

1 **SUMOylation regulates protein cargo in Astrocyte-derived small**
2 **extracellular vesicles**

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33 ABSTRACT

34 Recent studies have described a new mechanism of intercellular communication mediated by
35 various types of extracellular vesicles (EVs). In particular, exosomes are small EVs (sEVs) released to
36 the extracellular environment by the fusion of the endosomal pathway-related multivesicular
37 bodies (containing intraluminal vesicles) with the plasma membrane. sEVs contain a molecular cargo
38 consisting of lipids, proteins, and nucleic acids. However, the loading mechanisms for this complex
39 molecular cargo have not yet been completely elucidated. In that line, the post translational
40 modification SUMO (Small Ubiquitin-like Modifier) has been shown to impact the incorporation of
41 select proteins into sEVs. We therefore decided to investigate whether SUMOylation is a
42 mechanism that defines protein loading to sEVs. In order to investigate the role of SUMOylation
43 in cargo loading into sEVs, we utilized astrocytes, an essential cell type of the central nervous system
44 with homeostatic functions, to study the impact of SUMOylation on the protein cargo of sEVs.
45 Following SUMO overexpression, achieved by transfection of SUMO plasmids or experimental
46 conditions that modulate SUMOylation in primary astrocyte cultures, we detected proteins related
47 to cell division, translation, and transcription by mass-spectrometry. In astrocyte cultures treated
48 with the general SUMOylation inhibitor 2-D08 (2',3',4'-trihydroxy-flavone, 2-(2,3,4-
49 Trihydroxyphenyl)-4H-1-Benzopyran-4-one) we observed an increase in the number of sEVs and a
50 decreased amount of protein cargo within them. In turn, in astrocytes treated with the stress
51 hormone corticosterone, we found an increase of SUMO-2 conjugated proteins and sEVs from these
52 cells contained an augmented protein cargo. In this case, the proteins detected with mass-
53 spectrometry were mostly proteins related to protein translation. To test whether astrocyte-derived
54 sEVs obtained in these experimental conditions could modulate protein synthesis in target cells, we
55 incubated primary neurons with astrocyte-derived sEVs. sEVs from corticosterone-treated
56 astrocytes stimulated protein synthesis while no difference was found with sEVs derived from 2-
57 D08-treated astrocytes. Our results show that SUMO conjugation plays a fundamental role in
58 defining the protein cargo of sEVs impacting the physiological function of target cells.

59

60 **Keywords:** sEV; exosomes; SUMOylation; post-translational modification; astrocytes; neurons.

61

62 INTRODUCTION

63 Extracellular vesicles (EVs), i.e. extracellular structures enclosed by a lipid bilayer, are novel players
64 in intercellular communication (Mathieu, Martin-Jaular, Lavieu, & Théry, 2019; Van Niel, D'Angelo,
65 & Raposo, 2018). EVs that are released after the fusion of multivesicular bodies (MVBs) with the
66 plasma membrane, allowing the secretion of intraluminal vesicles (ILVs) of a diameter from 30 to
67 160 nm, are termed exosomes. In turn, when EVs are generated directly from the plasma
68 membrane, their diameter varies between 30 nm and 1 μ m, and they are called microvesicles. Given
69 the heterogeneity of EV preparations, exosome-containing fractions are now more precisely termed
70 small extracellular vesicles (sEVs) (Colombo, Raposo, & Théry, 2014; Witwer & Théry, 2019).
71 Remarkably, the molecular content (i.e. lipids, proteins and nucleic acids) of sEVs depends on the
72 physiological or pathophysiological state of the donor cell (Mathieu et al., 2019). Regarding proteins,
73 sEVs contain a common protein pattern (or protein markers), such as CD63, CD9, Flotillin or TSG101,

74 as well as a cell type-specific and physiological state-dependent protein cargo (Doyle & Wang, 2019;
75 Jeppesen et al., 2019; Kowal et al., 2016; Zhang, Liu, Liu, & Tang, 2019).

76 The transfer of sEVs to target cells can regulate the recipient cell function in a cargo-dependent
77 manner, e.g. influencing the development or progression pathophysiological processes (Isola &
78 Chen, 2016; Kalluri & LeBleu, 2020). It is therefore of great relevance to understand precisely the
79 mechanisms involved in the loading of biomolecules, which will define the biological effect of sEVs.

80 The mechanisms that participate in the biogenesis of sEVs may act independently or in coordination
81 with the classification/loading of molecules into the vesicles (Simons & Raposo, 2009). The first
82 mechanism described depends on the endosomal sorting complex required for transport (ESCRT),
83 composed of 4 multiproteic complexes (0 to III). Secondly, ceramides constitute a primary lipid
84 factor with physical and structural properties that facilitate the biogenesis of intra-luminal vesicles
85 (Holopainen, Angelova, & Kinnunen, 2000; Simons & Raposo, 2009). The third mechanism is through
86 tetraspanins, which are capable of modulating the formation of membrane microdomains, playing
87 a fundamental role not only in the formation of vesicles, but also in the selection of proteins that
88 are specifically incorporated into them (Zhang et al., 2019).

89 Post-translational modifications that contribute to the selective loading of proteins into sEVs include
90 protein modification by ubiquitin and ubiquitin-like proteins (UBLs) such as SUMO (Small Ubiquitin-
91 like Modifier) (Ageta & Tsuchida, 2019; Colombo et al., 2014). SUMO conjugation to lysine residues
92 impacts on the function of proteins and importantly, the formation of multi-protein complexes (J.
93 M. Desterro, Thomson, & Hay, 1997; Johnson & Blobel, 1997). Moreover, SUMO can interact in a
94 non-covalent manner to SUMO interactive motif, also known as SIM (Hecker, Rabiller, Haglund,
95 Bayer, & Dikic, 2006; Song, Durrin, Wilkinson, Krontiris, & Chen, 2004). There are three functional
96 SUMO homologs in mammals with a molecular weight of ~10kDa: SUMO-1, SUMO-2, SUMO-3 (J. M.
97 P. Desterro, Rodriguez, Kemp, & Ronald T, 1999; Liang et al., 2016; Melchior, 2000). SUMOylation
98 regulates the activity, stability, and sub-cellular localization of proteins, primarily modifying protein-
99 protein interactions (Geiss-Friedlander & Melchior, 2007). SUMO modification is involved in a wide
100 variety of cellular processes, affecting among others translation, transcription, replication,
101 chromosome segregation, DNA repair, differentiation, apoptosis, senescence, cell cycle, nuclear
102 transport and signal transduction (Hay, 2005; Hendriks & Vertegaal, 2016; Khan, Pandupuspitasari,
103 Huang, Hao, & Zhang, 2016; Pichler, Fatouros, Lee, & Eisenhardt, 2017).

104 This modification affects the ability of the ribonucleoprotein hnRNPA2B1 to export microRNAs
105 (miRNAs) in sEVs (Villarroya-Beltri et al., 2013). Moreover, α -synuclein is SUMOylated in the sEV
106 lumen while GFP is sorted into sEVs only when it is conjugated with SUMO-2 (Kunadt et al., 2015).
107 In the same context, in homogenized primary astrocyte cultures, the glycolytic enzyme Aldolase C
108 (ALDOC) is detected with two molecular weights: 36kDa (expected weight) and 55kDa (the
109 putatively SUMO-conjugated form). Importantly, in rat serum sEVs, only the high molecular weight
110 form of the enzyme can be found suggesting that SUMOylation determines the loading of this
111 enzyme (Gómez-Molina et al., 2019). In summary, these results strongly support the concept that
112 SUMO conjugation of proteins might dynamically regulate the classification and loading of proteins
113 in sEVs. Previously, the effect of SUMOylation as a general loading mechanism in sEVs had not
114 been described, neither were the proteins SUMOylated within sEVs. In the present work, we
115 describe that SUMOylation regulates the protein cargo of sEVs derived from HeLa cells and
116 astrocytes. Identification of proteins by mass spectrometry indicates that after stimulation of

117 SUMOylation in donor cells (by SUMO overexpression or corticosterone treatment), proteins related
118 to cell division, transcription and translation are enhanced in sEVs. Moreover, sEVs derived from
119 corticosterone-treated donor astrocytes increase *de novo* protein synthesis in target neurons.

120

121 **METHODOLOGY**

122 **Reagents and antibodies.** α ALDOA (#390733), α ALIX (#53540), α EF-2 (#166415), α SUMO-1 (#5308)
123 were purchased from Santa Cruz (Dallas, Texas). α FLOTILLIN (#610820) and α GM130 (#610823),
124 were purchased from BD Transduction Labs (New Jersey, USA). Goat anti mouse (#926-80010), Goat
125 anti rabbit (#926-80011) were purchased from Licor (Nebraska, USA). Donkey anti Sheep (#16041),
126 Alexa Fluor 488 mouse (#A21202), DMEM medium (#12100046), Fetal bovine serum (#26140079),
127 G418 (#11811023) Penicillin/Streptomycin (#15140122), neurobasal medium (#21103049), B27
128 (#17504044), OptiMEM (#31985062), Lipofectamine (#11668030), PBS (#14190), Total Exosome
129 Isolation kit (#4478359), chemiluminescence kit (#32106), DMSO (#85190), IL-1 β (#14-8018-62),
130 MOWIOL (#81381) were purchased from Thermo Fisher (Massachusetts, USA). N-Ethylmaleimide
131 (NEM, #34115), DAPI (4',6-diamidino-2-phenylindole; D9542), Poly-D-Lysine (#4174),
132 Corticosterone (#27840), 2-D08 (#1052) were purchased from Sigma (Missouri, USA). MAP2
133 (#MAB378) was purchased from Millipore (Massachusetts, USA). α GFAP (#G2032) were purchased
134 from US Biological (Massachusetts, United States). Cheap α SUMO-2 was generously donated by Dr.
135 Ronald T. Hay, University of Dundee, UK.

136 **Cell culture.** HeLa cells were maintained in DMEM containing 10% Fetal bovine serum with 100
137 units/ml of penicillin and 100 μ g/ml of streptomycin and 200 μ g/ml of G418 (for maintaining stable
138 cell lines) incubated at 37°C, with 5% CO₂ and 95% humidity. Astrocytes were obtained from the
139 telencephalon of post-natal rats (at postnatal day 1) as already described (Luarte et al., 2020).
140 Astrocytes were maintained in DMEM containing 10% Fetal bovine serum with 100 units/ml of
141 penicillin and 100 μ g/ml of streptomycin incubated at 37°C, with 5% CO₂ and 95% humidity. On days
142 4 and 8 *in vitro* (DIV), a total change of medium was made. After 15 DIV, the astrocytes were re-
143 plated to decrease the presence of microglia and seeded at a confluence of 70-80%. Cortical
144 neurons of the rat embryo brain (E18) were dissociated from the cerebral cortex and 20,000-40,000
145 cells were seeded on coverslips coated with Poly-D-Lysine using 35mm plates. Cortical neurons were
146 maintained *in vitro* for 15 days in neurobasal medium supplemented with B27 and 100 units/mL of
147 penicillin and 100 μ g/mL of streptomycin incubated at 37°C, with 5% CO₂ and 95% humidity. Cells
148 were cultured in plates until they reached 60% confluence or after 15DIV (astrocytes and cortical
149 neurons). At that time, some wells were transfected or treated with different reagent, as indicated
150 in each case. At the end of these treatments, cells were trypsinized, resuspended in phosphate-
151 buffered saline (PBS) and an aliquot was counted using a Neubauer chamber.

152 **Transfections.** Transfections were performed after reaching 60% cell confluence using a 3:1 ratio of
153 DNA: Lipofectamine in Opti-MEM. pCDNA3.1 HIS-SUMO-1 and HIS-SUMO-2 plasmid were
154 generously donated by Dr. Ronald T. Hay, University of Dundee, UK.

155 **Obtaining sEVs.** Cell cultures were grown in a sEV free culture medium for 72 hours (DMEM with
156 10% FBS, depleted by ultracentrifugation for 2 hours at 100,000xg). Then, the conditioned media of
157 these cells was harvested to isolate sEVs by ultracentrifugation. sEVs were isolated by serial
158 centrifugations as already described (Luarte et al., 2020; Théry, Amigorena, Raposo, & Clayton,

159 2006): After 30 minutes at 2,000g the supernatant was recovered, the centrifuged for 45 minutes
160 at 10,000g. Then the supernatant was centrifuged for 2 hours at 100,000g, the supernatant was
161 eliminated and the sEV enriched pellet was washed and re-suspended in PBS and stored at -80°C
162 until use. Or, using Total Exosome Isolation kit (Thermo-Fisher), the sEV containing fraction was
163 obtained by centrifugation for 30 minutes at 2,000g. The supernatant was resuspended with 0.5
164 volumes of the Total Exosome Isolation solution and incubated overnight at 4°C. Then the mix was
165 centrifuged for 1 hour at 10,000g and the pellet was resuspended in PBS and stored at -80°C until
166 use.

167 **Nanoparticle tracking analysis.** The nano particle tracking analysis (Nanosight NS300, Malvern
168 Instruments, Malvern, UK) was used to determine particle concentration and size distribution.
169 Samples were diluted 5 or 10 times in PBS to obtain ≥80 particles per field for analysis and 3 videos
170 were recorded with a duration of 30 seconds each.

171 **Western blots.** Cells were lysed in RIPA buffer (150mM NaCl, 25mM Tris-HCL pH 7.4, NP-40, 0.5%
172 sodium deoxycholate, 20mM NEM (N-Ethylmaleimide)). Protein quantification of the homogenates
173 and sEVs (sEV resuspended with with 0.1% SDS) were determined using the BCA method (Smith et
174 al., 1985). The samples were boiled with loading buffer and 20mM NEM (N-ethyl-maleimide, modify
175 cysteine residues in proteins and peptides) for 5 minutes at 100°C. Each lane was loaded with either
176 the same amount of total protein or the same number of vesicles, as depicted. Proteins were
177 separated in 12% polyacrylamide gels under denaturing conditions. Gels were stained with
178 Coomassie dye or transferred to nitrocellulose membranes. The membranes were incubated with
179 blocking solution (5% nonfat milk in PBS) for one hour, then incubated over night with primary
180 antibody. The membranes were washed with PBS. Finally, they were incubated with secondary
181 antibody for 45 minutes and then visualized with a chemiluminescence kit (Pierce ECL #32109).

182 **SIM-SUMO pulldown.** SIM-HALO resins consisting of the SIM sequence fused with a HALO tag and
183 linked to a resin were incubated with 200 µg of sEV protein with RIPA buffer and 20mM NEM for 16
184 hours at 4°C. They were centrifuged at 500xg for 5 min and washed 8 times with RIPA with 20mM
185 NEM. The supernatant was removed each time. Finally, the pellet was resuspended with loading
186 buffer and 20mM NEM, the samples were boiled, and centrifuged at 10,000xg for 10 minutes and
187 then analyzed by SDS-PAGE followed by Coomassie staining. 10% of the Input eluates were loaded.

188 **Proteomics.** sEV proteins were separated using polyacrylamide gradient gel electrophoresis. Each
189 lane was divided into 8 sections to perform in-gel digestion. Liquid chromatography followed by
190 tandem-mass spectrometry (MS/MS) of the sample fractions was performed on a hybrid dual-
191 pressure linear ion trap/orbitrap mass spectrometer (LTQ Orbitrap Velos Pro, Thermo Scientific)
192 equipped with an EASY-nLC Ultra HPLC (Thermo Scientific). Peptide samples were dissolved in 10 µL
193 of 2% acetonitrile/0.1% trifluoric acid and fractionated on a 75-µm i.d., 25-cm PepMap C18-column,
194 packed with 2 µm of resin (Dionex, Germany). Separation was achieved by applying a gradient of 2%
195 to 35% acetonitrile in 0.1% formic acid over 150 minutes at a flow rate of 300 nL/min. The LTQ
196 Orbitrap Velos Pro MS was exclusively used for CID fragmentation when acquiring MS/MS spectra,
197 which consisted of an orbitrap full mass spectrometry (MS) scan followed by up to 15 LTQ MS/MS
198 experiments (TOP15) on the most abundant ions detected in the full MS scan. The essential MS
199 settings were as follows: full MS (resolution, 60,000; mass to charge ratio range, 400–2000); MS/MS
200 (Linear Trap; minimum signal threshold, 500; isolation width, 2 Da; dynamic exclusion time setting,
201 30 seconds; and singly charged ions were excluded from the selection). Normalized collision energy

202 was set to 35%, and activation time was set to 10 milliseconds. Raw data processing and protein
203 identification were performed by ProteomeDiscoverer 1.4 (Thermo Scientific) and a combined
204 database search used the Sequest and Mascot algorithms. The false discovery rate was calculated
205 by the Percolator 2.04 algorithm and was set to <1%. An Ingenuity Pathway Analysis was used for
206 network analysis of functional interactions of proteins (Gómez-Molina et al., 2019). The bio-
207 informatic analysis was done by DAVID Bioinformatics Resources 6.8. pValue was represented as -
208 Log₁₀.

209 **Incubation of target cells with sEVs.** A total of 1000 vesicles was added per cell, and this was
210 repeated 24 hours later. Then, 48 hours later, the cells were used for the respective assays: MTT (3-
211 (4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), DAPI (4',6-diamidino-2-
212 phenylindole), PI (Propidium iodide) or FUNCAT assay (fluorescence non-canonical amino acid
213 tagging).

214 **Donor cell treatments.** The culture medium was completely changed to sEV free culture medium
215 and the following treatments added 1 hour later: 10ng/ml interleukin 1 β (IL-1 β), 1 μ M
216 Corticosterone (CORT) or 30 μ M 2-D08 (inhibitor of the enzyme UBC9, SUMO-conjugating enzyme)
217 or DMSO (as a control). This was repeated 24 and 48 hours later, and the conditioned medium was
218 collected 72 hours later. The medium was collected to isolate sEVs and the cells were homogenized
219 and stored at -80°C until further use.

220 **Morphological analysis.** Neurons incubated with astrocyte-derived sEVs obtained in the different
221 experimental conditions were analyzed using Sholl analysis using plugins from ImageJ software
222 (National Institute of Health, USA). The dendritic tree was examined in 3 μ m increments. The
223 following parameters were obtained: total dendrite length (i.e., the largest radius at which there is
224 an intersection with a neuronal process) and total number of intersections (i.e., the sum of all
225 intersections with each different radius) (Luarte et al., 2020).

226 **FUNCAT (fluorescent click chemistry) and immunofluorescence.** To visualize newly synthesized
227 proteins in cells, the FUNCAT method was used according to Daniela Dieterich (Dieck et al., 2012).
228 Cultures were incubated with the non-canonical amino-acid AHA (L-azidohomoalaine), which was
229 incorporated into newly synthesized proteins instead of methionine. As a negative control,
230 methionine was used. Then, the neurons were fixed and the FUNCAT assay was performed. Briefly,
231 cells were washed with cold PBS-MC (1 mM MgCl₂, 0.1 mM CaCl₂ in PBS pH7.4) and directly fixed
232 with 4% paraformaldehyde for 30 minutes, then washed 3 times with PBS pH 7.4 and incubated with
233 B-Block solution (10% normal horse serum, 5% sucrose, 2% BSA, 0.2% TritonX-100 in PBS pH 7.4) for
234 1 hour at room temperature. Cells were newly washed 3 times with PBS pH 7.8 and incubated
235 overnight with FUNCAT solution (0.2mM Triazole ligand (Tris[(1-benzyl-1H-1,2,3-triazol-4-
236 yl)methyl]amine), 0.5mM TCEP [Tris-(2-carboxyethyl)phosphine hydrochloride], TAMRA (red-
237 fluorescent tetramethylrhodamine) Alkyne tag, 40 μ g/ml CuSO₄ in PBS pH 7.8). The cells were then
238 washed with PBS pH 7.8 and then with PBS pH 7.4 and incubated with primary antibody in B-block+
239 0.2% TritonX-100 for 2 hours at room temperature, then were washed with PBS pH 7.4 and
240 incubated with secondary antibody in B-Block solution for 1 hour at room temperature. Finally, the
241 cells were incubated for 10 min with 300mM DAPI in PBS 7.4, washed with PBS pH 7.4 and mounted
242 using MOWIOL mounting media. The images were taken with a confocal LSM 800 Zeiss microscope.
243 The FUNCAT signal was visualized as a fire lookup table, to visually favor the range of expression of

244 the newly synthesized proteins through a range of colors from blue to white, where blue is the
245 absence of new protein synthesis.

246 **Statistical analysis.** One-way ANOVA were performed followed by a post-hoc Tukey test using
247 GraphPad Prism version 5 for Windows, GraphPad Software, La Jolla California USA.

248

249 **RESULTS**

250 **SUMOylation in astrocytes leads to a differential cargo in the derived sEVs**

251 Our initial studies were to characterize the vesicles obtained by two methods of sEV purification,
252 the ultracentrifugation method (UC) and a commercial vesicle isolation kit (IK). Both methods
253 produce samples with a similar protein pattern observed in the Coomassie staining. Western blots
254 revealed that the sEV marker protein Alix was enriched in the vesicles compared to the donor
255 astrocytes using both methods. Flotillin in turn was present, but not enriched, while a protein that
256 is not contained in sEVs, GM130, was not detected in the sEV fraction (Figure 1A). Using either
257 method nanoparticle tracking analysis (NTA) showed a similar particle size profile obtaining a mean
258 of 152 nm in the case of UC (Figure 1B) and of 156 nm after using the IK (Figure 1C) in the sEV
259 fractions isolated.

260 To evaluate the consequences of SUMOylation on the protein content of astrocyte-derived sEVs,
261 transfections of astrocytes with HIS-SUMO-1 (S1) or HIS-SUMO-2 (S2) were carried out, using GFP
262 as a transfection control. We obtained a transfection efficiency of 10-15% by lipofection, evaluated
263 by the detection of GFP-positive cells (data not shown). SUMO conjugation in astrocytes was
264 evaluated by Western blot (Supplementary figure 1A), and in both cases a slightly increased
265 SUMOylation was observed, but do not significant (Supplementary figure 1B and 1C). The UC
266 derived sEVs were subjected to Coomassie staining (Figure 2A) and analyzed by NTA (Figures 2B and
267 2C). No differences were found in the mean size and sEV yield. At that time, the protein content
268 (in ng) per vesicle was calculated (Figure 2D), and an increase in the protein load in S2 astrocyte-
269 derived sEVs was found. Then, sEV proteins were identified by mass spectrometry (Figure 3 and
270 Supplementary table 1). The Venn and subsequent GO analysis using the DAVID database indicate
271 that the 512 common proteins were significantly enriched in classical exosome components (Figure
272 3A), confirming that the analyzed fractions are enriched in sEVs (data not shown). When exclusive
273 proteins among conditions were compared, an enrichment in biological processes such as
274 transcription and cell division were found in S1 and S2 sEVs (Figure 3B). To directly identify
275 SUMOylated proteins, we performed a pulldown (PD) of astrocyte-derived sEVs with SIM-HALO
276 resins. Proteins contained in the sEVs (input) and proteins bound to SIM resin were shown by
277 Coomassie staining (Figure 3C). A prominent band was observed over 26kDa in pulled down samples
278 (SIM-bound). In the samples that did not bind to SIM-beads, a banding pattern similar to the input
279 was found at high molecular weights. The samples obtained in the PD were analyzed by mass
280 spectrometry (Supplementary table 1) and we identified an enrichment in biological processes such
281 as Chromatin organization, mRNA processing and cell division (Figure 3D).

282 Next, a metabolism-related protein ALDOLASE A, (ALDOA) and protein synthesis implicated protein
283 Eukaryotic elongation factor 2 (EEF-2) were validated by Western blot. Equal amounts of protein
284 were loaded per lane in the case of cell samples, while an equal number of vesicles was used in the

285 case of sEV samples. (Supplementary figure 2A). ALDOA in cell samples was found with a molecular
286 weight of ~36kDa, corresponding to the predicted molecular weight interestingly. In sEVs, ALDOA
287 was observed at ~55kDa, corresponding to possible SUMOylated form(s), in S1 and S2-derived sEVs
288 (Supplementary figure 2C). Another band is observed at a weight greater than 250 kDa, which could
289 be compatible with a polySUMOylated form of ALDOA. This form is consistently elevated in sEVs
290 from S1 astrocytes (Supplementary figure 2B). In turn, EEF-2 was detected at the predicted
291 molecular weight. This protein tends to have a higher content in sEVs from S1 astrocytes
292 (Supplementary figure 2D).

293 **Modulation of SUMOylation in astrocytes and-derived sEVs under pathophysiologic-like** 294 **conditions**

295 In order to change the environmental conditions of sEV donor cells, astrocytes were incubated
296 either with IL-1 β , a powerful pro-inflammatory cytokine reported to stimulate SUMOylation
297 (Hajmler et al., 2014; Miranda, Loeser, & Yammani, 2010), or with corticosterone as an *in vitro* stress
298 model, because stress *in vivo* enhances levels of SUMOylated proteins in sEVs (Gómez-Molina et
299 al., 2019). In the opposing direction, SUMOylation was inhibited with 2-D08, an inhibitor of the
300 enzyme E2 (Ubc9) (Kim, Keyser, & Schneekloth, 2014).

301 Astrocytes were treated one time per day for 3 days with 10ng/ml IL-1 β , 1 μ M Corticosterone
302 (CORT), 30 μ M 2-D08 or DMSO as control. We first analyzed the SUMOylation pattern of proteins by
303 Western blot (Figure 4A). Quantification showed that corticosterone increased the conjugation of
304 proteins with SUMO-2, while 2-D08 decreased protein conjugation with SUMO-1 and SUMO-2
305 (Figure 4B and C). To check whether astrocytes were affected after the treatments, the astrocyte
306 marker protein glial fibrillary acid protein (GFAP) was detected in these cultures: 2-D08 decreased
307 GFAP immunoreactivity, and a recovery of its levels was obtained after corticosterone plus 2-D08
308 (Figure 4D and E).

309 Then, we studied the possible effects of these sEVs, isolated by IK, on target cells. First, the same
310 number of vesicles were loaded in each lane of an SDS-PAGE gel to observe the general protein
311 pattern (Figure 5A). As confirmed later, the 2 D08-derived sEVs contained less proteins. There were
312 no differences in the average size of the vesicles derived from the three experimental conditions, as
313 revealed by NTA (Figure 5B). Subsequently, the number of vesicles released per one million cells
314 was quantified. The total number of cells was quantified at the time of sEV collection (Figure 5C). A
315 strong increase of sEVs released from cells treated with 2-D08 was observed, while a decrease in
316 the amount of proteins contained per sEV was observed after 2-D08 treatment (Figure 5D). This
317 result is compatible with the previous Coomassie staining. Interestingly, in HeLa cells a similar effect
318 of 2-D08 was found (Supplementary figure 3).

319 **Effect of astrocyte-derived sEVs on protein synthesis in neurons**

320 We next identified the sEV protein content by mass spectrometry derived from control,
321 corticosterone or 2-D08 treated astrocytes (Figure 6A and Supplementary table 3). In total, 149
322 exclusive proteins were identified in sEVs from corticosterone treated astrocytes. Of these, 19 were
323 related with protein synthesis (Table 1). Comparing protein synthesis-related hits (biological
324 processes) among conditions, an enrichment in rRNA transcription and transcription by RNA
325 polymerase I was found in sEVs from corticosterone-treated astrocytes. Then, we could predict
326 possible SUMOylation sites in 15 of these proteins using the GPS-SUMO database (Zhao et al., 2014).

327 Due to the large representation of protein synthesis regulatory proteins in sEVs after corticosterone
328 treatment, we decided to study whether astrocyte-derived sEVs could modulate protein synthesis
329 in target cells, i.e. in neurons. It was previously shown that astrocyte sEVs can be taken up by
330 neurons (Luarte et al., 2020). Moreover, the relationship between SUMOylation and gene
331 expression or transcription regulation has been described extensively (Liu & Shuai, 2008; Müller,
332 Ledl, & Schmidt, 2004; Rosonina, Akhter, Dou, Babu, & Sri Theivakadadcham, 2017), as well as its
333 relationship with protein translation (Hendriks & Vertegaal, 2016; Nie, Xie, Loo, & Courey, 2009; X.
334 Xu, Vatsyayan, Gao, Bakkenist, & Hu, 2010).

335 Mature cortical neurons (21DIV) were incubated with sEVs derived from astrocytes treated with
336 DMSO as a control, 2-D08 or CORT. To visualize the neurites of neurons in culture,
337 immunofluorescence using a MAP2 antibody was performed and Sholl analysis was made to
338 quantify dendritic arborization parameters (Figure 7A). We did not observe significant differences
339 in the total dendrite length (Figure 7B). When analyzing the total number of intersections of
340 dendritic branches (i.e. reflecting the dendritic arbor complexity), we found that neurons incubated
341 with sEVs of control astrocytes presented a significant decrease compared to neurons that were not
342 incubated with sEVs. This negative regulation is compatible with our previous results using 3 to 6
343 DIV neurons and suggest that astrocyte sEVs are implicated in dendritic pruning and/or in decreased
344 growth (Figure 7C). Interestingly, an increase in the synthesis of new proteins in the soma of neurons
345 incubated with sEVs derived from CORT-treated astrocytes was observed (Figure 7D and 7E). We
346 observed no changes in the FUNCAT signal in neurons incubated with control astrocyte sEVs or with
347 sEVs from astrocytes treated with the SUMOylation inhibitor (2-D08), compared with the signal in
348 untreated neurons. This confirms that sEVs derived from corticosterone-treated astrocytes contain
349 an increased number or level of proteins involved in the stimulation of protein synthesis.

350 **DISCUSSION**

351 In recent years, sEVs have acquired an important place in the sights of many researchers in the area
352 of neuroscience, due to the fact that they are secreted by most cells, can modulate target cell
353 function (such as neuronal function), are present in the cerebrospinal fluid, and cross the blood
354 brain barrier (Gómez-Molina et al., 2019; Liu et al., 2019). The presence of circulating sEVs in body
355 fluids has opened the possibility that their molecular content could be used as a non-invasive
356 diagnostic strategy to obtain biomarkers of physiological and pathological situations (Lin et al., 2015;
357 Saeedi, Israel, Nagy, & Turecki, 2019; Zhang et al., 2019). Given the outstanding characteristics of
358 these vesicles in the biomedical field, it was important for us to better understand the enigmatic
359 mechanism of cargo biomolecule determination in them. Literature background led us to propose
360 that SUMOylation corresponds to an important component that defines the proteins of sEVs.

361 **SUMOylation and its effect on astrocytes**

362 In astrocytes, the overexpression of SUMO-1 and SUMO-2 did not substantially increase the levels
363 of SUMOylation. This may be due to the low levels of transfection that we obtained in astrocytes,
364 of about 15%. This is consistent with publications that describe the low transfection efficiency of
365 astrocytes (Alabdullah et al., 2019), but also, that SUMOylation is a transient event that does not
366 increase steady state levels (Henley, Craig, & Wilkinson, 2014; Klug et al., 2013).

367 In order to complement our observations regarding the role of SUMO overexpression, we used the
368 SUMOylation inhibitor 2-D08. Other SUMOylation inhibitors such as Ginkgolic acid and Anacardic

369 acid had been previously described (Fukuda et al., 2009), while more recently, the 2-D08 inhibitor
370 was described (Kim et al., 2014). Several groups have already used this inhibitor to decrease
371 SUMOylation: for example, in U937 cells, 2-D08 restored the anti-proliferative activity of retinoids
372 (Baik et al., 2018; Lin et al., 2020; Lorente et al., 2019; Zhou et al., 2019). Accordingly, 2-D08 inhibited
373 SUMOylation by SUMO-1 or SUMO-2, although both proteins regulate different cellular pathways.
374 Specific SUMO inhibitors act at the level of non-covalent SIM interactions, thus providing an indirect
375 inhibition of SUMO-regulated proteins (Hughes et al., 2017).

376 Previously, we had found that in serum sEVs of rats, a possibly SUMOylated form of ALDOC
377 increased under stress conditions (Gómez-Molina et al., 2019; Ramírez, 2017). The stress response
378 might be positively associated with this post-translational modification, to trigger the incorporation
379 of a different set of proteins in sEVs. Thus, we decided to explore the effect of *in vitro* stress on
380 SUMO levels in our experimental model using corticosterone. Despite observing an increase in
381 SUMO-2ylation in astrocytes treated with corticosterone, we did not observe a significant increase
382 in SUMO-1ylation. It would be interesting to explore under other *in vitro* stress conditions such as
383 oxidative stress, heat stress, hypoxia, starving, or others whether this preference is a general stress
384 effect. Moreover, these results favor again the idea that SUMO-1 and SUMO-2 protein modifications
385 exert different biological roles or functions.

386 **SUMOylation and its effect on sEV cargo**

387 The idea that SUMOylation participates in the process of protein loading in sEVs, is supported by
388 the work showing that hnRNPA2B1 (Villarroya-Beltri et al., 2013) and α -synuclein (Kunadt et al.,
389 2015) sorting depend on SUMOylation. Here, we determined that this mechanism broadly affects
390 the loading of proteins into sEVs both in HeLa cells as well as in astrocytes.

391 Few studies focus on the total protein content in sEVs and/or the number of sEVs released under
392 different SUMOylation conditions. Ageta described that the ubiquitin-like 3 (UBL3)/membrane-
393 anchored Ub-fold protein (MUB) UBL3 is necessary for protein sorting in sEVs. In serum sEVs of UBL3
394 knock-out animals, a strong reduction of the total protein content in vesicles was found when gels
395 were loaded with the same volume of a rat serum derived sEV suspension (Ageta et al., 2018). In a
396 different study in cells transfected with siRNAs targeting TSG101 and Hrs, which are proteins of the
397 ESCRT system, a decrease in the number and the total amount of proteins contained in sEVs was
398 induced (Smith, Jackson, & Schorey, 2015). However, in this study the number of sEVs were not
399 related to the parent cell number, which is an important issue to be considered, because the number
400 of cells can vary according to the treatment.

401 Despite the low levels of SUMO expression in astrocytes, the modification by SUMO-2 caused an
402 increase in protein content in the derived vesicles. One of the possible explanations for such an
403 effect is the “SUMO paradox”, in general only a small fraction of the SUMO modified proteins is
404 detected at a particular time point. However, while SUMO modification is involved in dynamic
405 processes, many proteins will be modified at a given time. Thus, the enhancement or inhibition of
406 SUMOylation strongly impacts the biological outcome, this also known as the history of SUMO
407 modification (Hay, 2005) i.e. in our case, even low levels of SUMOylation is sufficient to influence
408 protein loading of sEVs (Geiss-Friedlander & Melchior, 2007).

409 We show for the first time that inhibition of SUMOylation using 2-D08 in both astrocytes and HeLa
410 cells could significantly increase the number of harvested sEVs. The release of other vesicle types,

411 such as of synaptic vesicles, is regulated by SUMOylation (Craig, Anderson, Evans, Girach, & Henley,
412 2015; Tang, Craig, & Henley, 2015). Interestingly, in pancreatic β cells and synaptic vesicles in
413 neurons, the SUMOylation of STXBP5 (Tomosyn1A), a protein related with docking and fusion of
414 vesicles is necessary for its binding with STX1A (Syntaxin-1A) and suppression of exocytosis. In such
415 a way, inhibition of STXBP5 SUMOylation leads to an increase of exocytosis (Ferdaoussi et al., 2017).
416 These proteins are also involved in the fusion of MVBs to the plasma membrane to release
417 intraluminal vesicles (Geerts et al., 2017; Zhang et al., 2006). We thus speculate that SUMOylation
418 of this protein could also be involved in the endo- exocytosis balance of MVB favoring the exocytosis
419 when SUMOylation is inhibited.

420 **Identification of sEV proteins derived from cells that over-express SUMO**

421 The SUMOylated proteins contained in sEVs and the biological processes in which these proteins
422 are involved have been poorly described. We found proteins related to the synthesis of proteins in
423 sEVs from astrocytes that overexpressed SUMO-1 or SUMO-2. Previously, the relationship of SUMO
424 with chromosome stabilization, DNA replication, mRNA splicing and transcription and translation in
425 cells had already been described in cells (Hendriks & Vertegaal, 2016; Nie et al., 2009; X. Xu et al.,
426 2010). Possibly SUMOylated proteins in astrocyte sEVs were detected after pull downs with SIM
427 domains. From them, EF-2, a protein related with protein synthesis was enriched in sEVs from S1
428 and S2 astrocytes, strongly suggesting that SUMOylation is a cellular process that defines EF-2
429 loading. However, the protein itself is detected at the expected molecular weight and thus, is
430 unlikely to be SUMOylated, possibly it could be loaded into sEVs by interaction with another
431 SUMOylated protein that acts as a “carrier”.

432 In turn, ALDOA was a common protein in sEVs from astrocytes related with cellular metabolism. We
433 detected this protein with high molecular weights in sEVs, 55kDa and over 130kDa. Both molecular
434 weights could coincide with different SUMOylation forms, the first is a mono-SUMOylation and the
435 second a polySUMOylation (Pichler et al., 2017). The 130kDa form is mostly detected in sEV of
436 astrocytes transfected with SUMO-1. It would be interesting to mutate the possible K residues that
437 are SUMO acceptors to demonstrate that ALDOA loading depends on SUMOylation.

438 **Functional effect of astrocyte-derived sEVs on neurons**

439 When neurons were incubated with control astrocyte sEVs, a decrease of dendritic arbor complexity
440 was observed. This can be interpreted as increased pruning and thus enhanced network maturation
441 during this time period or decreased growth (Luarte et al., 2020). Intriguingly, sEVs derived from
442 corticosterone treated astrocytes did not affect dendritic length or complexity, but stimulated
443 protein synthesis. The functional roles of the newly synthesized proteins need to be determined in
444 the future. As the dendritic architecture depends on many different signaling pathways and
445 molecular mechanisms (Arikkath, 2012; Skelton, Poquerusse, Salinaro, Li, & Luikart, 2020; J. Xu et
446 al., 2019; Ziegler & Tavasani, 2019). The contribution of specific molecules and proteins contained
447 in sEVs on it deserves many future studies.

448 The methodology used by us to detect de novo protein synthesis in neurons allows visualization of
449 newly synthesized proteins with high sensitivity (Dieck et al., 2012). The effect of sEVs on protein
450 synthesis was abolished when astrocytes had been treated with SUMOylation inhibitor 2-D08,
451 suggesting that SUMOylation is necessary to load proteins related with protein synthesis in sEVs.
452 The relationship between SUMOylation and with chromosome stabilization, DNA replication, mRNA

453 splicing and transcription has been widely described in cells (Hendriks & Vertegaal, 2016; Nie et al.,
454 2009; X. Xu et al., 2010).

455 In summary when astrocytes are under stress, they increase the conjugation of proteins with SUMO-
456 2 and allow the loading of proteins related to the synthesis of proteins within their sEVs. To confirm
457 that SUMOylation is necessary for protein loading in sEVs, by inhibiting of SUMOylation with 2-D08
458 in 2 different cell types (HeLa and astrocytes) protein load per sEV was decreased, confirming the
459 importance of SUMOylation in protein loading in sEVs. In turn, sEVs can cause changes in target cells
460 such as neurons by changing dendritic arborization and increasing *de novo* protein synthesis, when
461 the sEVs are from stressed astrocytes.

462

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469 **Author Contribution.** AF, AR and UW designed the experiments and wrote the manuscript. AF
470 prepared all figures of the manuscript. The experimental work was done by: Figure 1, generated by
471 AF; Figure 2, generated by AF; Figure 3, generated by AF and OS; TK supervised the mass
472 spectrometry; AF did the bio-informatic analysis. Figure 4, generated by AF and MM; Figure 5,
473 generated by AF. Figure 6 generated by AF; TK supervised the mass spectrometry; AF did the bio-
474 informatic analysis. Figure 7 generated by AF; PL supervised the FUNCAT. Supplementary Figure 1
475 generated by AF. Supplementary Figure 2 generated by AF. Supplementary Figure 3 generated by
476 AF. KC and TG prepared astrocytes primary cultures in all experiments.

477 **Competing Financial Interests.** All authors declare that they have no conflict of interests

478

479

480 **FIGURE LEGENDS**

481 **Figure 1. Characterization of small Extracellular Vesicles (sEVs) from astrocytes.** A) Coomassie
482 staining and sEVs-enriched proteins detected by Western blot of sEVs isolated by
483 Ultracentrifugation (UC) or sEV isolation kit (IK) compared to donor astrocytes (As). B) sEV size
484 distribution using Nanoparticle Tracking Analysis of sEVs isolated by UC, 152 ± 7.8 nm (mean \pm SEM).
485 C) sEV size distribution using sEV IK, mode 156 ± 10.7 nm (mean \pm SEM). n= 6 sEVs and cell culture
486 from different murine preparation, 3 technical repeats for each n.

487

488 **Figure 2. Increased protein loading in Small Extracellular Vesicle (sEVs) of SUMO-2 astrocytes.** A)
489 Coomassie staining of the corresponding sEVs. B) Mean sEV size in nm C) Quantification of the
490 number of vesicles obtained from the conditioned media of one million cells. D) Quantification of
491 protein content per sEV. All data are expressed as mean \pm SEM and for quantification one-way
492 ANOVA was performed, followed by a post-hoc Tukey test. Statistical significance is represented
493 with asterisks. n = 7-8, sEVs and cell culture from different murine preparation, 3 technical repeats
494 for each n. * $p < 0.05$.

495

496 **Figure 3. Proteomics of small Extracellular Vesicles (sEVs) from astrocytes expressing SUMO reveal**
497 **proteins related to transcription, translation and cell division.** A) Venn analysis of sEV proteins from
498 astrocytes transfected with GFP, SUMO-1 (S1) or SUMO-2 (S2), identified by mass spectrometry. B)
499 Comparison between biological processes present in exclusive proteins in each sample analyzed by
500 the DAVID database. C) Coomassie staining of 10% input of astrocyte-derived sEVs bound to SIM-
501 beads. D) Biological processes present in proteins bound to SIM-beads analyzed by the DAVID
502 database. Data obtained from n=2-3 6 sEVs from different murine preparation.

503

504 **Figure 4. Treatment with 2-D08 decreases and corticosterone increases SUMOylation in**
505 **astrocytes.** Astrocytes were treated 3 times with DMSO (control), 10 ng/ml IL-1 β , 1 μ M
506 corticosterone (CORT) or 30 μ M 2-D08 (UBC9 inhibitor), in 24-hour intervals. sEVs were prepared
507 24 hours after the last treatment. A) Detection by Western blot of proteins conjugated with SUMO-
508 1 or SUMO-2 in treated cells. B) Quantification of protein conjugated with SUMO-1 by densitometry,
509 expressed as fold change with respect to the control and corrected by the densitometry of
510 Coomassie staining. C) Quantification of protein conjugated with SUMO-2 in the same conditions.
511 D) Astrocytes were stained with GFAP antibody (green) and DAPI (blue). E) Quantification of the
512 intensity of GFAP signal. F) Quantification of astrocyte size. All data are expressed as mean \pm SEM
513 and for quantification one-way ANOVA was performed, followed by a post-hoc Tukey test. Statistical
514 significance is represented with asterisks. n = 5-8, sEVs and cell culture from different murine
515 preparation, 3 technical repeats for each n. * $p < 0.05$).

516

517 **Figure 5. SUMOylation inhibition prevents, and corticosterone increases protein load in small**
518 **Extracellular Vesicles (sEVs) from Astrocytes.** Astrocytes were treated 3 times with DMSO (control),
519 10 ng/ml IL-1 β , 1 μ M corticosterone (CORT) or 30 μ M 2-D08 (UBC9 inhibitor), in 24-hour intervals.
520 sEVs were prepared 24 hours after the last treatment. A) Coomassie staining shows the sEV
521 proteins contained in 10 million sEVs. B) Mean sEV size in nm. C) Number of vesicles released per
522 million cells. D) Quantification of protein content (in ng) per sEV. All data are expressed as mean \pm
523 SEM and for quantification one-way ANOVA was performed, followed by a post-hoc Tukey test.
524 Statistical significance is represented with asterisks (n = 9, sEVs and cell culture from different
525 murine preparation, 3 technical repeats for each n.***p<0.001).

526

527 **Figure 6. Proteomics of small Extracellular Vesicles (sEVs) from astrocyte treated with**
528 **corticosterone reveals proteins related with protein synthesis.** A) Venn analysis of sEV proteins
529 from astrocytes treated with DMSO (control), corticosterone (CORT) and SUMO inhibitor (2-D08),
530 identified by mass spectrometry. B) Comparison between Biological process present in exclusive
531 proteins in each sample analyzed by the DAVID database. Data obtained from n=4-6 sEVs from
532 different murine preparation.

533

534 **Figure 7. Neurons with small Extracellular Vesicles (sEVs) from astrocytes treated with**
535 **corticosterone increase newly synthesized proteins.** Cortical neurons untreated (-sEV) or incubated
536 with 1000 astrocyte-derived sEVs per neuron (two times in 24 hour intervals). Astrocytes were
537 previously treated with DMSO (Ctrl As-sEVs), corticosterone (CORT sEVs) and 2-D08 (2-D08 sEVs).
538 24 hours after the last incubation with sEVs, neurons were incubated with AHA or methionine added
539 to the culture medium for 3 hours. The FUNCAT assay and MAP2 staining was performed. A) Sholl
540 Analysis of neurons incubated with astrocyte-derived sEVs. B) Quantification of the total dendrite
541 length per neuron. C) Quantification of total number of intersections per neuron. D) Neurons were
542 stained with MAP2 antibody and newly synthesized proteins were detected by the AHA-TAMRA tag.
543 The intensity of the FUNCAT signal is inserted as a fire lookup table in the right panel. E)
544 Quantification of the intensity of the FUNCAT signal, TAMRA tagged-newly synthesized proteins
545 (AHA). All data are expressed as mean \pm SEM and for quantification one-way ANOVA was performed,
546 followed by a post-hoc Tukey test. Statistical significance is represented with asterisks (n = 3-7, sEVs
547 and cell culture from different murine preparation, 20 neurons was quantified by preparations
548 *p<0.05).

549

550 SUPPLEMENTARY FIGURE LEGENDS

551 **Supplementary figure 1. Transient transfection of SUMO plasmids in primary cultures of**
552 **astrocytes has no effect on SUMO-1 or SUMO-2 expression.** A) Proteins conjugated with SUMO-1
553 or SUMO-2 were detected by Western blot in cells transfected with GFP, SUMO-1 (S1) or SUMO-2
554 (S2). The corresponding Coomassie staining is shown. B) Quantification of the proteins conjugated

555 with SUMO-1 or C) SUMO-2 expressed as fold change with respect to the control (GFP). All data are
556 expressed as mean \pm SEM and for quantification one-way ANOVA was performed, followed by a
557 post-hoc Tukey test. n=3, cell culture from different murine preparation.

558

559 **Supplementary figure 2. ALDOA and EF-2 are contained in astrocyte-derived small Extracellular**
560 **Vesicles (sEVs).** A) ALDOA, EF-2 and FLOT1 were detected by Western blot in sEVs from astrocytes
561 transfected with GFP, SUMO-1 (S1) or SUMO-2 (S2). The same number of sEVs was loaded per lane.
562 B) Quantification of ALDOA 55kDa double band in sEVs. C) Quantification of ALDOA 250kDa form.
563 D) EEF-2 in astrocyte-derived sEVs. Fold change with respect to control (GFP). All data are expressed
564 as mean \pm SEM and for quantification one-way ANOVA was performed, followed by a post-hoc Tukey
565 test. Statistical significance is represented with asterisks (n = 3, sEVs and cell culture from different
566 murine preparation. *p<0.05).

567

568 **Supplementary figure 3. SUMOylation inhibition prevents proteins loading in small Extracellular**
569 **Vesicles (sEVs) of HeLa cells.** Cells were treated 3 times with DMSO (control), 10 ng/ml IL-1 β , 1 μ M
570 corticosterone (CORT) or 30 μ M 2-D08 (UBC9 inhibitor), in 24-hour intervals. sEVs were prepared
571 24 hours after the last treatment. A) Coomassie staining shows the sEV proteins contained in 10
572 million sEVs. B) Mean sEV size in nm. C) Number of vesicles released per million cells. D)
573 Quantification of protein content (in ng) per sEV. All data are expressed as mean \pm SEM and for
574 quantification one-way ANOVA was performed, followed by a post-hoc Tukey test. Statistical
575 significance is represented with asterisks (n = 9, sEVs and cell culture from different murine
576 preparation, 3 technical repeats for each n. *p<0.05, ****p<0.001).

577

578 TABLE LEGEND

579 **Table 1. List of the proteins involved in protein synthesis in sEVs derived from astrocytes treated**
580 **with corticosterone (CORT).** K (Lysine) is a possible site of SUMOylation. Data obtained from n=4-6
581 biological repeats.

582

583 SUPPLEMENTARY TABLE LEGENDS

584 **Supplementary Table 1:** List of proteins from astrocyte-derived sEVs. The columns contain the
585 protein name, the Uniprot ID and in the following columns, the number of times that each protein
586 was detected in sEVs samples from astrocytes transfected with GFP, SUMO-1 (S1) or SUMO-2 (S2).
587 n=2 determinations for each case. Mass spectrometry was performed in Otto von Guericke
588 University Magdeburg, Germany.

589 **Supplementary Table 2:** List of proteins pulled down with SIM beads from astrocyte-derived sEVs.
590 The columns contain the protein name, the Uniprot ID and in the following columns, the number of
591 times that each protein was detected in the starting material (input) and in the corresponding pull

592 downs (PD) from astrocyte-derived sEVs. n=3 determinations for each case. Mass spectrometry was
593 performed in Otto von Guericke University Magdeburg, Germany.

594 **Supplementary Table 3:** List of proteins from astrocyte-derived sEVs. The columns contain the
595 protein name, the Uniprot ID and in the following columns, the number of times that each protein
596 was detected in sEVs samples from astrocytes treated with DMSO (control), corticosterone (CORT)
597 or 2-D08 (SUMOylation inhibitor). n=4-6 determinations for each case. Mass spectrometry was
598 performed in Otto von Guericke University Magdeburg, Germany.

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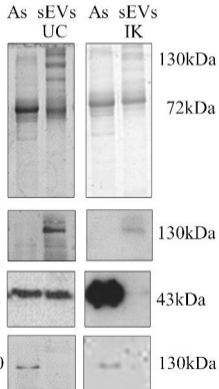
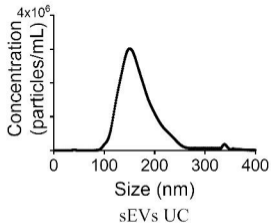
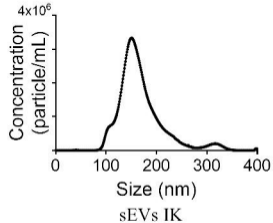
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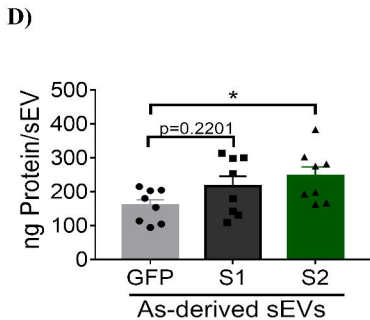
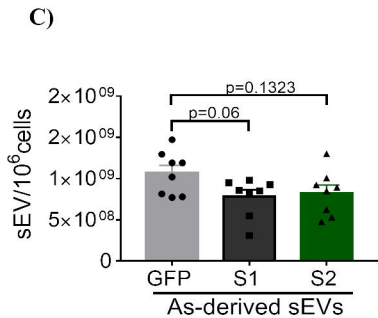
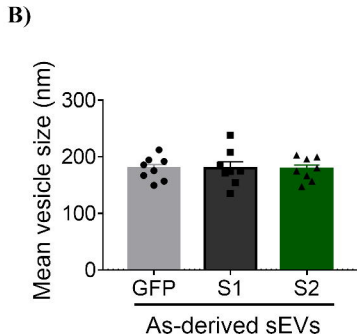
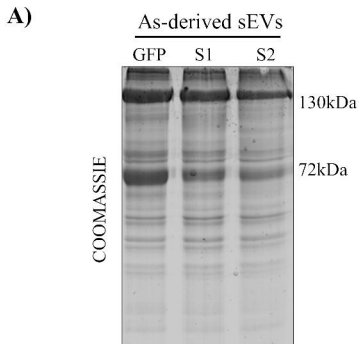
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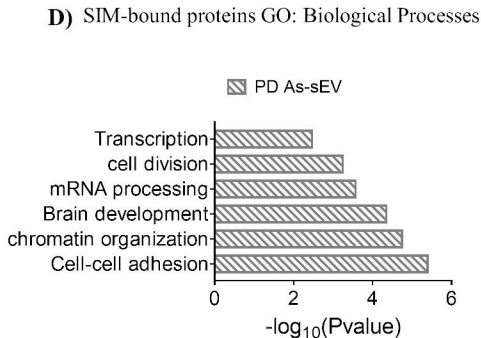
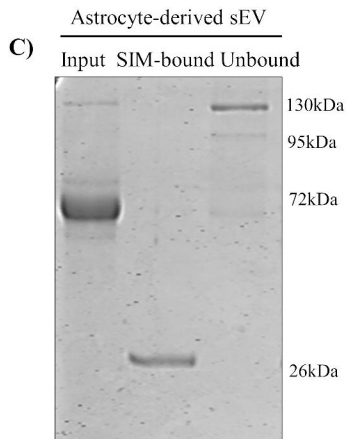
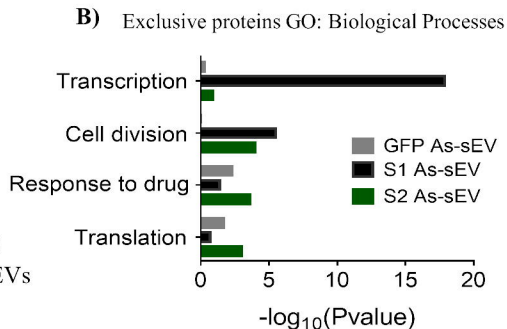
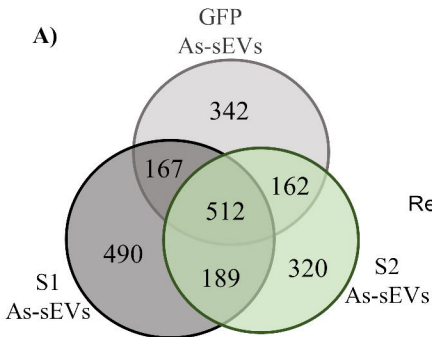
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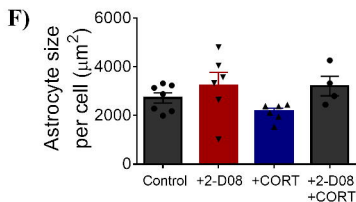
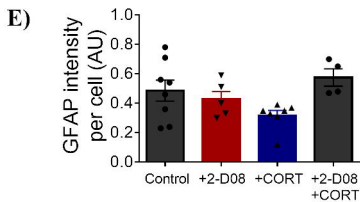
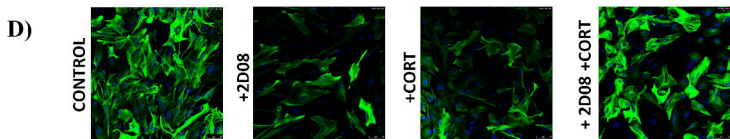
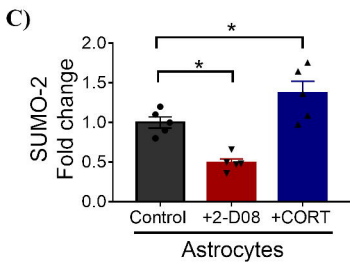
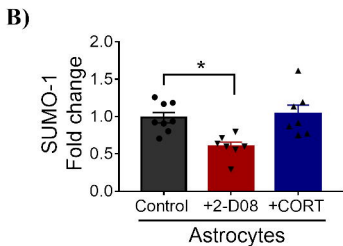
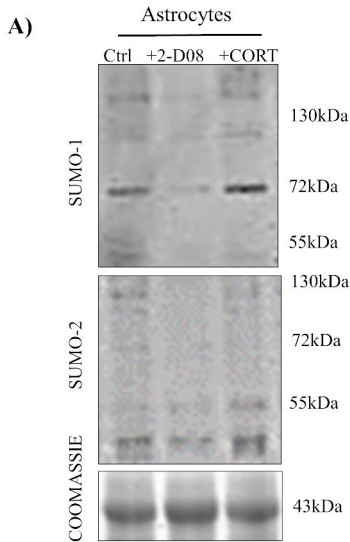
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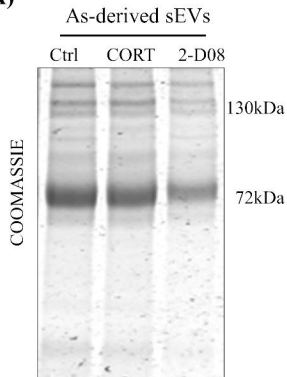
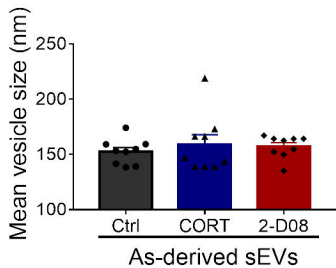
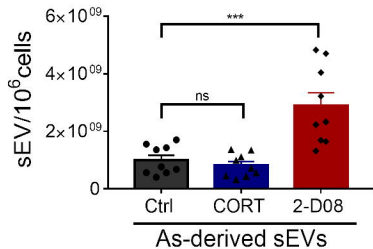
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