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Transparent carbon nanotubes promote the outgrowth of enthorino-dentate projections in lesioned organ slice cultures

*Original*

Transparent carbon nanotubes promote the outgrowth of enthorino-dentate projections in lesioned organ slice cultures / Pampaloni, Niccolò P; Rago, Ilaria; Calaresu, Ivo; Cozzarini, Luca; Casalis, Loredana; Goldoni, Andrea; Ballerini, Laura; Scaini, Denis. - In: DEVELOPMENTAL NEUROBIOLOGY. - ISSN 1932-8451. - 80:9-10(2020), pp. 316-331. [10.1002/dneu.22711]

*Availability:*

This version is available at: 20.500.11767/99008 since: 2020-04-24T17:11:45Z

*Publisher:*

*Published*

DOI:10.1002/dneu.22711

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27 network formation and function, an effect comparable to the one observed for their dark counterparts. We  
28 further adopt tCNTs to support the growth of intact or lesioned Entorhinal-Hippocampal Complex  
29 organotypic cultures (EHCs). Through immunocytochemistry and electrophysiological field potential  
30 recordings, we show here that tCNTs platforms are suitable substrates for the growth of EHCs and we  
31 unmask their ability to significantly increase the signal synchronization and fibre sprouting between the  
32 cortex and the hippocampus with respect to Controls. tCNTs transparency and ability to enhance recovery  
33 of lesioned brain cultures, make them optimal candidates to implement implantable devices in  
34 regenerative medicine and tissue engineering.

35 **Keywords:** nanomaterials, neural interfaces, hippocampus, injured brain, regeneration, synaptic  
36 enhancement

## 37 INTRODUCTION

38 In modern neuroscience, a large amount of (interdisciplinary) research is devoted to the development of  
39 novel therapeutic approaches to treat a variety of pathological conditions, ranging from neurodegenerative  
40 diseases (Perlmutter and Mink, 2006) to traumatic brain injuries (Girgis *et al.*, 2016; Maas *et al.*, 2002;  
41 Finnie *et al.*, 2002) and psychiatric disorders (Perlmutter and Mink, 2006). An attractive strategy involves  
42 the development of assistive implantable devices, such as electrodes or interfaces, aimed at restoring the  
43 lost functions (Guggenmos *et al.*, 2013). In the engineering of neuroprosthetic devices, nanotechnology  
44 demonstrated to play an important role (Cetin *et al.*, 2012), by enriching artificial scaffolds with controlled  
45 nano-sized features/cues, improving the interfacing stability with neuronal tissues at the cellular and  
46 subcellular level (Vidu *et al.*, 2014; Lee *et al.*, 2006; Wang *et al.*, 2017), and providing a potential  
47 regenerative guidance. In this framework, electrically conductive nanomaterials such as carbon nanotubes  
48 (CNTs) (Iijima, 1991), are still promising, because of their tunable physicochemical features (O'Connell  
49 *et al.*, 2006) and their ability to finely interact with neuronal cells (Lovat *et al.*, 2005; Cellot *et al.*, 2011)  
50 and neural tissues (Fabbro *et al.*, 2012; Usmani *et al.*, 2016). Because of these properties, CNT-endowed  
51 surfaces have been employed in the fabrication of diverse neural interfaces (Bareket-Keren *et al.*, 2013;  
52 Vidu *et al.*, 2014), such as retinal implants (Cyril *et al.*, 2017) or deep brain stimulators (Vitale *et al.*,  
53 2015). Intriguingly, CNTs were shown to improve axons regeneration and functional reconnection among  
54 segregated mammal spinal cord explants in vitro (Aurand *et al.*, 2017; Fabbro *et al.*, 2012; Usmani *et al.*,  
55 2016). However, their ability to trigger similar effects when challenged with other central nervous system  
56 (CNS) areas has yet to be shown. Besides, two significant factors that limit CNTs engineering in brain  
57 interfaces, namely their lack of optical transparency and their unstable adhesion to nanostructured films,  
58 need to be addressed. Until now, the opaqueness of CNT-films directly grown via chemical vapor  
59 deposition (CVD) (Rago *et al.*, 2019), hindering the passage of visible light, restrained the exploitation of

60 such substrates in live imaging or optogenetic applications. On the other hand, the limited mechanical  
61 stability of CNTs films prepared following the drop-casting procedure (Hokkanen *et al.*, 2017) to the  
62 supporting substrate may result in support detachment due to shear stresses induced by cell growth and  
63 motion (Nelson, 2017), muscular tissue contractility (Tscherter *et al.*, 2001) and/or culturing media  
64 replacement (Huber *et al.*, 2018).

65 In this study, we take advantage of direct growth of a thin layer of CNTs on fused silica slides, which  
66 results in transparent substrates endowed with tightly-bonded CNTs suitable for the assessment of  
67 functional reconnection in complex CNS organ explants. Through patch-clamp electrophysiology and  
68 immunocytochemistry experiments, we investigate whether the novel CNT-endowed substrates retain the  
69 ability to support the maturation and growth of dissociated neurons and glial cells from rat hippocampus  
70 and, more importantly, we evaluate their impact on the emerging circuit activity. We demonstrate that the  
71 novel CNT-endowed substrates can sustain the development of synaptic networks characterized by  
72 improved activity (Lovat *et al.*, 2005; Cellot *et al.*, 2009; Cellot *et al.*, 2011).

73 We further address the potential of tCNTs in supporting axons regeneration when coupled to complex  
74 Central Nervous System (CNS) structures (Usmani *et al.*, 2016), by interfacing tCNTs with entorhinal-  
75 hippocampal organotypic cultures (EHCs) containing the entorhinal cortex, the perforant path, and the  
76 dentate gyrus. To investigate the regenerative potential of tCNTs platforms, we mimic a CNS lesion by  
77 transecting the perforant-pathway, (Starega *et al.*, 1993; Li *et al.*, 1994; Steward and Vinsant; 1983; Del  
78 Turco and Deller 2007; Woodhams and Atkinson 1996; Woodhams *et al.*, 1993; Perederiy *et al.*, 2013;  
79 Parnavelas *et al.*, 1974). To better reproduce a severe mechanical injury (Finnie *et al.*, 2002), we  
80 introduced a remarkable gap between the two portions of tissue: the Hippocampus (H) was placed at 0.5  
81 mm far apart from the Entorhinal Cortex (EC). We show that tCNTs boost EHCs fibre sprouting ability,  
82 which ultimately leads to functional and anatomical reconnection of the two separated brain structures.

83

## 84 RESULTS

85 tCNTs synthesis and characterization.

86 Transparent CNTs (tCNTs) were synthesized via catalytic chemical vapor deposition (CCVD) directly on  
87 fused silica slices. The synthesis was done taking advantage of the catalytic effect of iron nanoparticles  
88 thermally obtained from a thin iron film deposited (without the employment of any adhesion layer) on the  
89 fused silica substrates. The thermal synthesis required just 90 seconds resulting in an ultra-thin layer of  
90 entangled CNTs decorating the slices. In our CCVD synthesis of CNTs, catalyst plays a crucial role since  
91 nanoparticles result from a thermal annealing treatment of the substrates and they act as starting sites for  
92 the subsequent CNTs growth (Shah and Tali, 2016). Size and density of these nanoparticles are strongly  
93 related to annealing treatment parameters (i.e., temperature and time) and the features of the initial catalyst  
94 layer (i.e., starting film thickness and its adhesion to the underneath substrate (Chiang and Sankarana,  
95 2007). In the attempt to enhance CNT synthesis yield, one or even more intermediate metallic layers could  
96 be used as adhesion and/or anti-diffusion layer between the catalyst and the underneath support (Michaelis  
97 *et al.*, 2014; Bayer *et al.*, 2011). Moreover, it was reported that by setting the annealing treatment  
98 conditions at 720 °C for 3 hours and the growth parameters at 720 °C for 1 hour, it is possible to obtain  
99 long vertically aligned CNTs (LVA-CNTs) on various supports (Morassutto *et al.*, 2016). Anyhow, we  
100 here demonstrated that, although any adhesion metal was employed and even if the growth time was  
101 limited to just 90 sec, the yield, reproducibility, and density of the as-produced CNTs are comparable with  
102 that of similar carbon nanostructures produced by using more time-consuming methods. Just the length of  
103 the resulting CNTs is limited and, consequently, the total thickness of the CNTs film covering the  
104 supporting substrate. Scanning electron microscopy (SEM) imaging was performed on CNTs mat to assess  
105 CNT dimensions, length, uniformity, and density. SEM micrographs (Figure 1A, left) showed a uniform

106 carpet of short CNTs (star mark) covering a flat supporting surface of fused silica (hash mark) exposed  
107 scratching CNTs away with a razor blade. The enlargement of the dashed-line marked area pointed out a  
108 crumpled portion of the CNTs film (Figure 1A, center) allowing to estimate a film thickness of about 1  
109  $\mu\text{m}$ . A high magnification image in correspondence of the star mark made visible the single CNTs  
110 constituting the carpet and their random orientation (Figure 1A, right) due to the absence of proximity  
111 effects. (Zhang *et al.*, 2006). Transmission electron microscopy (TEM) characterization was conducted  
112 on CNTs to explore their structure and crystallinity. It has been found that CNTs consist of multi-walled  
113 carbon nanotubes (MWNTs) with a variable number of walls. Specifically, Figure 1B shows an isolated  
114 MWNT with an outer diameter (OD) of less than 30 nm and inner diameter (ID) of approximately 10 nm.  
115 These measurements are consistent with 15 nanotube walls (Chiodarelli *et al.*, 2012). In addition, TEM  
116 analysis revealed the presence of structural defects (Figure 1B, right), generally imperfections of  
117 conjugated  $\text{sp}^2$  carbon along the tubes (i.e., breaks),  $\text{sp}^3$  hybridized carbon atoms, Stone-Wales defects  
118 (i.e., two heptagons and two pentagons), presumably ascribable to the low synthesis temperature used  
119 ( $730\text{ }^\circ\text{C}$ ) (Lee *et al.*, 2001; Charlier *et al.*, 2002). Interestingly,  $<2\text{ }\mu\text{m}$  thick CNT films covering the fused  
120 silica do not prevent the light from passing through the sample (see the transmittance plot for samples of  
121 CNT film with different thickness shown in Figure 1C, left) resulting in (quasi) transparent CNT  
122 substrates. An increase in the synthesis time (e.g., 4 minutes) gives rise to almost opaque CNT films  
123 (Figure 1C, right). In this work we used samples characterised by a CNT film thickness in the range of  
124  $0.2$  to  $2\text{ }\mu\text{m}$ , ultimately able to guarantee the needed optical transparency. The degree of structural ordering  
125 and the quality of our CCVD CNTs were evaluated by Raman spectroscopy. The two main bands typical  
126 of all graphite-like materials, including MWNTs, present in Raman spectra (Figure 1D) correspond to the  
127 G band at  $\sim 1583\text{ cm}^{-1}$ . This band related to the in-plane tangential vibration of  $\text{sp}^2$  carbon atoms resulting  
128 from the graphitic nature of CNTs and the D band at  $\sim 1330\text{ cm}^{-1}$  indicating the presence of amorphous

129 and/or low ordered carbon structure (carbonaceous impurities with  $sp^3$  bonding, and broken  $sp^2$  bonds in  
130 the sidewalls (Costa *et al.*, 2008). The ratio between the D ( $I_D$ ) and G ( $I_G$ ) band integral intensities was  
131 usually adopted as an indicator of CNTs quality. Specifically, similar intensities of these bands (Antunes  
132 *et al.*, 2007), as in our case, suggested the presence of non-graphitic carbon in nanotubes, typical for low-  
133 temperature CVD-grown CNTs (Bulusheva *et al.*, 2008). Together with the G band, the second-order  
134 Raman peak G' is characteristic of graphitic  $sp^2$  materials and is located at  $\sim 2700\text{ cm}^{-1}$ . The G' band, an  
135 overtone mode of the D band (Saito *et al.*, 2003), is associated with defect density, but not as crucially as  
136 the first order mode. It was also reported that the intensity of this peak depends significantly on the  
137 metallicity of CNTs (Kim *et al.*, 2007). Other peaks located at  $\sim 1698\text{ cm}^{-1}$  and  $\sim 1759\text{ cm}^{-1}$  are related to  
138 C=O bond vibration (Roeges, 1997; Long, 1997) and indicate possible partial oxidation of MWNTs. From  
139 the XPS survey spectrum of CNTs (Figure 1E) three elements can be discriminated: carbon (C1s), oxygen  
140 (O1s) and silicon (Si2s and Si2p). The atomic percentage of C and O are 87.6 at% and 10 at%, respectively.  
141 Only a small amount of Si was detected (2.4 at%). The presence of oxygen on CNTs surface is intrinsically  
142 related to our CVD procedure and, specifically, to defects originated during CNTs synthesis showing the  
143 tendency to adsorb oxygen when exposed to air. Figure 1F indicates the C1s core level for a  $\sim 8\text{ }\mu\text{m}$  thick  
144 CNT film. The most intense peaks located at 284.7 eV and 285.8 eV can be assigned to  $sp^2$ -hybridized  
145 graphitic carbon atoms located on CNTs walls and to amorphous carbon ( $sp^3$ -hybridized carbon atoms),  
146 respectively (Mattevi *et al.*, 2008; Hofmann *et al.*, 2009). The amorphous carbon is likely due to the CNTs  
147 synthesis process, as confirmed by the structural defects identified via TEM (Figure 1B) and Raman  
148 spectroscopy (Figure 1D). The peak at 290.8 eV corresponds to the electron energy loss peak due to  $\pi$ -  
149 plasmon excitations. These three peaks are characteristics of C1s core level from CNTs (Okpalugo *et al.*,  
150 2005; Mudimela *et al.*, 2014). The additional small peaks at 287.15 eV, and 288.4 eV were assigned to  
151 the presence of oxygen (Okpalugo *et al.*, 2005).



152

153 tCNTs biocompatibility: dissociated primary neurons growth and synaptic activity

154 CNTs carpets have been since long characterized as platforms enriched with nano-scaled topology able to  
155 support neural cultures development, and their effects on cultured hippocampal primary cells are well  
156 described (Lovat *et al.*, 2005; Cellot *et al.*, 2009; Cellot, *et al.*, 2011). Anyway, being the result of a novel  
157 fabrication process, our first concern was to understand if the new tCNTs carpets were biocompatible and  
158 able to sustain the development of healthy and functional neural networks, potentiating the emerging  
159 synaptic activity in respect to Control cultures, as reported for opaque CNTs interfaces (Lovat *et al.*, 2005;  
160 Mazzatenta *et al.*, 2007; Cellot *et al.*, 2011; Rago *et al.*, 2019). To this aim, we compared cultured  
161 dissociated primary neurons from rat hippocampus interfaced to tCNTs-decorated substrates with glass  
162 supported Controls. To evaluate if tCNTs were allowing the correct adhesion and growth of primary cells,  
163 we quantified the neuronal and glial cell densities after 8÷10 days of in vitro growth (DIVs). Neurons and  
164 glial cells were imaged by immunofluorescence of the specific cytoskeletal components  $\beta$ -Tubulin III, to  
165 visualize neurons, and glial fibrillary acidic protein (GFAP) to visualize glial cells; as shown in Figure 2A  
166 the cellular composition of the networks developed onto Controls (left) and tCNTs (right) substrates are  
167 qualitatively comparable. We quantify the number of neurons and astrocytes composing the networks and  
168 no statistical difference in terms of cell densities were pointed out (bar plots in Figure 2B) indicating that  
169 tCNTs can sustain hippocampal cells growth in a fashion similar to Control substrates. We further  
170 addressed network synaptic activity by means of single neuron, whole-cell patch clamp recordings. Figure  
171 2C shows sample current tracings of the basal spontaneous synaptic activity of Control and tCNTs  
172 neurons, characterized by the occurrence of heterogeneous events of inward currents, displaying variable  
173 amplitudes. (Mazzatenta *et al.*, 2007). We did not detect any significant variation in the mean amplitude  
174 values of the post-synaptic currents (PSCs) in tCNT-interfaced neurons (n=59 cells) when compared to

175 Controls (n=40 cells; Controls:  $30\pm 2.8$  pA; tCNTs  $44\pm 5$  pA;  $p=0.10$ ; box plots in Figure 2D, left), as well  
176 as in the membrane passive properties, such as the input resistance (Controls:  $790\pm 104$  M $\Omega$ ; tCNTs:  
177  $587\pm 67$  M $\Omega$ ;  $p=0.10$ ) and membrane capacitance (Controls:  $34\pm 2$  pF; tCNTs:  $39\pm 3$  pF;  $p=0.20$ ).  
178 Conversely, we measured a significant ( $p=0.03$ ) increase in the PSCs frequencies when comparing the  
179 two conditions (Controls:  $1.3\pm 0.1$  Hz; tCNTs:  $1.8\pm 0.1$  Hz; Figure 2D, right). By these preliminary tests,  
180 we concluded that the newly manufactured tCNTs allow hippocampal cell adhesion, viability, synaptic  
181 network development and promote enhanced synaptic activity, an effect reminiscent of what reported  
182 when interfacing neurons to CNT carpets (drop-casted or thick CVD growth films; Lovat *et al.*, 2005;  
183 Mazzatenta *et al.*, 2007; Cellot *et al.*, 2009; Cellot *et al* 2011; Rago *et al.*, 2019).

184

#### 185 Organotypic Entorhinal-Hippocampal cultures growth interfaced to tCNTs

186 In the second set of experiments, we tested tCNTs, characterized by transparency and strong adhesion to  
187 the underneath fused silica substrate, as growth interfaces for intact and injured CNS explants. In  
188 particular, we focused on the entorhinal-hippocampal system. As shown by low magnification  
189 immunofluorescence images in Figure 3A, intact entorhinal-hippocampal organotypic slices (EHC)  
190 successfully grew interfaced to tCNTs, in a way similar to Controls EHCs (Figure 3A, right and left,  
191 respectively). To challenge the regenerative potential of the new tCNTs, we simulated a severe mechanical  
192 lesion at the subicular level by surgical complete transection. After transecting the tissue, the Entorhinal  
193 Cortex (EC) and Hippocampus (H) components were cultured (8÷12 DIV) at a distance of  $500\pm 100$   $\mu$ m  
194 apart (Figure 3B; see Methods). Also after denervation, we detected adhesion, survival and growth of the  
195 organotypic cultures on both tCNTs and Control (Figure 3B, right and left, respectively). We adopted this  
196 configuration to reproduce in vitro a traumatic event due to mechanical injury, resulting in anatomical and  
197 functional disconnection of the two brain regions. A severe perforant pathway (PP) transection at the

198 subicular level is a widely exploited and generally accepted model to investigate neural circuits plasticity  
199 in response to brain injury, adopted in vivo and in organotypic slices (Perederiy and Westbrook, 2013;  
200 Vuksic *et al.*, 2011; Vlachos *et al.*, 2012). We next investigated the functional impairments following the  
201 lesion and the residual neuronal activity in both the EC and H slices (Perederiy and Westbrook, 2013).

202 tCNTs enhance the entorhinal-hippocampal field potential synchronization

203 We performed simultaneous local field potential (LFP) recordings by placing one electrode in the H within  
204 the molecular layer of the dentate gyrus (DG), and a second one within the deep layer (IV/V) of the EC.  
205 LFPs are voltage signals that reflect collective multiple neurons membrane activities. We compared the  
206 spontaneous basal activities emerging upon 8÷12 DIV between intact EHC and the lesioned one, in which  
207 the PP was totally resected and the two (emi)-portions of the EHC displaced (see the cartoon in Figure  
208 3C). Field recordings were performed in standard saline solution (see Methods) for intact and lesioned  
209 EHCs developed on glass substrates (sketched in Figure 4A; n=7 and n=9, respectively) or interfaced with  
210 tCNTs (sketched in Figure 4B; n=5 and n=6, respectively).

211 We quantified DG spontaneous activity when grown on Control glass substrates, by measuring the LFPs  
212 Inter-Event Intervals (IEIs). Upon prolonged denervation, IEIs show a significant (cumulative distribution  
213 in Figure 4C, top plot for DG;  $p < 0.001$ ) increase in duration in lesioned EHCs when compared to the  
214 intact slices, testifying a reduction in DG excitation. Similarly, LFPs in EC on Control substrates showed  
215 a significant increase in IEIs duration in lesioned EHCs when compared to the intact slices (cumulative  
216 distribution in Figure 4C, bottom plot;  $p < 0.001$ ). Thus, in Control conditions, denervation usually  
217 determined a reduction in the occurrence of LFPs. When analysing EHCs interfaced to tCNTs, in the intact  
218 organ slices we detected higher LFPs occurrence in DG when compared to glass Controls (cumulative  
219 distribution in Figure 4C, top plot;  $p < 0.001$ ). To note, in DG, LFPs activity was further enhanced after  
220 8÷12 DIV of denervation, even when compared to intact tCNTs cultures (i.e., lower IEI values; cumulative

221 distribution in Figure 4C, top plot;  $p < 0.01$ ). A similar behaviour was observed when measuring the  
222 distribution of IEIs values of LFPs recorded from the EC interfaced to tCNTs, in intact or injured EHC  
223 (cumulative distribution in Figure 4C, bottom plot;  $p < 0.001$ ). These results suggest that in intact slices,  
224 tCNTs interfacing promote an increase in spontaneous activity, reminiscent of the material effect detected  
225 in spinal slice cultures (Fabbro *et al.*, 2012), and presumably due to the reported ability of CNT-based  
226 interfaces to enhance synaptic networks (Lovat *et al.*, 2005; Mazzatenta *et al.*, 2007; Cellot *et al.*, 2011;  
227 Fabbro *et al.*, 2012).

228 Regardless of the intact EHCs, in lesion ones tCNTs interfacing has the ability to promote LFP occurrence  
229 in both DG and EC slices when compared to injured glass Controls (Figure 4D, blue and red box plots,  
230 respectively; Control:  $n=9$ , tCNT:  $n=6$ ;  $p < 0.001$ ), a result that might indicate the ability of tCNTs in  
231 promoting functional changes in excitatory synapses post-denervation, alternatively tCNTs might also  
232 favour regeneration and synaptic targeting of the injured PP axons (Usmani *et al.*, 2016).

233 To assess whether tCNTs have the ability to promote PP regeneration and synaptic targeting, we assessed  
234 the functional connectivity between the DG and the EC in intact and lesion EHC when interfaced to the  
235 two different substrates by cross-correlation analysis of the simultaneously recorded, spontaneous LFPs.  
236 Interestingly, in intact EHC, only 43% of Controls DG and EC displayed a Pearson Correlation Coefficient  
237 (CCF) that was significantly larger than that expected by chance (see Methods; Usmani *et al.* 2016), such  
238 a value was increased to 100% in intact tCNT recordings (summarized by bar plot in Figure 4E). In  
239 lesioned EHCs, correlated LFPs dropped to 11% of Controls, while 50% of tCNTs LFPs recordings were  
240 still correlated (bar plot in Figure 4E). Thus, injured EHC, upon 8÷12 DIV interfaced to tCNTs, displayed  
241 a lower impairment in spontaneous LFPs characterised by a larger connectivity, as supported by the higher  
242 synchronization of the two segregated EHC portions. These results suggest that tCNTs enhanced the  
243 regeneration of PP fibres and promote synaptic targeting when interfacing lesioned EHC.

244

245 tCNTs favour regrowth of active fibres in injured EHC slices

246 To assess whether tCNTs promoted new fibres sprouting leading to a more functional bridge between the  
247 EC and H sections, we tested the ability of stimulating EC superficial layers, where the PP is known to  
248 originate (Jacobson and Marcus, 2008; Witter and Amaral, 2004; Witter, 2007) in evoking LFPs in injured  
249 EHC. The two recording electrodes were positioned in the same configuration used for simultaneous  
250 recordings of DG and EC spontaneous LFPs, while an additional stimulating electrode was placed in the  
251 superficial layers of the EC (see sketches in Figure 5A, left). We, therefore, proceeded with the PP  
252 stimulation (see Methods), and we grouped the evoked LFPs into three categories: the first, when the  
253 stimulation evoked successful responses from both EC and DG, the second when the response was evoked  
254 only in the EC or, third, only in the DG. Tracings in Figure 5A, right panel, shows sample voltage tracings  
255 depicting these three responses (in blue, green, and magenta, respectively), for the lesioned EHC.

256 In intact ECHs, regardless the presence of tCNTs, PP stimuli always evoked LFPs in both EC and DG  
257 (Figure 5B, top; Control: n=4; tCNTs: n=4). On the opposite, in injured EHCs, evoked responses in the  
258 two groups diverged. In injured Control organ slices, only in 12.5% of cases PP stimulation evoked a LFP  
259 in both EC and DG, while in the majority of case (50%) only EC responses were evoked. Intriguingly, in  
260 12.5% of cases only LFP in DG was evoked, with the remaining 25% of slices unresponsive. Notably, in  
261 injured EHCs interfaced to tCNTs, we elicited evoked LFPs from both areas in 100% of cases, as in intact  
262 slices (Figure 5B, bottom; Control: n=8; tCNTs: n=6). This evidence further strengthens the hypothesis  
263 that the slices recovered a 1 (re)connection with similar evoked LFPs of the intact (i.e. not lesioned)  
264 structure when cultured onto tCNT platforms. Eventually, we investigated if the tCNT-related increase in  
265 EC/H synchronized activity and PP-stimulation evoked responses were attributable to an increased  
266 number of newly generated fibres interconnecting the EC and H sections and able to carry effective

267 electro-chemical signals. To address this point, we performed via immunohistochemistry a quantification  
268 of SMI32-positive axons (see Methods) crossing the gap separating H and EC (Figure 5C). In injured  
269 ECH interfaced to tCNTs we detected a significantly larger amount of SMI32-positive “crossing-fibres”  
270 sprouting into the lesioned area with respect to the Control counterparts (Control: n=7, tCNT: n=6; p=0.02;  
271 Figure 5D). Together with the previous electrophysiological findings, this result shows that tCNTs  
272 enhanced the regeneration of axons and their synaptic targeting between EC and DG, re-establishing an  
273 active crosstalk between the two separated areas of the sectioned tissue.

274

## 275 DISCUSSION

276 CNTs have contributed considerably to developments in tissue engineering (Edwards *et al.*, 2009) and  
277 nanomedicine (Erol *et al.*, 2017; Marchesan *et al.*, 2015) due to their unique physical features (O’Connell  
278 *et al.*, 2006) and hold the potential to further contribute to the design of novel nano-devices and neural  
279 interfaces (Pancrazio *et al.*, 2008; Bareket-Keren *et al.*, 2013). In this study, we report a novel CCVD  
280 based approach in CNT synthesis generating uniform carpets of entangled nanotubes on fused silica  
281 supporting substrates. Differently from commonly used CVD or drop-casting CNT decorating  
282 methodologies (Lovat *et al.*, 2005; Mazzatenta *et al.*, 2007; Rago *et al.*, 2019; Chena *et al.*, 2012) by our  
283 new approach we manufactured optically transparent (Anguita *et al.*, 2013) CNT substrates characterised  
284 by mechanical stability due to their strong adhesion to the underneath surface. These features made our  
285 novel films of tCNTs of particular interest in (neuro)-biology applications where the substrate mechanical  
286 stability and the use of techniques demanding transmission of visible light through the samples are  
287 required. Our main results are that tCNT-based substrates when challenged with dissociated and organ  
288 CNS cultures were biocompatible, allowing the development of neuronal synaptic networks, and  
289 maintained CNT characterising features of potentiating neural transmission at the interface (Lovat *et al.*,

290 2005; Cellot *et al.*, 2011; Rago *et al.*, 2019) and promoting axons regrowth (Usmani *et al.*, 2016; Fabbro  
291 *et al.*, 2012).

292 Hippocampal dissociated cultures interfaced to tCNTs were characterized by CNS cell densities and  
293 neuron/glia ratios comparable to Controls; the viability of neurons on tCNTs was also supported by the  
294 values of the cell passive membrane properties, accepted indicators of neuronal health (Carp, 1992; Gao  
295 *et al.*, 2015). Despite the similarities in network size, tCNTs neurons displayed increased synaptic activity,  
296 probably due to the described synaptogenic effects of CNTs, acting as artificial biomimetic clues (Cellot  
297 *et al.*, 2011; Pampaloni *et al.*, 2018; Rago *et al.*, 2019).

298 We scaled up the system by developing organotypic cultures, to investigate the regenerative potentials of  
299 tCNTs. Organotypic CNS explants are a well-established technique, such slice cultures maintain a three-  
300 dimensional organisation, preserve the cytoarchitecture and cell populations of the organ of origin and  
301 provide excellent experimental access to electrophysiology, live imaging and morphology analyses  
302 (Fabbro *et al.*, 2012; Usmani *et al.* 2016). Accordingly, EHC organ cultures are 3D explants of the CNS  
303 in which the overall functional and anatomical neuronal connections are preserved (Del Turco and Deller,  
304 2007; Vlachos *et al.*, 2012). In accordance with our previous studies (Fabbro *et al.*, 2012) interfacing  
305 EHCs to tCNTs improved spontaneous network activity and potentiated LFP synchronization. We  
306 hypothesize that these effects are ultimately related to an increase in synaptic efficacy due to increased  
307 synapse formation at the interface with the large surface, roughness and conductivity of tCNTs (Fabbro *et*  
308 *al.*, 2012), although we cannot exclude other mechanisms, such as ability of the conductive tCNTs to  
309 mediate a direct electrical transmission within the cultured EHC areas.

310 To address the regenerative ability of tCNTs interfaces we adopted the perforant patch lesion model, a  
311 brain injury model that disrupts the main excitatory input to the DG (Vlachos *et al.*, 2012). Upon a  
312 complete transection of the PP, we cultured surgically separated H and EC components to the end of

313 assessing denervation-induced regenerative activity reconnecting the two structures and eventually  
314 leading to functional recovery. Indeed in Controls such a procedure leads to a loss of activity in DG and  
315 EC structures, indicative of a limited regenerative ability. We did not detect any form of synaptic  
316 plasticity, such as homeostatic synaptic scaling, due to denervation (Vlachos *et al*, 2012). Although we  
317 cannot exclude that such changes need single cell recording approaches for being detected, it is also  
318 feasible that at the time of recordings (> 1 week after denervation) this transient adaptation to the loss of  
319 excitatory drive had returned to baseline values (Vlachos *et al.*, 2012). tCNTs, upon axonal regeneration  
320 re-established the appropriate excitatory connections, at least in part, as indicated by evoked LFPs,  
321 synchronization of spontaneous LFPs and frequency of LFPs. The latter increase in activity, even higher  
322 than in intact structures, might indicate an overall increase in excitability, potentially due to long lasting  
323 plasticity compensation, again sustained by the conductive substrates. In previous studies, we have shown  
324 that CNT-based interfaces possess regenerative abilities when interfaced to spinal cord explants (Fabbro  
325 *et al.*, 2012; Usmani *et al.*, 2016). In particular, an increased growth cone activity was associated to direct  
326 interactions among axons and CNTs, via formation of membrane/material tight junctions (Fabbro *et al.*,  
327 2012). Modulating mechanical forces and adhesion may activate cascades of biochemical signaling  
328 relevant to CNS reconstruction.

329

330 In conclusion, by introducing a new method to synthesize CNTs and demonstrating for the first time the  
331 benefits that this substrate is bringing to lesioned organotypic EHCs cultures, we strengthened the notion  
332 of the use of physical features alone to guide different biological responses: tCNTs, with their peculiar  
333 transparency coupled to the regenerative effects, stand as a promising material to be exploited in a broad  
334 range of applications, from the development of new research tools to the design of devices able to actively  
335 interface neural tissue reconstruction.



336

## 337 MATERIALS AND METHODS

### 338 tCNTs synthesis

339 Multi-walled carbon nanotubes were synthesized by the decomposition of acetylene (carbon source)  
340 catalyzed by iron nanoparticles (NPs). NPs were obtained thermally annealing a thin layer of iron  
341 evaporated on fused silica (SiO<sub>2</sub>) wafer chips, acting as transparent supporting substrates (Ward *et al.*,  
342 2003). Fused silica wafers were manually cleaved into 15x15 mm<sup>2</sup> slices using a diamond scribe and  
343 cleaned following the Radio Corporation of America (RCA) method (Kern and Puotinen, 1970).  
344 Subsequently a thin layer of iron (0.2÷1 nm in thickness) was deposited directly above the SiO<sub>2</sub> chips  
345 surfaces using an electron beam (e-beam) evaporator. Iron film thickness was monitored using an in-situ  
346 quartz crystal microbalance. Since catalyst layer uniformity plays a crucial role in CNTs synthesis and  
347 growth, an average deposition rate of 0.2 Å/sec was adopted. The as-evaporated substrates were placed  
348 above the heating element of a high vacuum reaction chamber. An annealing treatment (4 min at 660±10  
349 °C in H<sub>2</sub> atmosphere) was performed to: (i) reduce iron oxides resulting from the exposition of the  
350 samples to the atmospheric air during the transfer from the e-beam deposition system to the high-  
351 vacuum CVD reactor, and (ii) to induce the nucleation from the continuous iron layer of homogeneously  
352 distributed nanoparticles which will act as nucleation sites for the CNTs growth. Once this treatment  
353 process was over, acetylene was introduced in the reaction chamber up to a partial pressure of about  
354 10÷20 mbar. Sample temperature was increased to 730 °C and reaction time was limited to 90 seconds,  
355 resulting in the formation of a uniform carpet of CNTs of less than 2 μm in thickness. After that,  
356 samples were let to cool down to room temperature and employed as removed from the reaction  
357 chamber.

### 358 tCNTs characterization

359 Field Emission Scanning Electron Microscopy (FE-SEM) imaging was performed on the as-produced  
360 CNTs using a Gemini SUPRA 40 SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany) operating at an  
361 accelerating voltage of 5 keV. Transmission electron microscopy (TEM) of CNT carpets was performed  
362 using an EM 208-Philips TEM system equipped with Quemesa (Olympus Soft Imaging Solutions)  
363 camera. Before TEM imaging, samples were released from the substrates, dispersed in ethanol and drop-  
364 casted onto a commercial lacey-carbon TEM grid. Transmission spectra in the visible spectral range  
365 (400÷700 nm) were acquired with an Agilent Technologies Cary-60 UV-VIS spectrophotometer at a  
366 scan speed of 600 nm/min and 1 nm resolution. CCVD CNT film thicknesses were evaluated performing  
367 Atomic Force Microscopy (Solver Pro, NT-MDT, RU) across a scratch in the film done with a scalpel  
368 and exposing the underneath fused silica substrate. Raman spectroscopy was conducted on the as-  
369 produced CNTs at room temperature employing a Renishaw inVia Raman microscope with a 60x  
370 objective lens at 632.8 nm laser excitation and a laser power of about 2 mW. In order to evaluate the  
371 CNTs surface composition, X-ray Photoelectron Spectroscopy (XPS) was carried out on a VG Escalab  
372 II spectrometer, in constant pass energy mode. Non-monochromatized Al K $\alpha$  exciting radiation (1486.6  
373 eV, 225 W) was used. Core-level XPS data analysis was performed after the removal of nonlinear  
374 Shirley background and deconvolution into Gaussian/Lorentzian components using Igor Pro 6.36  
375 software (Wavemetrics Co., US).

#### 376 Ethics

377 All procedures were approved by the local veterinary authorities and performed in accordance with the  
378 Italian law (decree 26/14) and the UE guidelines (2007/526/CE and 2010/63/UE). The animal use was  
379 approved by the Italian Ministry of Health. All efforts were made to minimize suffering and to reduce the  
380 number of animals used.

#### 381 Primary cultures

382 Hippocampal neurons were obtained from neonatal Wistar rats (postnatal day: P2-P3) as previously  
383 reported (Lovat *et al.*, 2005; Cellot *et al.*, 2009). Briefly, cells were plated either on poly-L-ornithine-  
384 coated (Sigma Aldrich; Controls) or on tCNTs-coated glass coverslips and incubated at 37 °C, 5% CO<sub>2</sub>,  
385 in Neurobasal-A (Thermo Fischer) medium containing B27 2% (Gibco), Glutamax 10 mM and  
386 Gentamycin 0.5 μM (Gibco). Cultured neurons were used for experiments at 8÷10 days in vitro (DIV).

#### 387 Organotypic cultures

388 Organotypic slice cultures were prepared according to the roller-tube technique, previously described  
389 (Gähwiler, 1988; Mohajerani and Cherubini, 2005). Briefly, 400 μm thick EHCs slices were obtained  
390 from P6÷ P8 old Wistar rats (the perforant pathway is described to be fully developed in rats from P6;  
391 Fricke and Cowan, 1977) by means of a tissue chopper (McIlwan) and stored for 1h in cold (4 °C) Gey's  
392 Balanced Salt Solution medium (GBSS) enriched with Glucose and kynurenic acid to limit excitotoxic  
393 processes. The slices were subsequently plated onto glass Control coverslips or tCNTs covered fused silica  
394 slices and embedded in chicken plasma (16 μL; SIGMA), which was coagulated with the addition of a  
395 drop of thrombin (23 μL). The lesion was made with a scalpel and under microscopy at the subicular level  
396 and the entorhinal cortex portion was placed from 400 to 600 μm far from the hippocampus one. This was  
397 accomplished taking advantage of a graduated ruler placed below the coverslips during plating. Cultures  
398 were then left for 1 hour at room temperature and then placed in Nunc<sup>TM</sup> tubes filled with 750 mL of  
399 Neurobasal-A (Thermo Fischer) medium containing B27 2% (Gibco), Glutamax 10 mM and Gentamycin  
400 0.5 μM (Gibco). Tubes were incubated at 37 °C in a roller-drum (0.17 RPM) and used for experiments  
401 after 8÷12 days in vitro (DIV). The medium was completely replaced every 3 days.

#### 402 Patch-clamp experiments

403 Patch-clamp, whole cell, recordings were achieved with glass micropipettes with a resistance of 4 to 7  
404 MΩ. The intracellular pipette solution was the following: 120 mM K-gluconate, 20 mM KCl, 10 mM

405 HEPES, 10 mM EGTA, 2 mM MgCl<sub>2</sub>, 2 mM Na<sub>2</sub>ATP, pH 7.3. Cultures were positioned in a custom-  
406 made chamber mounted on an inverted microscope (Eclipse TE-200, Nikon, Japan) and continuously  
407 superfused with external solution at a rate of 5 mL/min. The external saline solution contained: 150 mM  
408 NaCl, 4 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM HEPES, 10 mM glucose, pH 7.4. Cells were voltage  
409 clamped at a holding potential of -56 mV (not corrected for liquid junction potential, that was calculated  
410 to be 13.7 mV at 20 °C in our experimental conditions). The (uncompensated) series resistance had values  
411 lower than 8 MΩ. All recordings were performed at room temperature (RT). Data were collected using a  
412 Multiclamp 700A patch amplifier connected to a PC through a Digidata 1440 (Molecular Devices, US)  
413 and subsequently analysed using Clampfit 10.4 software suite (Molecular Devices, US).

#### 414 Field potential recordings

415 Simultaneous extracellular field potential recordings from visually identified molecular layer of the DG  
416 and the superficial layers of the EC were performed on slices at 8÷12 DIV at RT using low resistance  
417 (4÷6 MΩ) glass micropipettes filled with extracellular solution. For each experiment, the organotypic  
418 slices were cultured onto Control glass coverslips and tCNT-decorated fused silica slides, positioned  
419 into a recording chamber, mounted onto an upright microscope (Eclipse TE-200, Nikon, Japan) and  
420 superfused with standard saline solution containing: 152 mM NaCl, 4 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM  
421 CaCl<sub>2</sub>, 10 mM HEPES, and 10 mM glucose. The pH was adjusted to 7.4 with NaOH. After a  
422 stabilization period of about 20 minutes, the recordings of the spontaneous activity were sampled for  
423 additional 45 minutes in standard saline solution. Finally, as control of the excitatory nature of the  
424 recorded neuronal signals, we evaluated the activity for 15 minutes in the presence of CNQX (10 μM)  
425 and no LFPs were detected. All data were collected using a Multiclamp 700A amplifier connected to a  
426 PC through a Digidata 1440 (Molecular Devices, US) and subsequently analyzed using Clampfit 10.4  
427 software suite (Molecular Devices, US). To stimulate the PP we placed a bipolar electrode, made by a

428 low-resistance patch pipette containing normal saline solution, into the EC superficial layers, no changes  
429 in the electrode position were made. Voltage pulses (from 200 to 1000  $\mu$ s) of increasing amplitude (from  
430 1 to 50 V) were delivered by an isolated voltage stimulator (DS2A; Digitimer Ltd.) until a response was  
431 evoked and detected. The synchrony between DG and EC LFPs was assessed through a MATLAB  
432 custom-made script, as previously described (Usmani *et al.*, 2016). Briefly, for each pair of voltage time  
433 series, the Pearson correlation coefficient (CCF) was assessed and its statistical significance was  
434 determined by performing a permutation test. This test measures the distribution of correlation  
435 coefficients that one would expect to observe if the voltage signals recorded from a pair of explants  
436 happened to correlate purely by chance. By measuring how likely it was for the values of this null  
437 distribution to be larger or equal than the real correlation coefficient, it was possible to understand  
438 whether the correlation between the pair of time series was significantly larger than expected by chance.  
439 This procedure allowed for determining what fraction of cocultured slices exhibited a significantly  
440 synchronous LFPs, for all the tested conditions (Usmani *et al.*, 2016; Aurand *et al.*, 2017).

#### 441 Immunocytochemistry and microscopy

442 To visualize dissociated hippocampal neurons, we fixed cultures in 4% formaldehyde (prepared  
443 from fresh paraformaldehyde; Sigma) in PBS for 20 min, permeabilized with 0.3% Triton X-100 and  
444 incubated with primary antibodies for 30 min at RT. After washing in PBS, cultures were incubated with  
445 secondary antibodies for 45 min and then mounted with Vectashield<sup>®</sup> (Vector Laboratories) on 1 mm  
446 thick microscope glass slides. To visualize neurons and glial cells we used the following: rabbit anti- $\beta$ -  
447 Tubulin III primary antibody (Sigma T2200, 1:250 dilution) and Alexa 594 goat anti rabbit secondary  
448 antibody (Thermo-Fisher, 1:500); anti-GFAP mouse primary antibody (SIGMA, 1:250) and Alexa 488  
449 goat anti mouse secondary antibody (Thermo-Fisher, 1:500). Cell nuclei were visualized with the  
450 nuclear marker DAPI (1:1000). Cultures were imaged with an epifluorescence microscope using 10 $\times$

451 and 20x objectives (DM 6000, Leica) and analysed with the open-source software ImageJ  
452 (<http://rsb.info.nih.gov/ij/>). To stain Organotypic cultures we fixed them for 1h at RT as described  
453 above. After PBS washes, cultures were incubated with mouse SMI32 (1:250) and rabbit NeuN  
454 (SIGMA; 1:200) primary antibodies, and Alexa 594 goat anti rabbit (Invitrogen, 1:500), Alexa 488 goat  
455 anti mouse (Invitrogen, 1:500) secondary antibodies and Hoechst (Invitrogen; 1:1000). Cultures were  
456 then mounted with Vectashield® (Vector Laboratories) on 1 mm thick microscope glass slides,  
457 visualized with a confocal microscope (Nikon Eclipse Ti-E; 10x objective) and analysed with the  
458 Volocity image analysis software (Perkin Elmer). To quantify the SMI32-positive “crossing-fibres”, we  
459 selected 3D region of interest –ROI- (500 µm x 50 µm x 15 µm) in the gap between the H and the HC  
460 (with the ROI longitudinal axis perpendicular to the segment connecting the centres of the two EHC  
461 emi-sections, see Figure 5C) in both Controls and tCNTs cultures. The amount of SMI32-positive voxel  
462 within each ROI was quantified for each image, and normalized to the overall ROI volume. All the  
463 image values from the same condition were then averaged together and plotted.

#### 464 Statistics

465 All reported values are expressed as means  $\pm$  SD, with n indicating the number of cultures, unless  
466 otherwise specified. Statistically significant differences between pairs of data sets were assessed by  
467 Student’s t test (after validation of variance homogeneity by Levene’s test) for parametric data and by  
468 either the Mann-Whitney U test or the Kolmogorov-Smirnov test for nonparametric data. When multiple  
469 groups were compared, Kruskal-Wallis test was used. Correlation and IEIs of local field potentials were  
470 measured through two different custom programs wrote in MATLAB (The MathWorks, Inc., Natick,  
471 Massachusetts, United States) (Usmani *et. al* 2016). Statistical significance was determined at  $p < 0.05$ .

472

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659 Acknowledgments

660 Funding: The authors acknowledge the t ByAxon No. 737116 to L.B.; A.G. would like to acknowledge  
661 the NATO for the project G5140.

662 Author contributions: D.S., L.B. and A.G. conceived the study and wrote the MS, N.P.P., I.R. and I.C.  
663 carried out the experiments and analysis. L.C. contributed to the XPS characterization and analysis.

664 The authors would like to thank TASC-IOM for Clean Room assistance, Alois Bonifacio for technical  
665 support and discussion in Raman spectroscopy experiments, Paolo Bertoncin for technical support in  
666 TEM characterization, Alessio Ansuini and Gianfranco Fortunato for MATLAB scripting and Beatrice  
667 Pastore for her technical support in the organotypic culturing.

668 Competing interests: The authors declare that they have no competing interests. Data and materials

669 availability: All data needed to evaluate the conclusions in the paper are present in the paper. Additional  
670 data related to this paper may be requested from the authors.

671

672 Figures Legends

673 Figure 1. Morphological, structural and chemical characterization of CNTs synthesized by CCVD on  
674 fused silica substrates.

675 A. SEM investigation of CCVD tCNT substrates reveals the uniformity of the so obtained films (left),  
676 characterized by a thickness of about 1  $\mu\text{m}$ , visible in the crumpled portion of the film (center) and a  
677 random orientation of the entangled nanotubes (right). B. TEM images of tCNTs reveals their multi-  
678 walled characteristic (left) with all the different walls constituting the tube and structural defects well  
679 visible (right). In C. transmittance analysis in the visible spectrum of four samples characterised by  
680 different CNT film thickness compared to the pristine fused silica substrate (left); on the right two  
681 representative optical images of a thin CNT film grown on fused silica (top, about 0.7  $\mu\text{m}$  in thickness)  
682 and of a thick CNT film (bottom, about 9  $\mu\text{m}$  in thickness), pointing out the good transparency of the  
683 former one. D. Raman spectra exhibiting the characteristic D, G and G' peaks of CVD grown MWNTs.  
684 E. XPS survey and C1s core level (F.) spectra of tCNTs grown on fused silica substrates.

685

686 Figure 2. tCNTs boost the spontaneous synaptic activity of hippocampal neurons.

687 A. representative fluorescent micrographs depicting dissociated primary cells networks grown on glass  
688 Control substrates (left) and on tCNTs substrates (right) stained against  $\beta$ -Tubulin III to point out  
689 neurons (in red), GFAP to highlight astrocytes (in green) and DAPI to stain cell nuclei (in blue). B. Bar  
690 plots summarize the density values for neuron and glia in the two growth conditions, note the absence of  
691 differences. C. Two representative current tracings from a Control neuron (in black) and from a tCNTs  
692 neuron (in blue). D. Box plots summarize PSCs amplitudes and frequency values. Despite no significant  
693 changes in PSCs amplitudes, a significantly higher frequency of the PSC currents related to the tCNTs  
694 condition is visible (right,  $p=0.03$ ).



695

696 Figure 3. tCNTs are suitable substrates for the development of healthy EHCs organotypic cultures.

697 A. Representative epifluorescence stitched images showing 8-days-old organotypic EHCs cultures

698 stained with Hoetsch to make visible all cell nuclei (blue) and NeuN to highlight just neuronal nuclei

699 (green) in the intact organotypic slice when cultured on glass Control (left) and tCNTs (right). B.

700 Representative images of 8-days-old lesioned EHCs organotypic cultures stained with Hoetsch (blue)

701 and NeuN (green) and cultured on glass Control (left) and tCNTs (right). Both intact and lesioned EHC

702 organotypic cultures displayed a similar morphology when grown on Control and tCNTs substrates. C.

703 Representative sketch depicting the experimental setup: entorhinal cortex (EC), the dentate gyrus (DG)

704 in the hippocampus (H) and a clear vision of the perforant pathway (blue path) together with the Shaffer

705 collaterals (black path) and mossy fiber pathway (red path). Field potential extracellular recordings were

706 simultaneously performed from the EC (left electrode) and the hippocampal DG (right electrode) in the

707 intact (left) and injured (right) EHC slice.

708

709 Figure 4. tCNTs enhance the EC-DG signal synchronization in EHCs.

710 A. A sketch of the intact EHC (left) and the lesioned one (right) when cultured on glass slide Controls.

711 Below, two representative voltage traces for the DG (black trace) and EC (red trace) are shown in standard

712 saline solution. B. A similar sketch of the intact EHC (left) and the lesioned one (right) when interfaced

713 to tCNTs. Below, two representative voltage traces for the DG (black trace) and EC (red trace) are shown

714 in standard saline solution. C. The cumulative distribution function of IEIs up to 5 seconds is shown for

715 DG (top) and EC (bottom). When interfaced to tCNTs, the activity of both DG and EC is accelerated, as

716 appreciable from the IEIs cumulative distributions (green and blue lines), characterized by a significantly

717 larger population of brief IEI when compared to glass Controls (black and red lines). D. Box plots of IEI  
718 values for the lesioned EHC shown in logarithmic scale for Controls and tCNTs, note the significant drop  
719 in IEIs duration in tCNTs-interfaced tissues. E. Bar plots summarize the correlated DG and H pair  
720 recordings in intact and injured EHCs, both in Control and tCNT substrates.

721

722 Figure 5. tCNTs induce the sprouting of functionally active fibres crossing the lesioned area.

723 A. A sketch (left) of the experimental configuration used to evaluate EC/DG intercommunication ability  
724 through the PP in intact and lesioned EHC using a stimulation electrode inserted into the EC superficial  
725 layer. Some representative traces from DG and EC recordings of a lesioned EHC were shown (right).  
726 Note the three kind of evoked responses we could observe: simultaneously from both areas (in blue),  
727 just from EC (in green), and just from DG (in magenta). B. Bar plots summarizing the distribution of the  
728 three categories of evoked responses in intact (top) and injured (bottom) EHC, both for Controls and  
729 tCNTs. C. Representative confocal images showing the sprouting of SMI32-positive fibres (in green)  
730 into the lesioned area. As summarized in the bar plot in (D.) cultures grown onto tCNTs displayed a  
731 significantly higher percentage of SMI32-positive volume with respect to Controls.

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