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24 **ABSTRACT**

25 Neural stem cells differentiate into several cell types that display distinct functions.
 26 However, little is known about how cell surface mechanics vary during the
 27 differentiation process. Here, by precisely measuring membrane tension and bending
 28 modulus, we map their variations and correlate them with changes in cell morphology
 29 along differentiation into neurons, astrocytes and oligodendrocytes. Both neurons and
 30 undifferentiated cells reveal a decrease in membrane tension over the first hours of
 31 differentiation followed by stabilization, with no change in bending modulus. Astrocytes
 32 membrane tension initially decreases and then increases after 72h, accompanied by
 33 consolidation of GFAP expression and striking actin reorganization, while bending
 34 modulus increases following observed alterations. For oligodendrocytes, the changes
 35 in membrane tension are less abrupt over the first hours but their values subsequently
 36 decrease, correlating with a shift from O4 to MBP expressions and a remarkable actin
 37 reorganization, while bending modulus remains constant. Oligodendrocytes at later
 38 differentiation stages show membrane vesicles with similar membrane tension but
 39 higher bending modulus as compared to the cell surface. Altogether, our results display
 40 an entire spectrum of how membrane elastic properties are varying, contributing to a
 41 better understanding of neural stem cell differentiation from a mechanobiological
 42 perspective.

43 INTRODUCTION

44   The surface of all mammalian cells is composed of the plasma membrane
 45 cushioned underneath by a cortical actomyosin cytoskeleton. This pair of structures
 46 forms the membrane-cytoskeleton complex, a key regulator of several cellular
 47 processes, ranging from shape control and cell migration to molecule presentation and
 48 signalling [1,2]. The plasma membrane is the direct interface between the cytoplasm
 49 and the extracellular matrix [3,4], while the cortical cytoskeleton, also known as cell
 50 cortex, is a dynamic actomyosin meshwork that gives support to the plasma membrane
 51 [1,2]. The membrane-cytoskeleton complex exerts and reacts against forces owing to
 52 its elastic properties [1,2,4]. For brevity, we shall just refer to this complex as “cell
 53 membrane” (CM).

54   Over the years, different micromanipulation tools have been employed to exert
 55 forces on CMs so as to characterize their elastic responses [5]. Membrane tether
 56 pulling assays using optical tweezers (OT) [6,7] or atomic force microscopy (AFM) [8,9]
 57 have been used to extract nanotubes or tethers from CMs in order to determine these
 58 properties. By measuring both the equilibrium force and tether radius, the cell
 59 membrane surface tension (CMT - σ_{eff}) and cell membrane bending modulus (CMBM -
 60 κ_{eff}) have been determined for different cell types [7,10-15]. CMT and CMBM result
 61 from joint contributions of cytoskeleton architecture, membrane composition and
 62 membrane-cytoskeleton attachment [14]. Moreover, these elastic properties
 63 (particularly CMT), as well as their changes, have been characterized as important
 64 regulators of cellular behaviors, specially regarding shape changes and force
 65 production [14,16]. Furthermore, it has been shown [13] that membrane elastic
 66 properties are correlated to cell function.

67   During development, neural stem cells, as neural progenitors (referred to just as
 68 NSCs) give rise to all neurons of the mammalian Central Nervous System (CNS).
 69 NSCs are also the source of two types of macroglial cells, the astrocytes and
 70 oligodendrocytes [17]. These three cell types are extremely different, not only in their
 71 morphological features but also in their functions. Neurons are highly anisotropic cells,
 72 with relatively quiescent and compact cell bodies (soma) containing the cell nucleus
 73 and dynamic protrusions (axons and dendrites), both susceptible to large structural
 74 changes [18]. Astrocytes are remarkably dynamic, constantly modifying their
 75 morphology during migration or when interacting with neurons [19]. Oligodendrocytes
 76 extend many protrusions that can ultimately form myelin sheaths, wrapping around
 77 axons to promote fast saltatory conduction of action potentials [20]. Several studies

78 have revealed the molecular mechanisms that govern the path of NSCs differentiation
 79 and fate specification. Diverse growth factors and cytokines, together with epigenetic
 80 alterations including DNA methylation, histone modifications, and non-coding RNAs
 81 have already been described as key elements in this regard [21], as well as gene
 82 expression changes [22]. However, little is known about how the CM elastic properties
 83 of NSCs vary over the course of their differentiation nor the correlation between such
 84 variations and the final morphological phenotype of differentiated cells.

85 In the present work, we combined OT-based tether extraction experiments with
 86 measurements of tether radius using scanning electron microscopy (SEM), as well as
 87 fluorescence microscopy observations, to investigate the roles of the cytoskeleton on
 88 the CM elastic properties of NSCs along their differentiation into neurons, astrocytes
 89 and oligodendrocytes. Our aim was to map and collect experimental evidences that
 90 may provide a basis for future studies on how modifications in CM elastic properties
 91 correlate with shape and phenotype changes, ultimately influencing NSC
 92 differentiation.

93 MATERIALS AND METHODS

94 Animals

95 For NSC cultures, embryonic (E14; 14-day embryonic) swiss mice, obtained from
96 pregnant females, were used. Mice were maintained at the Institute of Biomedical
97 Sciences, Federal University of Rio de Janeiro. Animal husbandry and experimental
98 procedures were performed in accordance with and approved by the Ethics Committee
99 of the Health Sciences Center, Federal University of Rio de Janeiro (Protocol Number:
100 001-16).

101

102 Cell Cultures

103 Primary cortical NSC cultures were prepared according to a modified protocol [23,24],
104 using 14-days embryonic swiss mice. Briefly, NSCs suspensions were obtained by
105 dissociating cells from the cerebral cortex and plated in Dulbecco's Modified Eagle's
106 (DMEM) F-12 medium containing 0,6% glucose, N2, G5 (with FGF and EGF) and B27
107 supplements, 2mM L-glutamine, 5mM HEPES, 0,11% NaHCO₃, and 1%
108 penicillin/streptomycin (all from Invitrogen, Thermo Fisher, Carlsbad, CA, USA). The
109 NSCs were cultured as neurospheres for 5 days. Then, they were mechanically
110 dissociated and plated onto coverslips previously coated with 0.01% poly-L-lisine
111 (Sigma-Aldrich, St. Louis, MO, USA). Five different cell conditions were used: (1) NSCs
112 in neurospheres, (2) dissociated NSCs, (3) neurons, (4) astrocytes and (5)
113 oligodendrocytes. All cells were obtained from NSCs that were induced to differentiate
114 with specific media, partially replaced every 3 days, for 10 days (240h) under optimal
115 culture conditions (37 °C and 5% CO₂). All experiments were carried out at the
116 following time points: 2, 24, 48, 72, 96, 120, 168 and 240h. NSCs in neurospheres and
117 dissociated NSCs were maintained in their specific media. NSCs induced to
118 differentiate into neurons were placed in Neurobasal media supplemented with 2mM L-
119 glutamine, 1% penicillin/streptomycin and B27 supplement. NSCs induced to
120 differentiate into astrocytes were placed in DMEM-F12 supplemented with 2mM L-
121 glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin. NSCs induced to
122 differentiate into oligodendrocytes were placed in DMEM-F12 supplemented with 2mM
123 L-glutamine, 0.5% fetal bovine serum, B27, 50µM T3, 5 µg/ml Insulin, 5 µg/ml
124 transferrin, 5 ng/ml sodium selenite and 1% penicillin/streptomycin. All reagents, unless
125 otherwise mentioned, were purchased from Invitrogen-Thermo Fisher Scientific
126 (Carlsbad, CA, USA).

127 **Confocal Fluorescence Microscopy**

128 Confocal fluorescence microscopy was performed for all the cell types and time points
 129 used in this study. Briefly, cells were fixed in PBS-paraformaldehyde 4% for 15 min,
 130 permeabilized with PBS-triton X100 0.2% for 5 min, blocked with PBS-BSA 5% (Sigma-
 131 Aldrich, St. Louis, MO, USA) for 1 hour and then incubated overnight at 4°C with
 132 primary antibodies for each of the specific markers: for NSCs, polyclonal antibody
 133 against brain lipid binding protein (BLBP) (Millipore, Merck KGaA, Germany), mouse
 134 antibody against nestin (Millipore, Merck KGaA, Germany), polyclonal antibody against
 135 sox2 (Invitrogen, Thermo Fisher, Carlsbad, CA, USA); for neurons, monoclonal
 136 antibody against β -tubulin III (Promega Corporation, Madison, WI, USA); for astrocytes,
 137 polyclonal antibody against glial fibrillary acidic protein (GFAP) (Dako, Denmark) and
 138 for oligodendrocytes, monoclonal antibody against oligodendrocyte marker O4 (R&D
 139 Systems, Minneapolis, MN, USA) and polyclonal antibody against myelin binding
 140 protein (MBP) (Abcam, UK). Then, secondary monoclonal and/or polyclonal Alexa
 141 antibodies conjugated with 546nm, 568nm or 633nm fluorophores (Molecular Probes
 142 Inc, Eugene, OR, USA) were incubated for 2h together with phalloidin-FITC (Molecular
 143 Probes Inc, Eugene, OR, USA). Coverslips were mounted on microscopy slides and
 144 visualized with a HC PL APO 63x/1.40 Oil CS objective lens attached to a Leica TCS-
 145 SP5 II confocal microscope (Leica Microsystems, Germany). Images were acquired
 146 using the LAS AF 2.2.0 Software (Leica Microsystems, Germany). Quantification
 147 analysis of F-actin and GFAP cytoskeleton networks was performed using FibrilTool
 148 [25], an ImageJ (National Institutes of Health, USA) plug-in capable of determining the
 149 average orientation of a fiber array, providing quantitative information about its
 150 anisotropy. The anisotropy value ranges from a maximum of 1, when all fibers point to
 151 the same direction in the array, to a minimum of 0, when they are all randomly oriented.
 152 Fluorescence quantifications for GFAP, MBP and O4 were performed using ImageJ.
 153 Briefly, an outline was drawn around each cell and the values for area, the mean grey
 154 (fluorescence) value and integrated density (ID) were obtained along with the
 155 corresponding measurements for the adjacent background. Then, the corrected total
 156 cell fluorescence (CTCF) was calculated for each experimental situation using the
 157 following equation:

$$158 \quad CTCF = ID - (A_c \times F_B); \quad (1)$$

159 where A_c is the selected cell area and F_B is the mean grey (fluorescence) value of the
 160 background.

161 For GFAP, direct CTCF values were plotted for all experimental conditions. For O4 and
162 MBP, the ratios between O4 CTCF and MBP CTCF were plotted for all experimental
163 conditions.

164

165 **Optical Tweezers Setup and Calibration**

166 The optical tweezers (OT) system employed an infrared Ytterbium linearly polarized
167 colimated laser beam with a wavelength of 1064 nm and maximum power of 5W
168 (model YLR-5-1064-LP) (IPG Photonics, NY, USA). The laser was coupled to an
169 inverted Nikon Eclipse TE300 microscope (Nikon, Melville, NY, USA) equipped with a
170 PLAN APO 100X 1.4 NA DIC H Nikon objective, used to create the trap. The OT
171 system was calibrated using the same procedure previously described [13,26,27]. The
172 trap transverse stiffness per unit power at the objective entrance was $k/P = (0.25 \pm$
173 $0.01) \text{ pN}\mu\text{m}^{-1}\text{mW}^{-1}$.

174

175 **Tether Extraction Experiments with Optical Tweezers**

176 Tether extraction experiments using optical tweezers were performed following the
177 same procedures previously described [7,10-13]. Briefly, NSCs in neurospheres and/or
178 dissociated NSCs were plated and allowed to attach to glass bottom dishes. NSCs in
179 neurospheres were allowed to attach only for 2h prior to experiments. Dissociated
180 NSCs were allowed to attach and differentiate for all time points, as described above.
181 Then, uncoated polystyrene beads (radius = $1.52 \pm 0.02 \mu\text{m}$) (Polysciences,
182 Warrington, PA) were added and each one of the glass bottom dishes containing the
183 cells was placed in the OT microscope. A bead was trapped and pressed against a
184 chosen cell for ~5 seconds, allowing its attachment to the cell surface. The microscope
185 motorized stage (Prior Scientific, Rockland, MA, USA) was then set to move with
186 controlled velocity ($1 \mu\text{m/s}$). Movies were collected at a frame rate of 10 frames/second
187 using a Hamamatsu C2400 CCD camera (Hamamatsu, Japan) coupled to a SCION
188 FG7 frame grabber (Scion Corporation, Torrance, CA, USA). Using the trap calibration
189 and the measured bead position displacement, obtained by analysis of images
190 extracted from the movie, the tether force, F_0 , was determined. All experiments in the
191 OT microscope were conducted in optimal culture conditions (37°C and 5% CO_2). Data
192 analysis and force calculations were performed using ImageJ and Kaleidagraph
193 (Synergy Software, Essex Junction, VT, USA) softwares.

194

195 **Measurements of Tether Radii**

196 After extracting the cell tethers, the beads used during the extraction were attached to
197 the coverslip. The samples were then fixed and prepared for SEM following the same
198 procedures previously described [7,10-13]. Thus, the mean values for tether radii, R ,
199 corresponding to each experimental situation were obtained.

200 However, the aforementioned procedure could not be employed to measure the tether
201 radii extracted from oligodendrocyte vesicles. To circumvent this issue we adopted
202 another previously validated method, already tested for the same purpose [7,13]. This
203 method is based on the force barrier theory for tether formation [28] and its theory gives
204 the following relation:

$$205 \quad \frac{F_{m(ves)}}{F_{0(ves)}} = 1 + \frac{1}{2} \frac{R_{p(ves)}}{R_{(ves)}} ; \quad (2)$$

206 where $F_{0(ves)}$ is the vesicle tether force, $F_{m(ves)}$ is the vesicle maximum force before
207 tether formation, $R_{(ves)}$ is the vesicle tether radius and $R_{p(ves)}$ is the radius of the
208 circular patch contact area between the bead and the vesicle membrane.

209 The values of $F_{m(ves)}$ and $F_{0(ves)}$ were experimentally determined; $R_{p(ves)}$ was
210 measured from the image of the bead and the deformed vesicle surface. The value of
211 $R_{(ves)}$ was then obtained from equation (2).

212

213 **Determination of Membrane Elastic Properties**

214 Once the values of F_0 and R were measured, it was then possible to determine the
215 values of the cell membrane tension (CMT - σ_{eff}) and the cell membrane bending
216 modulus (CMBM - κ_{eff}) [29,30].

217 The CMT (σ_{eff}) is given by:

$$218 \quad \sigma_{eff} = \frac{F_0}{4\pi R} ; \quad (3)$$

219 and the CMBM (κ_{eff}):

$$220 \quad \kappa_{eff} = \frac{F_0 R}{2\pi} . \quad (4)$$

221 The values of membrane tension, $MT_{(ves)}$, and bending modulus, $BM_{(ves)}$, for
222 oligodendrocyte vesicles' were also determined using the equations above.

223 **Statistical Analysis**

224 Some data are presented as mean $\pm$ standard error. For tether force and radius values,
225 Box-and-Whiskers plots were used. The boxes extend from the 25th to 75th
226 percentiles, with a black horizontal line at the median and a black cross at the mean;
227 black whiskers extend from 5th to 95th percentiles for all experimental conditions;
228 values outside these ranges are plotted as individual points. Data were analyzed using
229 GraphPad Prism statistics software (GraphPadSoftware, Inc. La Jolla, CA, USA).
230 Mann-Whitney U -tests were used for comparisons between each situation and the 2h
231 condition. *means $p < 0.05$; ** means $p < 0.01$; *** means $p < 0.001$ and **** means $p <$
232 0.0001 . The p-values and other numbers for all experiments are provided in the figure
233 legends.

234 RESULTS

235 OT as a screening tool to measure membrane elastic properties of differentiated 236 and undifferentiated NSCs.

237 Aiming to characterize the CM elastic properties of NSCs induced to differentiate into
238 astrocytes, oligodendrocytes and neurons and to compare the results with those found
239 for cells maintained with their stem capacity, either as neurospheres or isolated cells,
240 OT-based tether extraction experiments were performed for each cell type over periods
241 of 2 to 240h in culture. As an example of the experimental procedures, images
242 associated with a tether extraction experiment performed in a neuron differentiated cell
243 are shown in Figure 1A (extracted tether is indicated by a white arrow) and Figure 1B
244 (zoom of the extracted tether). The corresponding tether extraction force curve is
245 shown in Figure 1C. The tether force, F_0 , (referred to as the steady-state force in Figure
246 1C) and the tether radius, R , (Figures 1D – F) were carefully measured for each
247 experimental time point. The results are presented in the following sections.

248

249 Membrane elastic properties measured for undifferentiated neural stem cells 250 vary only in the initial hours after plating

251 NSCs, previously grown as neurospheres for 5 days (Figure S1A), were dissociated
252 and replated as isolated cells in stem cell medium to maintain their stemness (Figure
253 S1B). Their morphologies and the expression of specific markers, were followed from 2
254 to 240h after plating. Figure 2A displays the actin cytoskeleton of these cells (stained in
255 green for phalloidin-FITC, a molecule commonly used as a cytochemical marker of
256 polymerized actin (F-actin)) together with a specific marker, the brain lipid binding
257 protein (BLBP or FABP7 - stained in red), known to be expressed in radial glial cells
258 during development. It has been documented that radial glial cells not only participate
259 as scaffolds that allow neurons to migrate within the developing cortex [31,32], but they
260 also function as neural progenitors cells [33-35]. Thus, the NSCs used in this study
261 (Figure 2A) have the morphology and characteristics of radial glial cells. Moreover, to
262 double-check that these cells were kept undifferentiated throughout the experimental
263 procedures, NSCs were also stained, after 240h in culture, for nestin (in white) and
264 sox2 (in red) (typical stem cell markers) together with phalloidin-FITC (in green) and the
265 cell nucleus, stained with dapi (in blue) (Figure S1B).

266 The actin cytoskeleton architecture, with the exception of the 2h condition, appeared to
 267 not present any substantial variations throughout the days of culture, as verified by the
 268 images (Figure 2A). However, to better quantify these visual observations, we also
 269 performed a quantitative analysis using FibrilTool [25]. The results of this analysis
 270 (Figure 2B) showed that, overall, the cells present a similar F-actin anisotropy
 271 throughout the entire experiment, except for the cells at the 2h time point.

272 Next, tether extractions were performed on dissociated NSCs that were maintained
 273 undifferentiated in culture. The corresponding mean values of tether force are shown in
 274 Figure 2C. No statistically significant change in tether force values were found for
 275 NSCs from 24 to 240h in culture (Figure 2C). However, a ~1.5-fold decrease in tether
 276 force value (from 22 ± 1 to estimated 14 ± 1 pN) was found when comparing the 2h
 277 condition with all the other ones (Figure 2C).

278 The tether radii were also measured using SEM. Figure 2D shows the mean values for
 279 the NSCs from 2 to 240h in culture. No statistically significant change in tether radii
 280 values were found for NSCs from 24 to 240h in culture (Figure 2D). However, a ~1.3-
 281 fold increase in tether radii value (from 43 ± 3 to estimated 57 ± 4 nm) was found when
 282 comparing the 2h condition with all the other ones (Figure 2D).

283 In order to compare the CM elastic properties of dissociated NSCs versus NSCs in
 284 spheres, we cultured neurospheres for 5 days (Figure S1A – phase contrast images),
 285 plated and allowed them to attach for 2h in stem cell medium (Figure S1A - nestin (in
 286 white), sox2 (in red) and dapi (in blue)). Tether force and radii measurements were also
 287 performed on different cells around the neurospheres. The mean values of F_0 and R
 288 are shown in Figures 2C and 2D, respectively. No statistically significant changes in
 289 tether force and radius values were found between the 2h-dissociated NSCs and those
 290 grown as neurospheres. However, the similar ~1.5-fold decrease in tether force value
 291 and the ~1.3-fold increase in tether radius were still evident when comparing those
 292 neurospheres condition with the 24-240h conditions.

293 Taken together, the results confirm that NSCs remain undifferentiated over the entire
 294 experiment, with an initial 2-fold drop in their CMT values in the very early hours,
 295 followed by stabilization in the subsequent hours (Figure 7A, grey curve and dots). The
 296 CMBM values, on the other hand, remain constant over time (Figure 7A, red curve and
 297 dots). These variations in CMT are not attributed to the dissociation of neurospheres
 298 but may be related to cell spreading and acquisition of final morphological phenotype.

Membrane elastic properties measured for neurons show a similar pattern in comparison to undifferentiated neural stem cells.

301

302 When dissociated, some NSCs were kept undifferentiated, but others were plated in a
303 culture dish containing neurobasal medium, supplemented with factors that induce cells
304 to differentiate into neurons. Again, the cell morphology and the expression of specific
305 markers were followed from 2h to 240h after plating. Figure 3A shows the phalloidin-
306 FITC labeled actin cytoskeleton (in green), together with β -tubulin III (in red), known to
307 be expressed in neurons [36]. The results show that the cells acquire the morphology
308 and characteristics of neurons, with several neurites and growth cones, which began to
309 appear within the first 24h and increased in the subsequent hours of culture (Figure
310 3A), confirming that NSCs already differentiate into neurons after being in culture for
311 only a few hours.

312 OT-based tether extractions were performed on these neurons along the differentiation
313 period. The mean values of F_0 are shown in Figure 3B. No changes in tether force
314 values were found for neurons from 24h to 240h in culture (Figure 3B). However, a
315 ~ 1.5 -fold decrease in tether force values (from 21 ± 1 to estimated 14 ± 1 pN) was found
316 when comparing the 2h condition with all other ones (Figure 3B).

317 Regarding the tether radii results, no statistically significant change was detected
318 among the time points ranging from 24h to 240h in culture (Figure 3C). However, a
319 ~ 1.4 -fold increase in tether radii values (from 45 ± 2 to estimated 64 ± 3 nm) was found
320 when comparing the 2h condition with all other ones (Figure 3C).

321 In view of the prior demonstration that, regardless of the region (cell body, neurites and
322 growth cones) there is no significant difference in the tether force and radius [13], we
323 chose to pool all the measurement results together in the plots (Figures 3B and 3C).
324 The values found for the tether force and radius were also of the same order of
325 magnitude as those previously found for mouse cortical and ganglionic eminence
326 neurons [13].

327 The results confirm that NSCs are able to differentiate into neurons with an initial 2.1-
328 fold drop in their CMT values in the very early hours, followed by stabilization in
329 subsequent hours, while increasing the number and length of neurites and number of
330 growth cones over time. The CMBM values, on the other hand, do not vary during the
331 experimental time points. Finally, the values for the CMT (Figure 7B, grey curve and
332 dots) and CMBM (Figure 7B, red curve and dots) are very similar to those found for

undifferentiated NSCs (Figure 7A). Again, variations in CMT may be related to cell spreading and acquisition of final morphological phenotype.

Astrocyte differentiation process reveals interesting patterns that correlate cytoskeletal architecture remodeling with changes in membrane elastic properties

Dissociated NSCs were also induced to differentiate into astrocytes. Again, the cell morphology and the expression of specific markers were followed from 2h to 240h after plating. Figure 4A shows the actin cytoskeleton, stained for phalloidin-FITC (in green), together with a specific marker for astrocytes, GFAP (in red) [37]. The results demonstrate that even though the cells acquired the morphology and characteristics of astrocytes, the expression of GFAP began showing up as filaments, in some cells, only 48h after plating, yielding more stable filaments after 72h of culture (Figures 4A and 4B), confirming that the NSCs differentiated into astrocytes. Interestingly, the actin cytoskeleton also presented a morphological shift, from small actin filaments (in the first 48h to 72h) to very well-defined stress fibers after 96h (Figure 4A). These results were confirmed by the FibrilTool analysis, which showed that the anisotropy of both actin and GFAP cytoskeletal networks increased with time in culture (Figures 4B and 4C).

Tether extraction measurements were performed in these astrocytes throughout their differentiation in culture. The mean values of tether force are shown in Figure 4D. A clear decrease (~1.3-fold) in tether force was observed from 2h to 48h, followed by a slight recovery at 72h after plating, consistently correlated with the increase in GFAP expression and the shift in F-actin architecture described above (Figures 4A, 4B 4C and 4D). Moreover, the tether force values found after 96h (~30 pN) increased~1.4-fold when compared with the 2h result and also increased almost 2.0-fold when compared with the 24 – 48h conditions (Figure 4D).

Figure 4E shows the mean radii values measured for cells induced to differentiate into astrocytes. A clear increase (~1,3-fold) in radii values from 24h to 72h and a slight increase from 96h to 240h (~1.16-fold) when compared to 2h time point were also observed (Figure 4E).

Altogether, the results confirm that even though NSCs differentiate into astrocytes and remain differentiated, their differentiation process is different from the neuronal differentiation, since GFAP expression starts to appear only 48h after induction, whereas in neurons the expression of β -tubulin III occurs in the very first hours. The shift in actin cytoskeleton architecture together with the increase in GFAP expression

are both correlated with the changes in CM elastic properties observed for astrocytes after 48 – 72h. The results show that there is a ~1.3-fold increase in CMT (Figure 7C, grey curve and dots) and a 1.7-fold increase in CMBM (Figure 7C, red curve and dots) in differentiated astrocytes (astrocytes from 96 – 240h) when compared to the undifferentiated cells (Figure 7A).

The values of CMT and CMBM found for astrocytes at later differentiation stages are within the same order of magnitude of those previously found for differentiated astrocytes obtained from newborn mice [13].

Oligodendrocyte differentiation reveals interesting patterns that correlate cytoskeletal remodeling and expression of specific markers with changes in membrane elastic properties

Finally, NSCs were also induced to differentiate into oligodendrocytes. Likewise, we followed the cell morphology and expression of specific markers during the differentiation process. Figure 5A shows the actin cytoskeleton (in green), together with two specific markers for oligodendrocytes: O4 (in red) and MBP (in white) [38]. The results show that the cells acquired the morphology and characteristics of oligodendrocytes. Interestingly, the expression of O4 increased in the first hours of differentiation and then decreased after the 96h time point. Conversely, the expression of MBP, which was low in the first hours, increased in the last hours, especially after the 96h time point (Figure 5A). The antagonistic expression patterns between O4 and MBP become more evident in Figure 5B, where the ratios between O4 and MBP fluorescence intensities were plotted. The cell morphology and actin cytoskeleton organization also dynamically changed over time in culture. Cells that were induced to differentiate into oligodendrocytes started to present a star-like branched morphology during the first 48h, with several F-actin containing protrusions (Figure 5A). However, this ramified morphology changed to a more flat-like lamellar shape, with the formation of membrane extensions, after 72 – 96h. These changes were correlated with the increase in MBP expression and the simultaneous decrease in O4 expression. Interestingly, in the same time frame, the actin cytoskeleton also presented peculiar changes in its architecture. These changes first appeared in the outermost region of the membrane extensions surrounding the cell as a sort of ring and then disappeared in the subsequent hours (168h and 240h) (Figure 5A). This morphological behavior had already been documented in other studies [39-41].

Tether extraction experiments were performed in oligodendrocytes along their differentiation process. The mean values of F_0 are shown in Figure 5C. No differences in tether force were observed in the first 72h as compared to the 2h time point (Figure 5C). However, a statistically significant decrease in tether force was observed in the time points between 96h and 240h (Figure 5C). This result can be well correlated with the increase in MBP expression, the change in oligodendrocyte morphology and the shift in actin cytoskeleton architecture described above (Figures 5A). The values for 120, 168 and 240h (on the order of ~12pN of force) were ~1.7 times lower than those found within the first 48h (on the order of ~21pN of force).

Concerning the mean radii values, Figure 5D depicts how these numbers changed along the oligodendrocyte differentiation process. We observed a clear increase (~1,3-fold) in tether radii from 120 to 240h when compared to the 2h condition (Figure 5D).

Another feature that appeared in the last hours of differentiation (168 – 240h) was the presence of spherical vesicle-like membrane protrusions stemming from the oligodendrocytes surfaces (Figure 6A, images 1 and 2). These vesicles had been previously documented several years ago in ultrastructural characterizations of cultured oligodendrocytes [42], but apparently neglected since that time.

In order to explore the elastic properties of these vesicles and to compare the results with those obtained from the oligodendrocyte surface, we next extracted tethers from these vesicles (Figure 6). Examples of the oligodendrocyte vesicles are shown in Figure 6A, with bright field images of three different situations: (image 3) when the bead is attached to the vesicle surface, (image 4) when the force is the maximum force $F_{m(ves)}$ (before tether formation), and (image 5) when the tether is already formed and the measured force is the steady-state tether force $F_{0(ves)}$. Figure 6B shows the force curve, with the numbered points corresponding to the numbered images in Figure 6A (images 3, 4 and 5). Figure 6C represents the mean values for $F_{m(ves)}$ and $F_{0(ves)}$ at 168 and 240h of differentiation. No differences among force values were observed for both time conditions. The values found were 14 ± 2 pN and 15 ± 2 pN for $F_{0(ves)}$ and 50 ± 12 pN and 53 ± 13 pN for $F_{m(ves)}$, respectively at 168 and 240h conditions. The bead/membrane contact patch radius, $R_{p(ves)}$, was also measured by image analysis. Figure 6A (image 4) represents an example of a chosen frame and indicates how $R_{p(ves)}$ was obtained in that case. $R_{p(ves)}$ was measured for both conditions (168 and 240h). No statistically significant differences were observed (Figure 6D). The values

438 found were 540 ± 7 nm and 545 ± 8 nm, respectively for the 168 and 240h conditions.
 439 Using the values of $R_{p(ves)}$, $F_{m(ves)}$ and $F_{0(ves)}$, we calculated the values of the
 440 oligodendrocyte vesicles tether radius, $R_{(ves)}$, using equation (2). The values found
 441 were 104 ± 36 nm and 112 ± 44 nm, respectively for the 168 and 240h conditions. Finally,
 442 the membrane tension, $MT_{(ves)}$, and bending modulus, $BM_{(ves)}$, for vesicles that
 443 appeared at the surface of oligodendrocytes at 168h and 240h were calculated.

444 Altogether, the results confirm that NSCs differentiate into oligodendrocytes; however,
 445 for this specific situation, the process seems to be slower than the others: although O4
 446 appeared in the first hours of induction, MBP expression only appeared in very late
 447 hours. The change in actin cytoskeleton architecture, the increase in MBP expression
 448 and the formation of vesicles around the oligodendrocytes' surface are all correlated
 449 with the decrease in CMT observed for these cells after 120h and until 240h (Figure
 450 7D, grey curve and dots). The oligodendrocytes CMBM (Figure 7D, red curve and dots)
 451 values do not vary significantly. Finally, vesicles that appeared at the surface of
 452 oligodendrocytes at 168h and 240h present similar membrane tension values but
 453 higher bending modulus values when compared to those found for the oligodendrocyte
 454 cell surface (Figure 7D insert – yellow and blue curves and dots).

455 DISCUSSION

456 The elastic properties of cell surfaces are increasingly recognized as key
457 regulators of cell functions [1,14,16,43,44]. Here, we have mapped the variations in CM
458 elastic properties during NSC differentiation into neurons, astrocytes and
459 oligodendrocytes. The results show that these variations are correlated with
460 morphological phenotype changes that can affect the differentiation process and
461 ultimately the cell functions.

462 CMT has been considered an ideal candidate for global cell signaling, in view of
463 its fast equalization around the cell in response to a stimulus [14], although
464 controversies exist [45]. Conversely, CMBM is locally adjustable by a variety of
465 mechanisms and is important for dynamic cell remodeling and movement [4]. These
466 considerations emphasize the need to precisely determine CMT and CMBM for
467 different cell types and in different biological contexts. Tether pulling experiments are a
468 reliable choice method for such purpose [14]. However, it is important to measure not
469 only the tether force but also the tether radius. Tether force can be measured with OT
470 [6,7] or AFM [8,9]. However, measurement of tether radius is a challenge, as they are
471 typically below the resolving limit of conventional optical microscopes. Some studies
472 just assume a standard value for the CMBM (~ 0.27 pN. μ m – first obtained by [46]) and
473 calculate CMT from direct measurements of tether force alone. This assumption that
474 CMBM has a quasi-universal value for all cell membranes is unwarranted as CMBM
475 itself is also dependent on tether force and radius [29,30]. Therefore, a correlative
476 microscopy-based method previously established [7,13], was used in the present study.
477 In this method, one extracts a tether and measures its force with OT and its radius with
478 SEM. The intrinsic difficulty in performing the method is the main obstacle for this
479 technique. Novel super-resolution live-microscopy techniques, capable of measuring
480 simultaneously the tether radius and force, should be undertaken to move the field
481 forward. Non-invasive methods have been recently developed [47,48] but still need
482 further tests before they may be used as standards. Therefore, even if there might be
483 some difficulties related with the correlative experiments performed in this work, they
484 are still the gold-standard method to precisely determine the elastic properties of cell
485 membranes.

486 Previous studies have performed tether extraction measurements from different
487 stem cell types, such as human mesenchymal stem cells [49,50], mouse embryonic
488 stem cells [51,52] and even mouse neural stem cells [53]. However, to the best of our
489 knowledge, the present study is the first to measure both the steady-state tether force

490 and tether radius for precise determination of the CM elastic properties of murine NSCs
491 over the course of their differentiation into neurons, astrocytes and oligodendrocytes.
492 We not only measured and mapped the changes in elastic properties of these cells
493 during differentiation but also correlated these changes with morphological phenotype
494 modifications acquired by these cells along this process.

495 Undifferentiated mouse NSCs used in this study have an embryonic origin and
496 presented a low-density and isotropic actin network over the course of the entire
497 experiment. This feature was previously documented for mouse embryonic stem cells
498 [54] and conjectured to be necessary to maintain the balance between cortical and
499 nuclear stiffness such that the rigidity of the cortex never exceeds the rigidity of the
500 nucleus. This is important because, otherwise, the nucleus would end up sensing the
501 cell's own stiff cortex instead of the external mechanical environment. It is well
502 established that nuclear stiffening follows stem cell differentiation [55]. The observed
503 low-density isotropic actin network is correlated with low elastic constant values, as
504 observed in the present study, suggesting that mouse NSCs have a weak interaction
505 between the plasma membrane and the adjacent cortical cytoskeleton. Indeed, these
506 cells must be prepared to give rise to the three cell types studied herein. Therefore,
507 NSCs low CM elastic constants and less structured actin cytoskeleton can be important
508 for allowing fast conversion to neurons, astrocytes and/or oligodendrocytes. More
509 recently, researchers have shown in mice that NSCs can contribute to embryonic, early
510 postnatal and adult neurogenesis in the hippocampus, and that these cells are
511 continuously generated throughout a lifetime [56], which reinforces the importance of
512 determining their CM elastic properties.

513 In our study we induced NSCs to differentiate into neurons. This induction
514 occurred quickly and may be related to the period in which these cells were isolated,
515 the neurogenesis window [17], with its peak happening between 12 and 16 days of
516 embryonic development in mice. Thus, cells with neuronal phenotypes appeared in the
517 first hours of differentiation and remained differentiated throughout the entire period,
518 increasing the number and length of their protrusions. Interestingly, the CM elastic
519 properties of neurons were also low and remained constant for almost the entire
520 experiment. These mechanical features have been previously described for
521 differentiated mouse cortical and ganglionic eminence neurons [13]. The fact that these
522 cells have low elastic constant values may be also related to a weak interaction
523 between the plasma membrane and the cortical cytoskeleton.

524 Despite being practically constant in neurons and NSCs over the observed
525 period of this study, the CM elastic properties, particularly the CMT, changed
526 dramatically in the first hours, decreasing to almost half its value before stabilization.
527 This change in CMT is correlated with the cell spreading behavior that changes from
528 initially round cells (grown in non-adherent spheres) to a final state in which the cells
529 are fully spread/attached within the first 24 hours of experiment. Though never
530 demonstrated before during NSCs differentiation, this behavior has been previously
531 described for fibroblasts [57]. The CMT decreased during spreading and it was
532 conjectured that the addition of new membrane and the increase in membrane area
533 were mainly responsible for such phenomenon [57]. More recently, using embryonic
534 stem cells, two different groups described, in quite recent studies yet to be published,
535 that the decrease in CMT occurred when embryonic stem cells changed from their
536 round and naïve state to a spread and primed state, and that the observed decrease in
537 CMT was correlated with a decrease in membrane-cytoskeleton attachment [51,52] via
538 GSK3 β -driven β -catenin degradation [52], which in turn controls membrane tension and
539 allows exit from naïve pluripotency. Our results are in agreement with all the above
540 descriptions, even if the murine NSCs used in this study no longer display the transition
541 between naïve and primed states. Moreover, all mentioned studies [51,52,57] assume a
542 standard value for the CMBM and calculate CMT from direct measurements of tether
543 force, without measuring the tether radius. As we have discussed, one should be
544 careful when assuming that CMBM has a quasi-universal value for all cell membranes,
545 since it depends on both tether force and radius [29,30]. In line with this, the results of
546 the present study and previous observations [7,10,13] confirm that CMBM can vary
547 depending on the cell type and cell context.

548 We also induced NSCs to differentiate into astrocytes. Although astrocytes
549 share with neurons the same ectodermal origin, they are considerably more dynamic
550 and mobile. Moreover, we noticed that the *in vitro* differentiation into astrocytes
551 occurred more slowly, possibly owing to the fact that NSCs were isolated during the
552 neurogenesis peak, between 12 and 16 days of murine embryonic development [17],
553 while gliogenesis occurs only in late-gestation to perinatal periods [21]. Thus, cells with
554 an astrocytic phenotype only appeared around 48-72 hours of differentiation after the
555 consolidation of GFAP expression. The increase in CM elastic properties values
556 followed the consolidation of GFAP expression and actin cytoskeleton reorganization
557 that strikingly changed from a low-density isotropic meshwork to a more organized
558 stress fiber pattern.

559   Lastly, we also induced NSCs to differentiate into oligodendrocytes. Similarly to
560 what happened for astrocytes, the *in vitro* differentiation process also occurred more
561 slowly for oligodendrocytes. This behavior partially recalls what happens during mouse
562 embryonic development. Although oligodendrocyte progenitor cells are generated from
563 NSCs in different locations and times during CNS development, the formation of
564 mature myelinated oligodendrocytes only occurs around 5-7 days after birth in mice
565 [38]. In our *in vitro* differentiation model, cells with the mature oligodendrocyte
566 phenotype appeared around 120 hours after plating. This differentiation coincided with
567 a decrease in O4 and an increase in MBP expression levels, and it correlated with a
568 striking actin cytoskeleton reorganization, changing from initially actin-rich tubular
569 protrusions to a more lamellar morphology, with actin moving towards the cell periphery
570 until almost disappearing at later stages. This morphological behavior has already been
571 documented in other studies [39-41]. Moreover, Nawaz et al. [40] performed tether
572 pulling experiments in oligodendrocytes after being in culture for 48 hours (without
573 myelin) and 120 hours (with myelin sheath). They found a ~1.3-fold decrease in tether
574 force, similarly to that found in our measurements. However, again, these authors did
575 not measure the tether radius but concluded, only from the tether force, that the CMT
576 was decreasing. This decrease was associated with actin depolymerization via
577 ADF/cofilin and induced membrane spreading and myelin sheet growth [40]. We
578 performed tether force and radius measurements for each experimental time point
579 ranging from 2 to 240 hours of differentiation and demonstrated that indeed CMT was
580 decreasing, while CMBM did not vary. Another feature that appeared in the last hours
581 of differentiation (168 and 240 hours) was the presence of membrane vesicles at the
582 oligodendrocytes surfaces. These vesicles were previously documented several years
583 ago in ultrastructural characterizations of cultured oligodendrocytes [42], but have been
584 neglected since that time. The formation of these vesicles can be well explained based
585 on the excess membrane production and the huge actin depolymerization associated
586 with an increase in membrane-cortex detachment already described at latter stages of
587 oligodendrocyte differentiation [39-41]. The elastic properties of these membrane
588 vesicles were determined and compared with the elastic properties of the
589 oligodendrocyte CM at these time points. The vesicle membrane tension was slightly
590 lower when compared to the CMT but the vesicle bending modulus increased ~2.3-fold
591 when compared to the CMBM. These differences may be associated with the total
592 decoupling of the plasma membrane from the underneath cortical cytoskeleton during
593 vesicle formation. It is noteworthy that even with the increase in actin depolymerization,
594 the plasma membrane of oligodendrocytes still has some connection with the cortical
595 cytoskeleton, but this connection may be lost during vesicle formation.

In conclusion, we have measured the elastic constants of NSCs membranes over the course of their differentiation into neurons, astrocytes and oligodendrocytes. We have also presented experimental evidence supporting the conjectured correlation between cells' surface elasticity and the functions of these cells during the differentiation process. All tether radii and forces were measured. The values, determined under uniform and controlled experimental conditions, not only reinforce confidence but can also be taken as data for future studies. The importance of CMT and CMBM is now established for a variety of cells. However, the mechanisms allowing cells to set and regulate their CM elastic properties remain to be elucidated. The present study contributes to the knowledge that the CM elastic constants not only differ among cell types, but also depend on the cell state at the time of measurement. A variety of cellular processes are already known to affect the CM elastic properties, but how they all manage to determine and maintain their values still remains a challenging question for the field.

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Author Contributions

Conceptualization: LR, HMN and BP. Formal analysis: JS and BP. Funding acquisition: HMN and BP. Investigation: JS, GRSA, CF, DM and BP. Methodology: GRSA, MF, SF, NBV, LR and BP. Project administration: BP. Resources: VMN, MF, SF, NBV, LR, HMN and BP. Supervision: HMN and BP. Validation: JS, GRSA and BP. Visualization: BP. Writing – original draft: HMN and BP. Writing – review & editing: HMN and BP. All authors have read and agreed to the published version of the manuscript.

630   **Conflict of interest**

631   The authors declare that they have no conflict of interest and no competing financial
632   interest.

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821 **FIGURE CAPTIONS**

822 **Figure 1: Typical tether force and radius measurements.** (A) Representative bright
823 field image of a 48h-differentiated neuron extracted tether (indicated by a white arrow).
824 ImageJ shadow north processing filter was applied to better visualize the tether. (B)
825 Zoom of the tether in A. Scale bar for A is 10 μm and for B is 5 μm . (C) A typical tether
826 extraction force curve. F_0 is the steady-state tether force. (D) SEM representative
827 image of tethers extracted from a typical 24h-differentiated neuron. (E) Zoom of one of
828 the tethers indicated by the white arrows in D. Scale bar for D is 2 μm and for E is 1
829 μm . (F) Grey level plot profile of the tether in E.

830 **Figure 2: Tether extraction experiments and radii measurements for**
831 **undifferentiated neural stem cells.** (A) Representative images of undifferentiated
832 NSCs stained for BLBP (red) and F-actin, with phalloidin-FITC, (green) from 2h to 240h.
833 Scale bars are all 10 μm . (B) Plot of the mean anisotropy values of F-actin after
834 FibrilTool analysis. At least 10 different cells for each differentiation time condition were
835 imaged and analyzed. They all show similar behaviors as the ones represented in A.
836 Standard errors were used as error bars. (C-D) Plot of tether force values (at least 32
837 different measurements) (C) and tether radii values (at least 12 different
838 measurements) (D) for each experimental group. The colored boxes extend from 25th
839 to 75th percentiles, with a black horizontal line at the median and a black cross at the
840 mean; black whiskers extend from 5th to 95th percentiles; values outside these ranges
841 are plotted as individual points. Tether force and radius values for cells in
842 neurospheres are also represented (highlighted with a purple rectangle) for comparison
843 purposes. * means $p < 0.05$, ** means $p < 0.01$, **** means $p < 0.0001$ in *Mann-*
844 *Whitney U*-test statistics when comparing each time point with the 2h condition.

845 **Figure 3: Tether extraction experiments and radii measurements for NSCs**
846 **induced to differentiate into neurons.** (A) Representative images of cells stained for
847 β -tubulin III (red) and F-actin (green) from 2h to 240h. Scale bars are all 10 μm . (B-C)
848 Plot of tether force values (at least 18 different measurements) (B) and tether radius
849 values (at least 10 different measurements) (C) for each experimental time point. The
850 colored boxes extend from 25th to 75th percentiles, with a black horizontal line at the
851 median and a black cross at the mean; black whiskers extend from 5th to 95th
852 percentiles; values outside these ranges are plotted as individual points. * means $p <$
853 0.05 , ** means $p < 0.01$, *** means $p < 0.001$, **** means $p < 0.0001$ in *Mann-Whitney*
854 *U*-test statistics when comparing each time point with the 2h condition.

Figure 4: Tether extraction experiments and radii measurements for NSCs induced to differentiate into astrocytes. (A) Representative images of cells stained for GFAP (red) and F-actin (green) from 2h to 240h. Scale bars are all 10 μm . (B) Plot of the mean CTCF values for GFAP over the course of differentiation from 2h to 240h. (C-D) Plot of the mean anisotropy values of F-actin staining (C) and GFAP staining (D) after FibrilTool analysis. At least 10 different cells for each condition were analyzed. Standard errors were used as error bars in B, C and D. They all show similar behaviors as the ones represented in A. (E-F) Plots of tether forces (at least 16 different measurements) (E) and tether radii values (at least 10 different measurements) (F) for each time point. The colored boxes extend from 25th to 75th percentiles, with a black horizontal line at the median and a black cross at the mean; black whiskers extend from 5th to 95th percentiles; values outside these ranges are plotted as individual points. * means $p < 0.05$, ** means $p < 0.01$, *** means $p < 0.001$, **** means $p < 0.0001$ in *Mann-Whitney U*-test statistics when comparing each time point with the 2h condition.

Figure 5: Tether extraction experiments and radii measurements for NSCs induced to differentiate into oligodendrocytes. (A) Representative images of cells stained for O4 (red), F-actin (green) and MBP (white) from 2h to 240h. Scale bars are all 10 μm . (B) Ratio of O4 and MBP CTCF values over the course of differentiation from 2h to 240h. At least 10 different cells for each condition were imaged and analyzed in B. They all show similar behaviors as the ones represented in A. (C-D) Plots of tether forces (at least 20 different measurements) (C) and tether radii values (at least 12 different measurements) (D) for each condition. The colored boxes extend from 25th to 75th percentiles, with a black horizontal line at the median and a black cross at the mean; black whiskers extend from 5th to 95th percentiles; values outside these ranges are plotted as individual points. * means $p < 0.05$, ** means $p < 0.01$, *** means $p < 0.001$ in *Mann-Whitney U*-test statistics when comparing each time point with the 2h condition.

Figure 6: Tether extraction experiments and patch radii measurements for oligodendrocyte vesicles. (A) Typical bright field images of oligodendrocytes at 168h (Image 1) and 240h (Image 2) of differentiation, presenting several vesicles along their surfaces. (Images 3-5) Selection of images of a typical tether extraction experiment from the surface of oligodendrocyte vesicles. (Image 3) Initial moment, bead is being pressed against the oligodendrocyte vesicle with the optical trap, (Image 4) moment when the force reaches the maximum value – schematics indicating how $R_{p(ves)}$ is obtained – and, (Image 5) membrane tether already formed, indicated as a white

891 arrow. Scale bar for Images 1-2 is 10 μm and for Images 3-5 is 5 μm . (B) Force curve
 892 of a tether extracted from the oligodendrocyte vesicle. Numbers 3, 4, and 5 in the plot
 893 correspond to the images of the same numbers in A. $F_{m(ves)}$ means the maximum force
 894 before tether formation and $F_{0(ves)}$ means the steady-state tether force. (C) Plot of the
 895 mean values of $R_{p(ves)}$ for each experimental condition, as indicated by the plot
 896 legends. Standard errors were used as error bars in C and D. At least 20 different
 897 experiments were performed for each situation.

898 **Figure 7:** Schematic representation correlating the variations in morphological
 899 phenotype with CMT and CMBM over the course of (A) undifferentiated NSCs, (B)
 900 NSC differentiation into neurons, (C) NSC differentiation into astrocytes and (D) NSC
 901 differentiation into oligodendrocytes. Insert in D represents the membrane tension and
 902 bending modulus for oligodendrocyte vesicles.

903

904 **Supplementary Materials**

905 **Figure S1: Confirmation that NSCs remain undifferentiated in culture.** (A) Phase
 906 contrast image of neurospheres in culture and a representative neurosphere stained for
 907 nestin (in white) and sox-2 (in red), together with DAPI (in blue). Scale bars are
 908 respectively 100 μm and 50 μm . (B) Representative image of a dissociated neural stem
 909 cell (radial glia) after 240h in culture stained for nestin (in white) and sox-2 (in red)
 910 together with DAPI (in blue) and actin, with phalloidin-FITC (in green). Scale bar is 20
 911 μm .

A **B** **C**

A **B** **C** **D**

A**Undifferentiated NSC**

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B**Neuron** **C****Astrocyte** **D****Oligodendrocyte**

— F-actin — Cell membrane (CM) — GFAP filaments ○ Oligodendrocyte vesicles ● Cell nucleus