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

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

Short title: Interfacing axon regrowth by transparent carbon nanotube

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Abstract

The increasing engineering of carbon-based nanomaterials as components of neuro-regenerative interfaces is motivated by their dimensional compatibility with subcellular compartments of excitable cells, such as axons and synapses. In neuroscience applications, carbon nanotubes (CNTs) have been used to improve electronic device performance by exploiting their physical properties. Besides, when manufactured to interface neuronal networks formation in vitro, CNT carpets have shown their unique ability to potentiate synaptic networks formation and function. Due to the low optical transparency of CNTs films, further developments of these materials in neural prosthesis fabrication or in implementing interfacing devices to be paired with in vivo imaging or in vitro optogenetic approaches are currently limited. In the present work, we exploit a new method to fabricate CNTs by growing them on a fused silica surface, which results in a transparent CNT-based substrate (tCNTs). We show that tCNTs favour dissociated primary neurons

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

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

INTRODUCTION

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

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

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

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

RESULTS

tCNTs synthesis and characterization.

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

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

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

tCNTs biocompatibility: dissociated primary neurons growth and synaptic activity

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

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

Organotypic Entorhinal-Hippocampal cultures growth interfaced to tCNTs

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

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

tCNTs enhance the entorhinal-hippocampal field potential synchronization

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

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

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

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

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

tCNTs favour regrowth of active fibres in injured EHC slices

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

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

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

DISCUSSION

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

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

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

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

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

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

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

MATERIALS AND METHODS

tCNTs synthesis

Multi-walled carbon nanotubes were synthesized by the decomposition of acetylene (carbon source) catalyzed by iron nanoparticles (NPs). NPs were obtained thermally annealing a thin layer of iron evaporated on fused silica (SiO_2) wafer chips, acting as transparent supporting substrates (Ward *et al.*, 2003). Fused silica wafers were manually cleaved into 15x15 mm^2 slices using a diamond scribe and cleaned following the Radio Corporation of America (RCA) method (Kern and Puotinen, 1970). Subsequently a thin layer of iron (0.2÷1 nm in thickness) was deposited directly above the SiO_2 chips surfaces using an electron beam (e-beam) evaporator. Iron film thickness was monitored using an in-situ quartz crystal microbalance. Since catalyst layer uniformity plays a crucial role in CNTs synthesis and growth, an average deposition rate of 0.2 Å/sec was adopted. The as-evaporated substrates were placed above the heating element of a high vacuum reaction chamber. An annealing treatment (4 min at 660±10 °C in H_2 atmosphere) was performed to: (i) reduce iron oxides resulting from the exposition of the samples to the atmospheric air during the transfer from the e-beam deposition system to the high-vacuum CVD reactor, and (ii) to induce the nucleation from the continuous iron layer of homogeneously distributed nanoparticles which will act as nucleation sites for the CNTs growth. Once this treatment process was over, acetylene was introduced in the reaction chamber up to a partial pressure of about 10÷20 mbar. Sample temperature was increased to 730 °C and reaction time was limited to 90 seconds, resulting in the formation of a uniform carpet of CNTs of less than 2 μm in thickness. After that, samples were let to cool down to room temperature and employed as removed from the reaction chamber.

tCNTs characterization

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

Ethics

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

Primary cultures

Hippocampal neurons were obtained from neonatal Wistar rats (postnatal day: P2-P3) as previously reported (Lovat *et al.*, 2005; Cellot *et al.*, 2009). Briefly, cells were plated either on poly-L-ornithine-coated (Sigma Aldrich; Controls) or on tCNTs-coated glass coverslips and incubated at 37 °C, 5% CO_2, in Neurobasal-A (Thermo Fischer) medium containing B27 2% (Gibco), Glutamax 10 mM and Gentamycin 0.5 µM (Gibco). Cultured neurons were used for experiments at 8÷10 days in vitro (DIV).

Organotypic cultures

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

Patch-clamp experiments

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

HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM Na_2ATP, pH 7.3. Cultures were positioned in a custom-made chamber mounted on an inverted microscope (Eclipse TE-200, Nikon, Japan) and continuously superfused with external solution at a rate of 5 mL/min. The external saline solution contained: 150 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4. Cells were voltage clamped at a holding potential of −56 mV (not corrected for liquid junction potential, that was calculated to be 13.7 mV at 20 °C in our experimental conditions). The (uncompensated) series resistance had values lower than 8 MΩ. All recordings were performed at room temperature (RT). Data were collected using a Multiclamp 700A patch amplifier connected to a PC through a Digidata 1440 (Molecular Devices, US) and subsequently analysed using Clampfit 10.4 software suite (Molecular Devices, US).

Field potential recordings

Simultaneous extracellular field potential recordings from visually identified molecular layer of the DG and the superficial layers of the EC were performed on slices at 8÷12 DIV at RT using low resistance (4÷6 MΩ) glass micropipettes filled with extracellular solution. For each experiment, the organotypic slices were cultured onto Control glass coverslips and tCNT-decorated fused silica slides, positioned into a recording chamber, mounted onto an upright microscope (Eclipse TE-200, Nikon, Japan) and superfused with standard saline solution containing: 152 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$,10 mM HEPES, and 10 mM glucose. The pH was adjusted to 7.4 with NaOH. After a stabilization period of about 20 minutes, the recordings of the spontaneous activity were sampled for additional 45 minutes in standard saline solution. Finally, as control of the excitatory nature of the recorded neuronal signals, we evaluated the activity for 15 minutes in the presence of CNQX (10 µM) and no LFPs were detected. All data were collected using a Multiclamp 700A amplifier connected to a PC through a Digidata 1440 (Molecular Devices, US) and subsequently analyzed using Clampfit 10.4 software suite (Molecular Devices, US). To stimulate the PP we placed a bipolar electrode, made by a

low-resistance patch pipette containing normal saline solution, into the EC superficial layers, no changes in the electrode position were made. Voltage pulses (from 200 to1000 μs) of increasing amplitude (from 1 to 50 V) were delivered by an isolated voltage stimulator (DS2A; Digitimer ltd.) until a response was evoked and detected. The synchrony between DG and EC LFPs was assessed through a MATLAB custom-made script, as previously described (Usmani *et al.*, 2016). Briefly, for each pair of voltage time series, the Pearson correlation coefficient (CCF) was assessed and its statistical significance was determined by performing a permutation test. This test measures the distribution of correlation coefficients that one would expect to observe if the voltage signals recorded from a pair of explants happened to correlate purely by chance. By measuring how likely it was for the values of this null distribution to be larger or equal than the real correlation coefficient, it was possible to understand whether the correlation between the pair of time series was significantly larger than expected by chance. This procedure allowed for determining what fraction of cocultured slices exhibited a significantly synchronous LFPs, for all the tested conditions (Usmani *et al*., 2016; Aurand *et al*., 2017).

Immunocytochemistry and microscopy

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

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

Statistics

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

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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 μm, 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 μm in thickness)
682 and of a thick CNT film (bottom, about 9 μm 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 β-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, $p=0.03$).

Figure 3. tCNTs are suitable substrates for the development of healthy EHCs organotypic cultures. A. Representative epifluorescence stitched images showing 8-days-old organotypic EHCs cultures stained with Hoetsch to make visible all cell nuclei (blue) and NeuN to highlight just neuronal nuclei (green) in the intact organotypic slice when cultured on glass Control (left) and tCNTs (right). B. Representative images of 8-days-old lesioned EHCs organotypic cultures stained with Hoetsch (blue) and NeuN (green) and cultured on glass Control (left) and tCNTs (right). Both intact and lesioned EHC organotypic cultures displayed a similar morphology when grown on Control and tCNTs substrates. C. Representative sketch depicting the experimental setup: entorhinal cortex (EC), the dentate gyrus (DG) in the hippocampus (H) and a clear vision of the perforant pathway (blue path) together with the Shaffer collaterals (black path) and mossy fiber pathway (red path). Field potential extracellular recordings were simultaneously performed from the EC (left electrode) and the hippocampal DG (right electrode) in the intact (left) and injured (right) EHC slice.

Figure 4. tCNTs enhance the EC-DG signal synchronization in EHCs. A. A sketch of the intact EHC (left) and the lesioned one (right) when cultured on glass slide Controls. Below, two representative voltage traces for the DG (black trace) and EC (red trace) are shown in standard saline solution. B. A similar sketch of the intact EHC (left) and the lesioned one (right) when interfaced to tCNTs. Below, two representative voltage traces for the DG (black trace) and EC (red trace) are shown in standard saline solution. C. The cumulative distribution function of IEIs up to 5 seconds is shown for DG (top) and EC (bottom). When interfaced to tCNTs, the activity of both DG and EC is accelerated, as appreciable from the IEIs cumulative distributions (green and blue lines), characterized by a significantly

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

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

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

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