 mM) and finally re-suspended in 50 μL
212 of the same solution in order to avoid contribution of phosphate and sugar groups to the IR
213 absorbance spectra. The IR measurements were carried out at the BL10.2-IUVS beamline at
214 Elettra synchrotron Trieste. The spectra were collected in ATR mode using a MIR DLaTGS
215 detector and a KBr-broadband beam-splitter. For each IR measurement, 2 μL of sample solution
216 were spread over the whole area of a monolithic diamond ATR plate and left to dry forming a
217 thin film. For each sample, 20 spectra were collected in the range $4000\text{-}800\text{ cm}^{-1}$, accumulating
218 256 scans for each spectrum reaching a resolution of 4 cm^{-1} . Each spectrum was corrected for the
219 background, aqueous vapor, CO_2 and not normalized to any absorbance band.

220 UVRR measurements were performed at the BL10.2-IUVS beamline at Elettra synchrotron
221 Trieste using the experimental set-up reported.[25] 5 μL of vesicles solution were drop-casted
222 onto an aluminum foil, allowed to dry and kept under nitrogen purging. All the measurements
223 were performed at RT, using an excitation wavelength of 244 nm and tuning the power of the
224 incoming radiation to 50 μW . The outgoing radiation was collected in backscattering geometry
225 by using a triple stage spectrometer (Trivista, Princeton Instrument) with a spectral resolution of
226 8 cm^{-1} . Samples were continuously oscillated horizontally in order to avoid photodamaging.

227 *2.12 Electrophysiological Recordings*

228 Patch-clamp recordings (whole-cell, voltage clamp mode) were performed from visually
229 identified (under differential interference contrast - DIC - microscopy) cortical neurons (DIV 8-
230 10) placed in a recording chamber, mounted on an inverted microscope (Eclipse Ti-U, Nikon,
231 Japan) and superfused with control physiological saline solution of the following composition (in
232 mM): 152 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES and 10 Glucose (pH adjusted to 7.4 by
233 NaOH 1M; osmolarity 300 mOsm). Cells were patched with glass pipettes (4-7 M Ω) containing
234 (in mM): 120 K gluconate, 20 KCl, 10 HEPES, 10 EGTA, 2 MgCl₂ and 2 Na₂ATP (pH adjusted
235 to 7.35 by KOH; osmolarity 298 mOsm). All electrophysiological recordings were performed at
236 RT and the spontaneous, basal synaptic activity was recorded by clamping the membrane voltage
237 at -70 mV (corrected for liquid junction potential, which was -14 mV). To investigate the acute
238 effect on synaptic activity of glia-derived MVs, an injection pipette (patch pipette with resistance
239 of 1-4 M Ω) filled with MVs previously isolated glial cultures as described above and re-
240 suspended in 100 μ L of extracellular saline solution was positioned at 200 μ m from the cell soma
241 and connected to a pico-spritzer (PDES-02DX, npi Electronics) with 0.3 psi in-line pressure.[19]
242 On the basis of MVs quantification obtained by NTA measurements, and considering that MVs
243 isolation was performed using the same protocol in all the experiments described, we calculated
244 that the concentration of MVs used for these tests was approximately of 6.64×10^9 for MVs
245 obtained by bzATP stimulation and 1.64×10^{10} for MVs isolated from s-GO treated cultures.
246 Baseline spontaneous synaptic activity was recorded for the 10 min prior delivering the puff
247 (500 ms duration) of MVs and followed up for 20 min to verify changes in post synaptic current
248 (PSC) frequency and amplitude induced by the fusion of MVs with neuronal membranes.

249 Data were collected by Multiclamp 700B patch amplifier (Axon CNS, Molecular Devices) and
250 digitized at 10 kHz with the pClamp 10.2 software (Molecular Devices LLC, USA). All

251 recorded events were analyzed offline with the AxoGraph 1.4.4 (Axon Instrument) event
252 detection software (Axon CNS, Molecular Devices).

253 *2.13 Statistical Analysis*

254 Data sets found to follow a non-normal distribution, were represented as box plot. The central
255 thick horizontal bar in the box plots indicates the median value, while the boxed area extends
256 from the 25th to 75th percentiles with the whiskers ranging from the 2.5th to the 97.5th percentiles.
257 Statistically significant differences between two non-parametric data sets were assessed by
258 Mann-Whitney's test, while to assess statistically significant differences among three data-set we
259 used the Kruskal-Wallis test and Dunn's post hoc test. $P < 0.05$ was considered at a statistically
260 significant.

261

262 **3. Results and discussion**

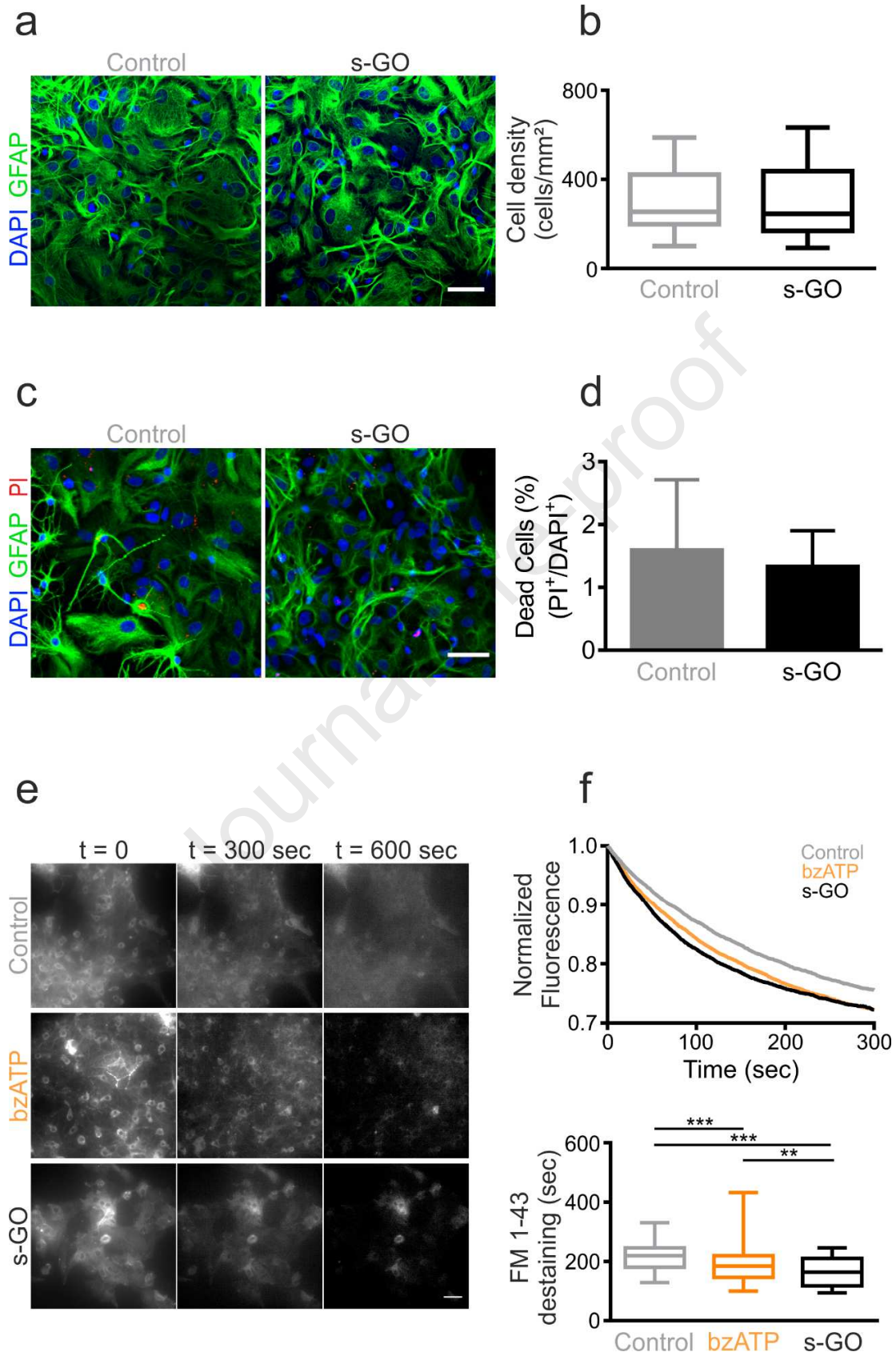
263 Astrocytes were isolated from postnatal (2-3 days) rat (Wistar) cortices, as previously
264 described.[19,20,23] We used visually homogenous s-GO dispersions containing s-GO
265 nanosheets with lateral dimensions predominantly between 50 – 500 nm.[19,20] We treated pure
266 glial cell cultures with s-GO (10 $\mu\text{g}/\text{mL}$) for 6 days.[19] Immunofluorescence labeling by antigen
267 against glial-fibrillary acidic protein (GFAP), an intermediate filament protein that is highly
268 specific for cells of the astroglial lineage, was used to visualize control and s-GO-treated
269 neuroglial cultures (GFAP, in green; Fig. 1a).[26] At the low concentrations used, s-GO
270 treatment did not impair astrocyte morphology and cell density when compared with matched
271 control cultures (box-plot in Fig. 1b).[19,27] Viability of glial cells was confirmed by propidium
272 iodide (PI) cell death assay. Control and s-GO treated cultures were incubated with PI, which
273 stain dead cells nuclei (Figure 1c, in red) and the percentage of PI-positive nuclei was calculated

274 (bar plot in Fig. 1d). Graphene nanosheets cytotoxicity is a largely debated issue, due to the
275 variable impact on cell toxicity of several material's features, related either on the GO physical-
276 chemical properties (thickness, size, surface functionalization, aggregation state and
277 concentration) or on the synthesis method[28,29], the experimental conditions adopted here and
278 in our previous works[19,20] exclude any cytotoxic effect on glial cells or neurons, both *in vitro*
279 and *in vivo*.

280 MVs are released into the extracellular space by direct budding from the plasma membrane of
281 astrocytes.[30] To explore the dynamics of MVs release in control, in s-GO treated and in ATP
282 treated (see below) astrocytes, we measured the presence of changes in membrane trafficking by
283 briefly incubating cultures with the fluorescent styryl dye FM1-43 and then quantifying the
284 astrocyte-membrane fluorescence decay to provide a cumulative measure of exocytosis in the
285 different growth conditions.[19,30]

286 FM dyes are fluorescent probes that reversibly stain membranes, and are largely used for
287 optical real-time measurements of membrane dynamics and secretory processes.[31–33]
288 Incubation with the FM dye (2 μ M, 2 min) resulted in clear surface membrane staining of
289 control, bzATP, an agent known to evoke massive MVs release (100 μ M, 30 min) and s-GO
290 treated cultures (10 μ g/mL, 6 days), highlighted in Figure 1e (left panels).[30] Brighter spots
291 were considered as adherent debris and were excluded from the analysis. Besides these, both
292 bright and weak FM-stained plasma membrane domains were present along the whole
293 cytoplasmic surface and became visible within 2 min incubation (Fig. 1e). Due to this initial
294 variability in the intensity of the membrane staining, all FM de-staining measures were
295 normalized to the relative time 0. Once astrocyte membranes were labeled by the fluorescent dye
296 FM1-43 we measured the plasma membrane de-staining over a fixed time (10 min) in control, in

297 s-GO treated cultures, or during acute exposure to bzATP, under the same culturing
298 conditions.[19] Representative fluorescence intensity traces are shown in Figure 1f (top plot); the
299 dynamic of the fluorescence decay observed in control cultures, indicates the presence of
300 physiological bleaching of fluorophore over the acquisition time course, however s-GO and
301 bzATP groups, despite the same bleaching-induced loss of fluorescence, showed a faster de-
302 staining rate in respect to controls. This is also visualized by the time-lapse images framed at
303 time 0 s, 300 s and 600 s of the crude recordings (Figure 1e middle and right panels). We
304 quantified the fluorescence decay time constant (τ) values (box plot of Figure 1f) and detected
305 shorter decay values in both bzATP and s-GO groups ($\text{median}_{\text{control}} = 219$ s; $\text{median}_{\text{bzATP}} = 184.2$
306 s; $\text{median}_{\text{s-GO}} = 163.9$ s). This result suggested that the membrane de-staining was actually related
307 to MVs release, as expected in bzATP treated cells, more than to other membrane turnover
308 activities. Such a release was comparable between bzATP and s-GO, both significantly faster
309 than controls ($P_{\text{bzATP}} < 0.001$; $P_{\text{s-GO}} < 0.001$).



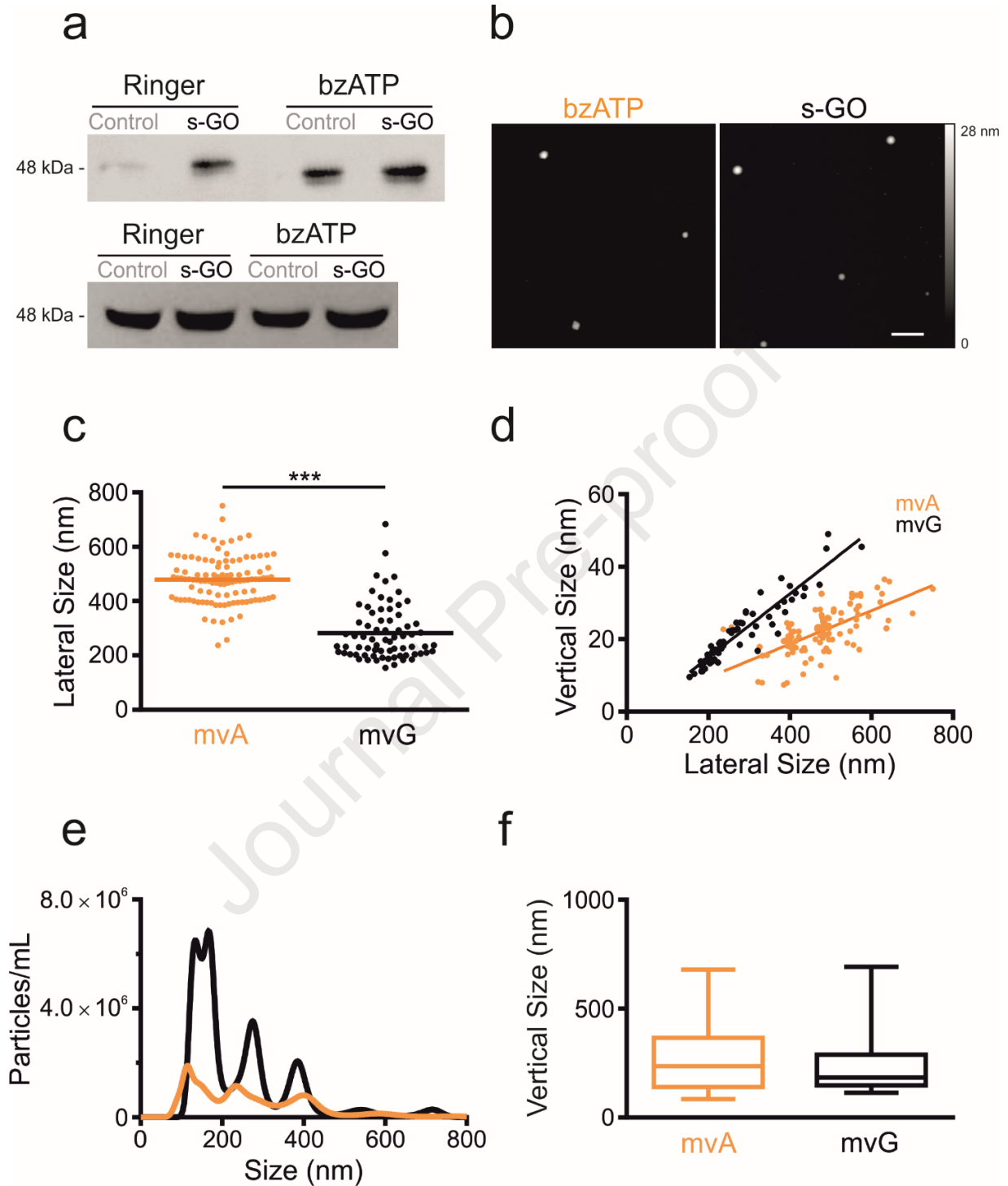
311 **Fig. 1. Graphene oxide nanosheets does not affect astrocytes vitality.** Cultured astrocytes release microvesicles
 312 (MVs) upon ATP or s-GO stimulation. a) Confocal images visualize cultured astrocytes in control and after s-GO
 313 (10 $\mu\text{g}/\text{mL}$; 6 days) treatment; anti-GFAP, in green, and DAPI (to visualize nuclei), in blue; scale bar 50 μm . b) Box
 314 plot summarizes the cell density measures; note the similar values in both groups. c) Confocal images visualize
 315 cultured astrocytes in control and after s-GO (10 $\mu\text{g}/\text{mL}$; 6 days) treatment. Cultures were treated with propidium
 316 iodide (PI) to visualize death cells. Anti-GFAP, in green, DAPI (to visualize nuclei), in blue and PI in red; scale bar
 317 50 μm . d) Histogram summarizes the percentage of death cells followed the s-GO exposure and in control condition;
 318 note that there are no significant difference between two groups e) Surface membrane staining and activity
 319 dependent de-staining of FM1-43 in cultured astrocytes, scale bar 25 μm . f) Normalized FM1-43 de-staining traces
 320 (top) in control astrocytes (light grey), in bzATP treated once (orange) and in s-GO treated once (black). The box
 321 plot (bottom) summarizes the decay time constant τ of FM1-43 de-staining in the three conditions (median_{control} =
 322 219.2 s; median_{bzATP} = 184.2 s; median_{s-GO} = 163.9 s). Thick horizontal bars in the box plots indicate median value;
 323 boxed area extends from the 25th to 75th percentiles, whiskers from 2.5th to the 97.5th percentiles. Significance: **P <
 324 0.01 ***P < 0.001, Kruskal-Wallis test, Dunn's post hoc test).

326 The release of MVs suggested by FM1-43 measures, was confirmed by immunoblot analysis
 327 for the biomarker flotillin-1 of the supernatant collected from control and treated cultures (Fig.
 328 2a). As expected, bzATP stimulation and s-GO incubation induced the appearance of a thick
 329 band corresponding to flotillin-1 (Figure 2a), a signature of MVs release by astrocytes, with an
 330 additive effect between s-GO exposure and pharmacological stimulation with bzATP (s-GO_{Ringer}
 331 is quantified as 100 % more than Control_{Ringer}; bzATP_{Ringer} is quantified as 360 % more than
 332 Control_{Ringer}; bzATP_{s-GO} is quantified as 2900 % more than Control_{Ringer}. Calculated over three
 333 independent experiments).[19,30,34] In control conditions only a weak band was perceived,
 334 indicating that MVs constitutive release in culture was poorly detectable. Atomic force
 335 microscopy (AFM) topographic reconstruction of re-suspended MVs pellet (Fig. 2b) confirmed
 336 the presence of MVs detected by the immunoblot in both bzATP (mvA) and s-GO (mvG)
 337 groups. When investigating the effect of shorter (3 days) exposure to s-GO, western blot
 338 experiments (supplementary Fig. S2) showed the absence of a significant increase in MVs
 339 constitutive release when compared to control. Yet, bzATP release of MVs was potentiated by 3

340 days s-GO (supplementary Fig. S2) suggesting that s-GO already modulated MVs release, but
341 longer time of s-GO exposure are needed to enhance basal release in the absence of additional
342 stimuli. In an additional set of western blot experiments, we tested the sensitivity of bzATP and
343 s-GO MVs release to extracellular calcium deprivation (supplementary Fig. S3). Differently
344 from bzATP, s-GO release was apparently not affected by calcium removal. In control condition,
345 a thick band appeared upon calcium removal, suggestive of an increase in constitutive release
346 (supplementary Fig. S3). These preliminary results hint at release mechanisms differently tuned
347 by calcium among control, bzATP and s-GO and require further studies.

348 We systematically investigated and compared the MV size distribution by means of AFM and
349 nanoparticle tracking analysis (NTA) measurements. AFM images show the presence of
350 roundish protrusions of dimensions compatible with the size of MVs. No other kind of
351 contaminant was present, to indicate that the procedure for isolating and collecting MVs from the
352 medium was clean and effective. When analyzed by AFM (Fig. 2c) s-GO-derived MVs (mvG)
353 lateral size were significantly smaller ($n = 72$, $\text{median}_{\text{mvG}} = 244 \text{ nm}$) than bzATP-derived ones
354 (mvA; $n = 107$, $\text{median}_{\text{mvA}} = 479 \text{ nm}$) ($P < 0.001$). Conversely, we detected no differences in MV
355 height values ($\text{median}_{\text{mvG}} = 19 \text{ nm}$; $\text{median}_{\text{mvA}} = 22 \text{ nm}$; $P = 0.17$). Within each group, the
356 distribution of size values detected was not negatively correlated to the height, as shown in
357 Figure 2d (left; $r_{\text{mvG}} = 0.8808$ and $r_{\text{mvA}} = 0.4039$; $P < 0.001$). However, AFM experiments were
358 performed in air, thus a not specific flattening of MVs caused by vesicle collapsing might have
359 influenced these measurements. In principle differences in MV elastic properties, potentially
360 related to diverse membrane components, might lead to a variable collapsing of MVs when
361 measured in air.

362 Since AFM measurements are affected by the reduced size of the analyzed samples, and might
363 not reflect the entire MVs population, we decided to use nanoparticle tracking analysis (NTA) to
364 perform bulk analysis of vesicles in aqueous suspension. NTA tracks single particle Brownian
365 motion within a dark field microscope, derives mean square vesicles velocity and translates them
366 into size distribution.[35] NTA revealed a more complex pattern of size distribution (Fig. 2e): in
367 the case of mvA we observed three subpopulations of vesicles at 115 nm, 235 nm and 400 nm
368 respectively while in the case of mvG we found two partially overlapping peaks at 135 nm and
369 168 nm, plus two distinct peaks at 275 nm and 385 nm (Fig. 2e). The diameter analysis revealed
370 a slight, but not significant, difference between the two populations with the diameter of mvG
371 smaller and less distributed, compared to those of mvA ($\text{median}_{\text{mvA}} = 235.4 \text{ nm}$; $\text{median}_{\text{mvG}} =$
372 183.6 nm) (Fig. 2f). These results convincingly suggested a comparable size distribution in both
373 MV populations and subpopulations. However, we detected a significant difference in the
374 number of vesicles released within the same time window (Fig. 2e) to indicate that cultures
375 treated with s-GO produced more MVs when compared to cultures stimulated with bzATP (mvA
376 $= \sim 3.32 \times 10^8 \text{ vesicle/mL}$; mvG $= \sim 8.19 \times 10^8 \text{ vesicles/mL}$), consistently with our results obtained
377 by MV release analysis and immunoblot (but see also Visnovitz et al. 2019[36]). Therefore, even
378 though the overall size of the MV population produced did not change, s-GO was more efficient
379 in generating MVs from astrocytes.



380

381 **Fig. 2. Graphene oxide nanosheets and bzATP induce MVs release in Astrocytes.** Microvesicle released by glial
 382 cells *via* bzATP or s-GO stimulations characterized by ultra-resolution approaches. a) Western blotting of the pellets
 383 (top row) and cell lysates (bottom row) for the MV marker flotillin-1 (N=3). Pellets were obtained from the medium
 384 of glial cultures treated or untreated (control) with s-GO under two different conditions: not stimulated (ringer) or

385 stimulated (bzATP) by 100 μM bzATP. s-GO_{Ringer} is quantified as 100% more than Control_{Ringer}; bzATP_{Ringer} is
386 quantified as 360% more than Control_{Ringer}; bzATP_{s-GO} is quantified as 2900% more than Control_{Ringer}. Calculated
387 over three independent experiments. b) AFM topographic reconstruction of MVs isolated from cultured primary
388 astrocytes treated with bzATP (100 μM) and s-GO (10 $\mu\text{g}/\text{mL}$) and performed in air (semi-contact mode). Scale bar
389 500 nm. c) Lateral size values distribution and median values for both groups, note that mvG lateral size is
390 significantly smaller than that of mvA (median_{mvA} = 479 nm; median_{mvG} = 244.1 nm; ***P > 0.001, Mann-Whitney
391 test). d) AFM measures of lateral size are plotted against AFM measures of height of MVs isolated from glial cells
392 treated by bzATP (100 μM ; mvA; in orange) or by s-GO (10 $\mu\text{g}/\text{mL}$; mvG; in black). e) Size distribution of MVs
393 isolated from glial cells treated by bzATP (100 μM ; in orange) or by s-GO (10 $\mu\text{g}/\text{mL}$; in black) measured by
394 nanoparticle tracking analysis (NTA). Values of the peaks are expressed in nm. f) Lateral size values distribution
395 and median values for both groups, obtained by NTA measurement.

396

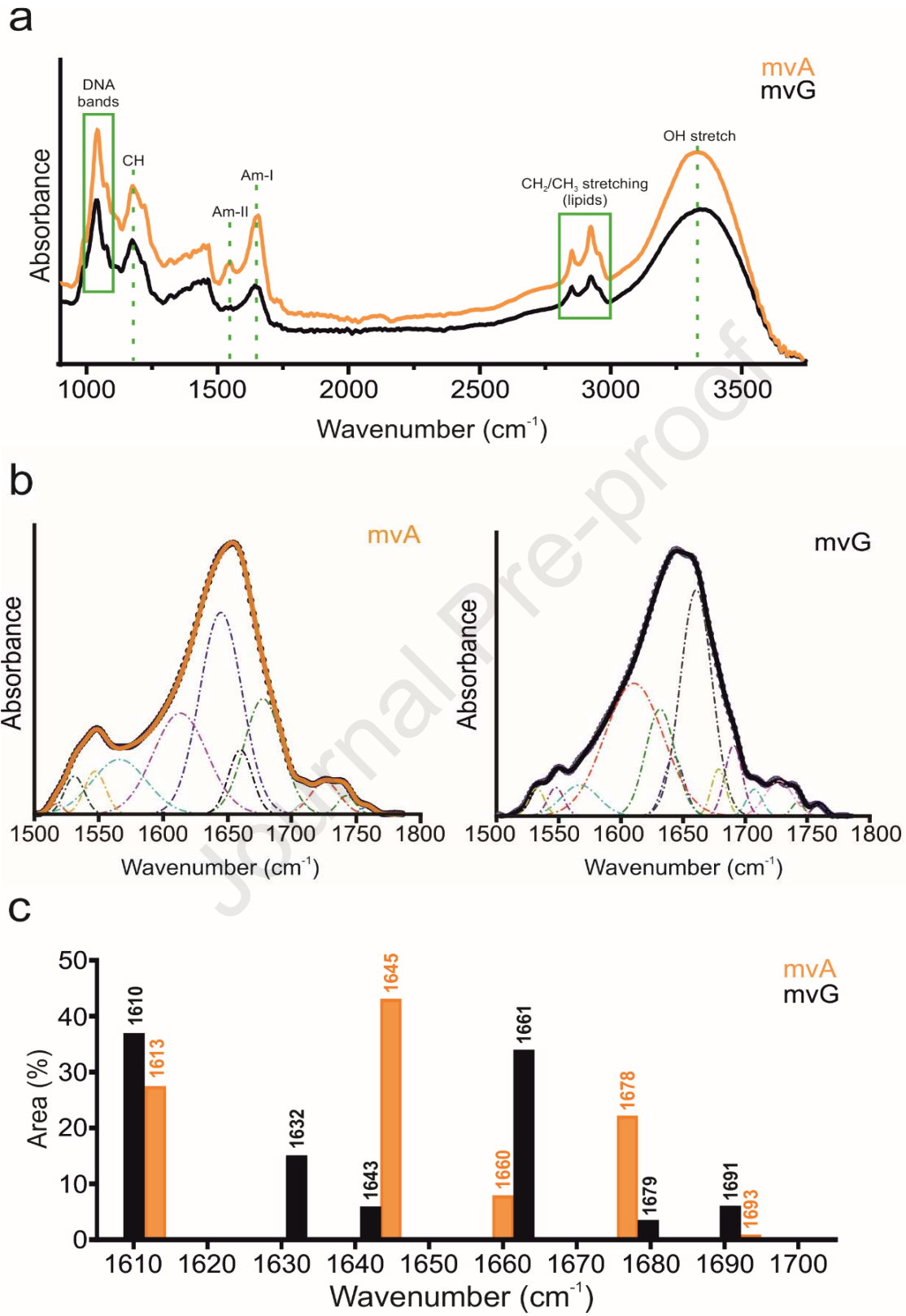
397

398 In order to analyze the macromolecular composition of MVs we took advantage of two
399 complementary techniques: FTIR-ATR and UVRR spectroscopy. For these measurements, MVs
400 were isolated by differential centrifugation as described before (see Material and Methods). To
401 avoid any spurious effect due to the absorption of phosphate groups from the buffer, we washed
402 and re-suspended the MVs pellet with a NaCl solution (150 mM). The infrared (IR) absorbance
403 spectra (Fig. 3a) revealed a clear contribution of the CH and the phosphate bands linked to DNA
404 in both samples, mvA and mvG, as well as the lipid signatures at 2900-3000 cm^{-1} , arisen from
405 the CH₂ and CH₃ stretching mode. The amide I and amide II bands between 1500 and 1785 cm^{-1} ,
406 the two major protein bands in the IR spectrum, show more pronounced differences between the
407 two populations of vesicles. The amide I band, which is primarily related to the C=O stretching
408 in the peptide bonds and modulated by the proteins' secondary structures, displayed similar
409 shapes for mvA and mvG. The amide II band, primarily due to C-N stretching and N-H in plane
410 bending vibrations, also reflecting the protein secondary structure, was instead clearly depleted
411 in the case of mvG. To gain insights into the contribution of different protein secondary
412 structures in the two families of vesicles, we analyzed the second derivative of the IR signal in

413 the amide I + amide II region (Fig. 3b) and used the position of the minima to guide a
414 multicomponent gaussian fit of the bands in the region 1500 and 1785 cm^{-1} . The % area
415 contributions obtained by the fit are reported in Fig. 3c. The low-energy flank of amide I of both
416 mvA and mvG is characterized by the presence of the 1610-1613 cm^{-1} vibrational peak which
417 could be addressed to a mixed contribution arisen from β -sheets and side-chains vibrations.
418 Additionally, the band at 1660 cm^{-1} might also be derived from the presence of RNA in the
419 vesicles.[37] Noteworthy, the relative populations of the peaks at approximately 1645 cm^{-1} ,
420 1660 cm^{-1} and 1678 cm^{-1} are inverted between mvA and mvG. According to the literature[37],
421 the 1645 cm^{-1} peak (depleted in mvG) might be assigned to random structures and/or helices; the
422 1660 cm^{-1} peak (depleted in mvA) to flexible helices (as 3_{10} helices); the 1678 cm^{-1} peak
423 (depleted in mvG) to beta structures such as b-turns. The bands at 1632 cm^{-1} and at 1693 cm^{-1} ,
424 both present in the mvG only, are ascribed to anti-parallel beta sheet, as found in aggregates in
425 tissues.[37,38] We can conclude that mvGs contain proteins with perturbed secondary structure,
426 characterized by beta structure-based aggregates and flexible helices. No significant variations in
427 the DNA phosphate bands were measured as well as the nature or localization of the DNA
428 signature detected. It is interesting to note that in previous measures the DNA delivered by EVs
429 has been reported to be stocked either inside the vesicles or on their surface[39].

430 UV Resonant Raman (UVRR) measurements taken using an excitation wavelength of 244 nm
431 (see experimental section and supplementary Fig. S4) confirmed the amide I signal depression in
432 mvG absorption spectrum. Due to an overlap between the UVRR s-GO band, we could not
433 confirm or discard the presence of s-GO inside the vesicles. Thus, we cannot exclude that the
434 changes in protein native structure might be due to s-GO altering the MV
435 micro(nano)environment or the possibility of a general interference with the measurements. The

436 absence of astrocytes cytotoxicity, even upon prolonged exposure to s-GO, together with the
437 functional measures of increased MVs release kinetic obtained by live imaging, are against a
438 mere alteration in protein integrity due to denaturation of membrane proteins in the presence of
439 s-GO.[40–42] FTIR-ATR spectroscopy is a powerful tool to assess the disordered character of
440 proteins, and the absence of a well-defined structure under native conditions is a peculiar
441 property of intrinsically disordered proteins (IDP).[43,44] In this framework, IDPs might
442 represent a specific signal vehiculated by mvG and the lack of protein structural constraints
443 could facilitate several, yet unknown, biological processes.[44]



445 **Fig. 3. MVs characterization by infrared spectroscopy.** Microvesicles produced by glial cells *via* bzATP or s-GO
446 stimulation and characterized by infrared spectroscopy. a) Infrared spectra of microvesicles obtained by bzATP
447 stimulation (mvA, in orange) or by s-GO exposure (mvG, in black) in the region 950-3600 cm^{-1} . Contributions
448 arisen from nucleic acids, proteins and lipids characterize the spectra. The two boxes in green and the green dashed
449 lines are used as eye-guides to highlight nucleic acids, $\text{CH}_2\text{-CH}_3$ stretching of lipids and protein amide bands,
450 respectively. b) Fitting procedure applied to mvA (on the left) and mvG (on the right) amide bands spectra.
451 Multicomponent Gaussian curves were used to actually reproduce the experimental data. The centres of the
452 Gaussian curves were chosen as the minimum of the 2nd derivative of the spectrum and kept free to vary within 4
453 cm^{-1} around its maximum. c) Histograms representing the areas of the Gaussian curves used to reproduce the
454 experimental data of mvA (in orange) and mvG (in black) in the region 1500-1785 cm^{-1} . The areas of each band has
455 been weighted respect to the total amide band area which they belongs to (i.e. 1640 cm^{-1} band has been weighted
456 with respect to the total Am-I band area).

457 It is clearly visible that mvA and mvG vesicles have a different secondary structure: the former is mainly
458 characterized by an intense peak at 1645 cm^{-1} (43%), which can be assigned to random structures/a-helix structure,
459 and at 1678 cm^{-1} (22%) usually assigned to b-turn and at 1613 cm^{-1} (27%), which could be assigned to side chains
460 vibrations; in contrast, mvG are mainly characterized by anti-parallel b-sheets structure (1632 (15%) and 1693 cm^{-1}
461 (3%) bands) with a strong contribution of flexible 3-10 helix (1661 cm^{-1} peak (34%)) and of side chain band at 1610
462 cm^{-1} (37%).

463

464 Finally, we set up a functional test to compare the impact of mvG delivery with that of mvA on
465 synaptic activity, when neuronal networks are acutely and transiently exposed to MVs. To this
466 aim, we isolated cortical neurons and glial cells from postnatal rat cortices and cultured them for
467 10 days. Fig. 4a shows confocal high magnification microscopy images of cortical cultures
468 where neurons were visualized by labeling class III β -tubulin (in red), a microtubule component
469 expressed exclusively by neurons, while astrocytes were visualized by GFAP labeling (in
470 green).[45] We patch-clamped visually identified cortical neurons (in voltage clamp
471 configuration, holding potential – 70 mV), while a second pipette for the local delivery of saline
472 solution was positioned at a distance of 200 μm (under microscopy visual control) from the
473 recorded cell (sketched in Fig. 4a, right). We estimated that, at this distance, the application of a
474 brief (500 ms) pulse of pressure should result in a local (i.e. on the recorded neuron) and

475 transient delivery of standard saline solution alone or containing mvG or mvA (re-suspended in
476 saline). A typical feature of these cultures is the prominent expression of spontaneous synaptic
477 activity, represented by heterogeneous postsynaptic currents (PSCs) of variable frequency and
478 amplitude (box plots in Fig. 4b). Baseline PSCs were recorded before (10 min) and after (15
479 min) the local saline, mvG or mvA ejection. Fig. 4c shows representative current tracings where
480 standard saline was pressure ejected (light grey, top), or where mvA solution (orange, middle)
481 and mvG (black, bottom) were administered.

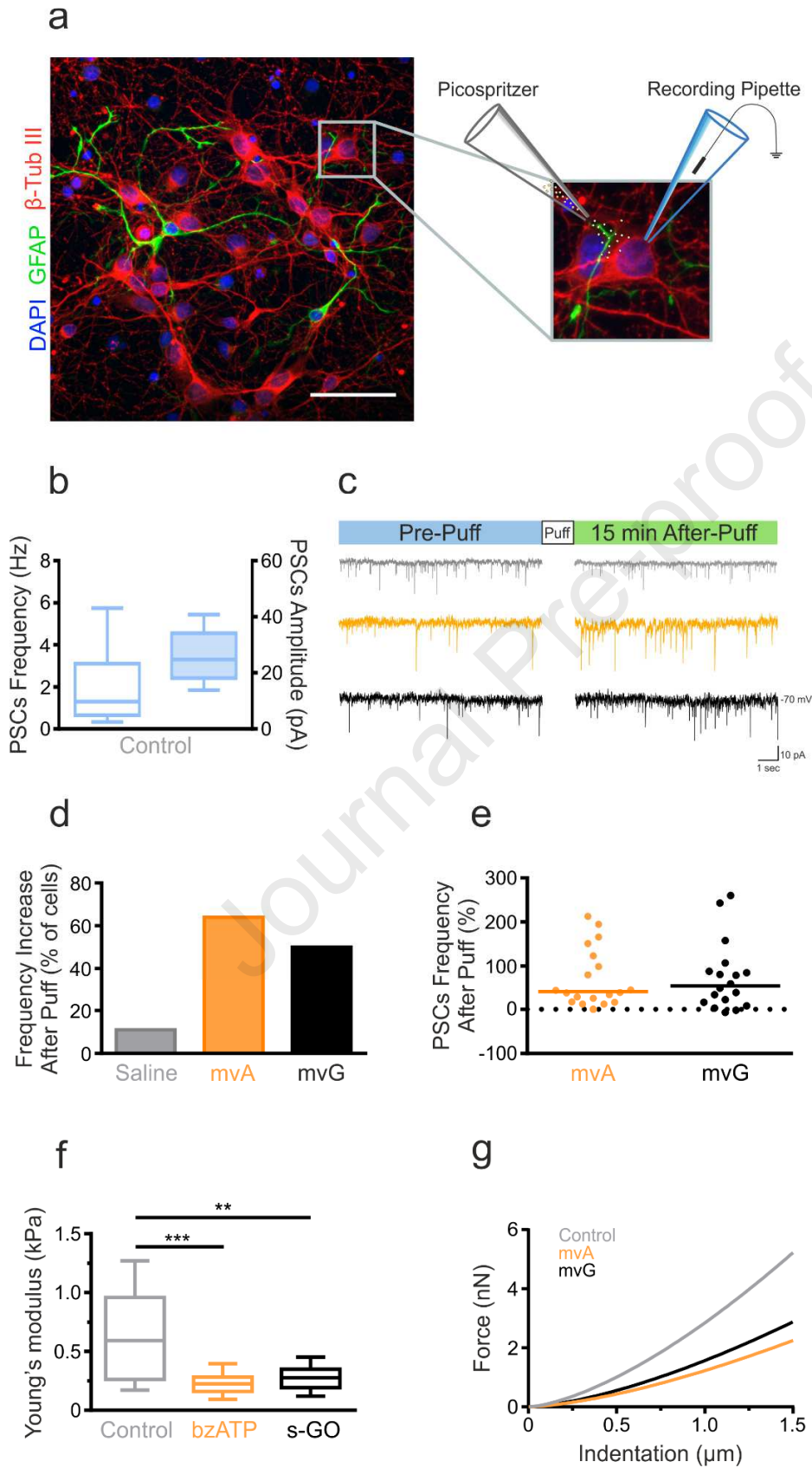
482 Since spontaneous fluctuations in PSCs frequency $\leq 15\%$ of baseline values were frequently
483 detected, we took this as the threshold value to estimate changes when comparing PSCs before
484 and after pressure ejections of saline. In the large majority (88 %, $n = 16/18$ neurons; histograms
485 in Fig. 4d) of neurons exposed to saline solution alone, spontaneous PSCs frequency did not
486 change. On the contrary, within 5-8 min from the acute mvA and mvG ejections, PSCs frequency
487 was stably increased in 64 % ($n = 16/25$ neurons, mvA) and 54 % ($n = 13/24$ neurons, mvG;
488 summarized in the histograms of Fig. 4d) of recorded neurons. Fig. 4e shows the increases in
489 PSCs frequency in individual experiments and highlights the variability of such changes when
490 administering MVs, with increased frequencies ranging from 25 % to 200 %. Since we could not
491 experimentally control the amount of MVs collected by primary astrocytes and delivered by
492 pressure ejection, neurons were exposed to different amounts of MVs and this can in part explain
493 the detected variability. PSCs frequency increases due to MVs exposures were not reversible
494 upon 20 min washout. From such functional investigation, glial-signaling generated by ATP or s-
495 GO affected similarly neuronal synapses upon transient, direct exposure.

496 To our knowledge, this is the first time that the functional effects of MVs generated by
497 astrocytes on synaptic activity upon local delivery have been electrophysiologically documented.

498 Neuroglia extracellular vesicles have been described to provide support on synaptic activity, with
499 the majority of studies focused on microglia and inflammation, apparently regulating neural
500 transmission at the pre-synaptic level.[5,46–48] Astrocytic MVs have been proposed to exert
501 neuroprotective effects in neuropathology and in physiology, however the role of astrocytes or of
502 discrete astrocyte populations in delivering different messages via MV release has yet to be
503 elucidated.[49,50]

504 After assessment of the ability of MVs released by glial cells to affect cortical neuron
505 physiology within min after their interaction with the targeted neuron, we asked if the presumed
506 fusion of vesicles with neuronal plasma membrane could also affect their mechanical properties.
507 To investigate this aspect, we delivered MVs obtained from glial cultures previously treated with
508 bzATP or s-GO to cortical neurons by re-suspending the isolated MVs in 100 μ L of neuronal
509 culture medium and adding them to neuronal cultures. 24 hours after the exposure, force
510 spectroscopy measurement on treated neuronal cultures were performed with AFM by
511 positioning the tipless cantilever with a borosilicate glass bead previously glued on it[51], at the
512 center of randomly chosen neurons. As showed by the boxplot in Fig. 4f, the exposure to mvA
513 caused a significant softening of neuronal soma, when compared to controls ($\text{median}_{\text{mvA}} = 0.22$
514 kPa, $\text{median}_{\text{control}} = 0.59$ kPa; $P_{\text{mvA}} < 0.001$). A similar result was observed also in the case of
515 mvG exposure even if the effect exerted on neuronal stiffness is less pronounced than that
516 induced by mvA ($\text{median}_{\text{mvG}} = 0.28$ kPa, $\text{median}_{\text{control}} = 0.59$ kPa; $P_{\text{mvG}} < 0.01$). The reported
517 effect of mvA and mvG on neuronal cell mechanical properties is presumably a consequence of
518 vesicular fusion with the cellular plasma membrane, which may affect its lipid composition.
519 Since the mechanical properties of a cell are mostly defined by plasma membrane features and
520 the ones of the underlying cytoskeleton, a change in neuronal plasma membrane lipid

521 composition can partially justify the observed reduction in mvA and mvG-treated neurons
522 stiffness.[52] In particular, glia-derived MVs are able to transport the enzyme Acid
523 sphingomyelinase (A-SMase) involved in the metabolism of sphingomyelin (SM), a precursor of
524 the phospholipid sphingosine (sph). Sph and its metabolites have been already reported to play a
525 fundamental role in facilitating synaptic vesicles release by changing the membrane composition
526 at pre-synaptic level.[48,53] This intrinsic capacity of MVs to participate in membrane lipid
527 metabolism may therefore modulate the contribution of plasma membrane to neuronal rigidity.
528 In this context, the slight difference of stiffness values reported among mvA and mvG-treated
529 neurons, although not statistically significant ($P_{mvA-mvG} > 0.05$) should not be
530 underestimated.[54,55] It is tempting to speculate that this difference can be explained by the
531 presence of specific proteins in the vesicles, that are unstable in mvG. Based on our measures,
532 we cannot exclude the presence of residual s-GO flakes either inside or on the vesicle surface. It
533 is known that GO is reportedly able to interact with the hydrophobic region of biological and
534 model lipid membranes, even though the nature of the interaction is strictly dependent to its size
535 and degree of surface oxygenation.[56,57] Assuming that the GO nanosheets, once added to the
536 culture medium, can adsorb on plasma membrane or pierce it and being embedded in the lipid
537 bilayer, there is a concrete possibility that MVs, which directly originate from plasma membrane,
538 can include those flakes in their structure.[30] The horizontal transfer of s-GO from glial cells to
539 cortical neurons, mediated by MVs, may therefore affect plasma membrane rigidity of targeted
540 neurons. Regardless of this, we can exclude a direct effect of s-GO in synaptic transmission,
541 reported to be transient and reversible upon acute exposure, in view of the persistent modulation
542 of synaptic current frequency brought about by MVs.[19,20]



543

544 **Fig. 4. MVs released by astrocytes affect cortical neuron post-synaptic activity and mechanical properties.**
 545 Potentiation of synaptic activity upon local applications of MVs in cortical neurons. a) Confocal micrograph
 546 visualizing cortical primary cultures at 8 days in vitro; anti-class III β -tubulin is used to visualize neurons (in red),
 547 anti-GFAP for astrocytes (in green) and DAPI (in blue) to visualize neurons. Scale bar 50 μ m. On the right, a
 548 representation of the experimental setting for the simultaneous MVs pressure-release (*puff*) and the cell patch-clamp
 549 recording from cultured neurons. b) Box plot summarizes the PSCs frequency and amplitude values in control
 550 cortical neurons. c) Top: diagram of the experimental protocol. Bottom: representative current tracings of the
 551 spontaneous synaptic activity detected prior and after puff applications of control saline (in light grey) or mvA (in
 552 orange) or mvG (in black). d) Bar plots of pooled data summarize the % of cells displaying PSCs frequency increase
 553 upon delivery of pressure ejected saline (light grey), mvA (orange) and mvG (black). Note that in control (saline)
 554 the large majority (88 %) of neurons did not increase their basal activity. e) The plot summarizes the distribution of
 555 the % of increase in PCSs frequency detected within the three groups. f) Elastic moduli of cortical neurons, grown
 556 on glass, and exposed to MVs isolated from glial cells previously treated with bzATP (orange) or s-GO flakes
 557 (black). Neurons treated with mvA and mvG are significantly less rigid if compared with control ($P_{mvA} < 0.001$;
 558 $P_{mvG} < 0.01$). Thick horizontal bars in the box plots indicate median value; boxed area extends from the 25th to 75th
 559 percentiles, whiskers from 5th to the 95th percentiles. Significance: **P < 0.01 ***P < 0.001, Kruskal-Wallis test,
 560 Dunn's post hoc test) g. Indentation curves of cortical neurons previously treated with mvA or mvG.
 561

562 4. Conclusion

563 Astrocyte-derived MVs may play significant roles in propagating signaling molecules, in CNS
 564 physiology and disease. Despite the increasing knowledge on extracellular vesicles (in particular
 565 exosomes) ability to promote inflammation or contribute in spreading of pathogenic proteins in
 566 neurodegenerative disorders (from Amyotrophic Lateral Sclerosis to Alzheimer disease [58,59])
 567 little is currently known on their properties (mechanical, biochemical, lipid membrane
 568 composition, cargo nature etc.), in particular when focusing on the smaller class of such natural
 569 vectors, the MVs. Nevertheless, MVs emerge as key players in neuronal and synaptic
 570 physiology, able to influence neurotransmission, or to support neurons.[48,60–62] In our study,
 571 we report the ability of artificially generating MVs by s-GO transient exposure. MVs generated
 572 by s-GO were apparently characterized by altered protein content when compared to the ATP-
 573 driven ones. Intriguingly, the tuning of synaptic activity by mvG or mvA was similar,

574 supposedly being related to features diverse from MV protein content. The ability of s-GO to
575 interfere with exo-endocytotic membrane dynamics is not surprising, indeed we have described
576 the ability of s-GO nanoflakes to interfere with presynaptic vesicle release *in vitro* and *in*
577 *vivo*. [19,20] In the current work, we describe the direct interference of MVs with synaptic
578 activity, presumably due to MVs fusion with the target neuron plasma membrane. Such an
579 approach holds the potential to open new opportunities in engineering MVs for synaptic
580 targeting. In this framework, it is tempting to speculate that s-GO interactions with the cell
581 membrane mimic extracellular mechanical signaling at the nanoscale sufficient to enable the
582 release of MVs, thus representing unconventional tools to exploit the physics governing vesicle
583 release. We feel pertinent here to consider the fact, that the enormous potentiality of graphene-
584 based materials in nanomedicine has already promoted the development of new generation-
585 nanocarriers for either gene or drug delivery [17,63]. In this framework, we may speculate on
586 future developments where engineered cells are mechanically induced to release MVs, carrying
587 GO nanoflakes properly functionalized to deliver genes or drugs of interest and thus representing
588 either the trigger and the cargo.

589

590 ASSOCIATED CONTENT

591 **Supporting Information**

592 Supporting experimental section

593 Supporting results, Figures S1–S4 and Table S1.

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600 **Author Contributions**

601 M.M. performed cell biology, electrophysiology, and immunofluorescence experiments and
602 analysis; M.M. and P.P. designed and performed AFM experiments; M.P. performed IR and
603 UVRR experiments and analysis. CM and GDM performed biology and WB experiments; N.L.
604 and K.K. contributed to the synthesis and characterization of thin graphene oxide of biological
605 grade. BB performed SEM micrographs of GO. L.B. and L.C. conceived the study; L.B.
606 conceived the experimental design and contributed to the analysis of data; L.B. wrote the
607 manuscript.

608 **Notes**

609 The authors declare no competing financial interest.

610

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Journal Pre-proof

HIGHLIGHTS

Graphene oxide interferes with cell membrane dynamics and enhance astrocytes' release of MVs

MVs driven by graphene oxide stimuli display a different protein profile from chemically driven ones

MVs released upon graphene oxide exposure affect neuronal signaling and membrane stiffness

Journal Pre-proof

Shedding Plasma Membrane Vesicles Induced by Graphene Oxide Nanoflakes in Brain Cultured Astrocytes

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Conflict of interest

Authors declare no competing interests.