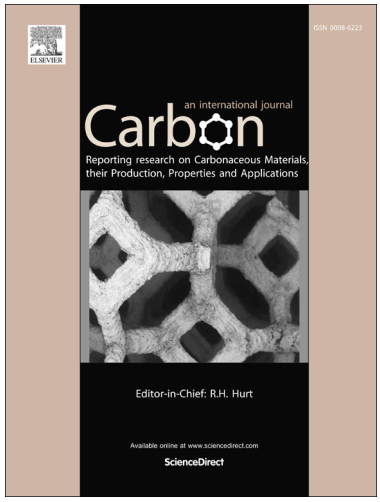

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Carbon Based Substrates for Interfacing Neurons: Comparing Pristine with Functionalized Carbon Nanotubes Effects on Cultured Neuronal Networks

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ABSTRACT

Pristine (as prepared) carbon nanotube (CNT) based substrates have been widely used to grow and interface neurons in culture. Nerve cells normally differentiate on CNTs and the synaptic networks, newly formed at the interface with this material, usually show an improved robustness in signal transfer. However manipulation of pristine CNTs is often prevented by their low dispersibility and tendency to aggregate in most solvents. This issue can be at least partially solved by adding solubilizing groups to the surface of CNT, which also helps improving their biocompatibility. It becomes therefore of crucial importance to determine whether chemically manipulated CNTs may maintain their performance in improving nerve signaling. Here we study and compare the impact *in vitro* on neuronal signaling of two classes of chemically modified multiwalled CNTs in reference to pristine CNTs, which are known to be a substrate able to boost neuronal growth and communication. We found that the extent of functionalization and the nature of the functional groups on MWNT sidewalls affect the conductivity and the biological effects of the final derivatives. This information is important for the future design of biointegrated devices.

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1. INTRODUCTION

Among the possible biological applications of carbon nanotubes (CNTs), tissue engineering seems to be one of the more promising [1]. In particular, due to their peculiar features of flexibility, electrical conductivity and mechanical strength, CNTs appear to be really ideal for the interaction with electrically active tissues, like neuronal and cardiac tissues. During the last two decades many studies have demonstrated that CNT substrates are able to sustain neuronal survival and to promote neuronal process outgrowth [2-5]. We have recently shown that CNT-based substrates are indeed able to affect neuronal physiology from the electrical signaling point of view. Hippocampal neuronal networks directly grown on pure multi walled carbon nanotube (MWNT) substrates show an increased frequency of spontaneous synaptic events (postsynaptic currents) through a promoted generation of synaptic contacts and potentiation of synaptic and non-synaptic responses [6,7,8]. In brief, it has been demonstrated that CNT are able to favor synaptogenesis and thus the global neuro-connectivity of a neural circuit and that the synapses that are newly-formed in the presence of CNT display different short-term plasticity features, namely the capacity of neural transmission to transiently change in strength when synapses are repeatedly activated [7]. Moreover, the impact of CNT on central nervous system 3D tissue has been tested by co-culturing embryonic spinal cord and dorsal root ganglia explants chronically interfaced to a film of purified MWNT [9]. CNT scaffolds are able to improve the outgrowth of neurites (enriched with more growth cones) expanding radially from the explants, thus positively influencing axonal re-growth [9]. When analyzing network activities by patch clamp recordings of single neurons, we observed a shift towards potentiation in spinal network communication dynamics in spinal explants interfaced to carbon nanotubes.

The manipulation of properly functionalized MWNTs is much easier in comparison to unmodified, pristine MWNT (pMWNT), because after functionalization the MWNTs become more easily dispersible in organic and aqueous solvents. Moreover it was demonstrated that the toxicity of these

carbon nanomaterials can be dramatically alleviated increasing their water solubility obtained through debundling of long CNT fibers [10]. The functionalization versatility might also improve neuronal affinity if we imagine to use nerve growth factors in the structure modification process. In literature, functionalized CNTs interfaced with neuronal cells are often intended to be variously oxidized CNTs that are functionalized with other groups after the oxidation [3, 13, 11]. This approach involves a quite invasive process that can alter the pristine features of CNTs and could not be predictive of the real effects that their peculiar structure can produce when interfaced to neuronal networks.

Few studies of the effects of covalent modification of CNT sidewalls on neuronal cultures have been reported so far, except for papers that describe the effect of variously oxidated derivatives. Liu et al. analyzed how chemical surface functionalization (both covalent and non-covalent) of MWNT arrays affects neuronal adhesion and network organization. They found that the affinity and the morphology of neurons changes dramatically from one substrate to the other. The authors remark that the hydrophilicity and the chemical nature of the substrate are able to tune the modalities of growth of the cultures (formation of clusters vs. regular networks, poor vs good neurites extension that implies poor vs. good intercellular communication). As a conclusion, it turns out that the control and choice of the functionalization strategy is crucial for the impact on cell growth and development [10]. To further explore the impact of functionalized CNT scaffolds on neuronal networks, and to improve the versatility of the materials used, we here report the effects of two kinds of covalently modified MWNTs on neuronal communication. In particular we have chosen two well known chemical approaches like 1,3-dipolar cycloaddition of azomethine ylides [15,16] and the radical diazonium salt arylation [17].

2. EXPERIMENTAL PART

2.1 MATERIALS

MWNT were purchased at Nanoamor Inc. (stock # 1237YJS, 95%, OD 20/30 nm, length 0.5-2 μm). All the chemicals and solvents were purchased at Sigma Aldrich and used without any further purification.

2.2 SYNTHESIS

MWNT1: 100 mg of pristine MWNT (MWNTp) are dispersed in 100 mL of ortho-dichlorobenzene (ODCB) and sonicated in a sonic bath for 15 minutes. 1.4 g (4 mmol) of N-phtalimido-amino-diethoxyethyl acetic acid [11] and 750 mg (160 mmol) of paraformaldehyde are added portionwise in 4 portions every 2 hours while the mixture is refluxing at 180°C. The reaction is then refluxed at 180°C for other 16 hours (22 hours in total). The crude is filtered on Millipore JHWP filters (0.45 μm) and washed several time with dimethylformamide, methanol and finally diethyl ether. The powder is then dried in vacuo and weighs 108 mg.

100 mg of the black powder are sonicated for 20 minutes in 100 ml of ethanol and then 625μl (0.012 mmol) of hydrazine monohydrate are added. The mixture is stirred at room temperature for 16 hours. The crude is then filtered on Millipore JHWP filters (0.45 μm) and washed several times with methanol and diethyl ether. The dried powder weights 105 mg.

HL (high loading)- (and LL (low loading)-) MWNT2: 100 mg of pMWNT are dispersed in 100 mL of water and sonicated for 20 minutes. 2 g (0.25 g for LL) of 4- [(N-Boc)aminomethyl]aniline and 2ml (0.25 ml for LL) of isopentyl nitrite are added. The mixture is stirred at 80°C for 8 hours. The crude is filtered on Millipore JHWP filters (0.45 μm) and washed several times with water, dimethylformamide, methanol and finally diethyl ether. The powder is then dried in vacuo and weights 114 (112 for LL) mg.

100 mg of the black powder are dissolved in 100 ml of chloridric acid 4M in dioxane and stirred at room temperature for 16 hours. The crude is then filtered on Millipore JHWP filters (0.45 μm) and washed several time with methanol and diethyl ether. The dried powder weights 104 (102) mg.

2.3 CHARACTERIZATION METHODS

UV–vis–NIR spectra were recorded on a Cary 5000 Spectrophotometer (Varian), using 1cm path quartz or optical glass cuvettes. Thermogravimetric analyses were performed using a TGA Q500 (TA Instruments). Conductivity measurements were performed using a Jandel resistivity meter (RM-3000) and a Jandel four point probe. Electrical conductivities on MWNT powder were measured after filtration of 1 mg of the material on a PTFE filter (Millipore 0.45µm pores, 90 mm diameter). The average membrane thickness was measured with a micrometer(High-Accuracy Digimatic Micrometer 293-100, Mitutoyo). SEM imaging was performed on a SUPRA 40 (Carl Zeiss AG).

2.4 CELL CULTURES

Primary dissociated cultures from newborn rat hippocampi were obtained as previously described [6]. Neurons were seeded on control (see below) substrates, on MWNT1, HL-MWNT2and on LL-MWNT2. Neuronal and glial cells density, quantified by immunofluorescence measures (see Supporting Information and Figure 1a) was similar in all substrates (for control, MWNT1 and HL-MWNT2, respectively: 177 ± 17, 169 ± 21 and 150 ± 20 neurons/mm^2, n=12, 11 and 8 visual fields; 166 ± 17, 147 ± 12 and 140 ± 11 glial cells/mm^2, n=12, 14 and 9 visual fields; n=2 culture series). Electrophysiological recordings were performed by the patch-clamp technique [6] (see Supporting Information for details).

Data are expressed as mean ± S.E.M. .n is the number of neurons, if not otherwise indicated. Student's t test was used for parametric data, and Mann-Whitney and Fisher tests for non-parametric data. A P value <0.05 has been taken as indicative of statistically significant difference.

3. RESULTS AND DISCUSSION

We used two well known reactions to functionalize MWNTs: a) the 1,3-dipolar cycloaddition of azomethine ylides that, starting from an aldehyde and an α-aminoacid, generates a pyrrolidine ring on the MWNT surface (Figure 1a) [15, 16], and b) the radical diazonium salt arylation of MWNTs using a substituted aniline and isoamyl nitrite as shown on Figure1b [17]. The latter reaction is quite invasive, in terms of functionalization degree, giving rise to MWNTs with a high loading of functionalizing groups (HL-MWNT). In order to check the effect of the functionalization degree on neuronal behavior, we used both the already known protocol [17] and controlled concentrations of the starting materials in order to have a lower functionalization degree (low loading, LL-MWNT). In order to have the half quantity of functionalization (table 1), we have found that it is necessary to use 8 times less concentrated reagents (see Synthesis paragraph). Chemical reactions of MWNTs are not easy to control and the protocol reported here is the result of an optimization process aimed at producing half functionalization degree (for a commentary on control of functionalization reactions, please, see ref. 18).

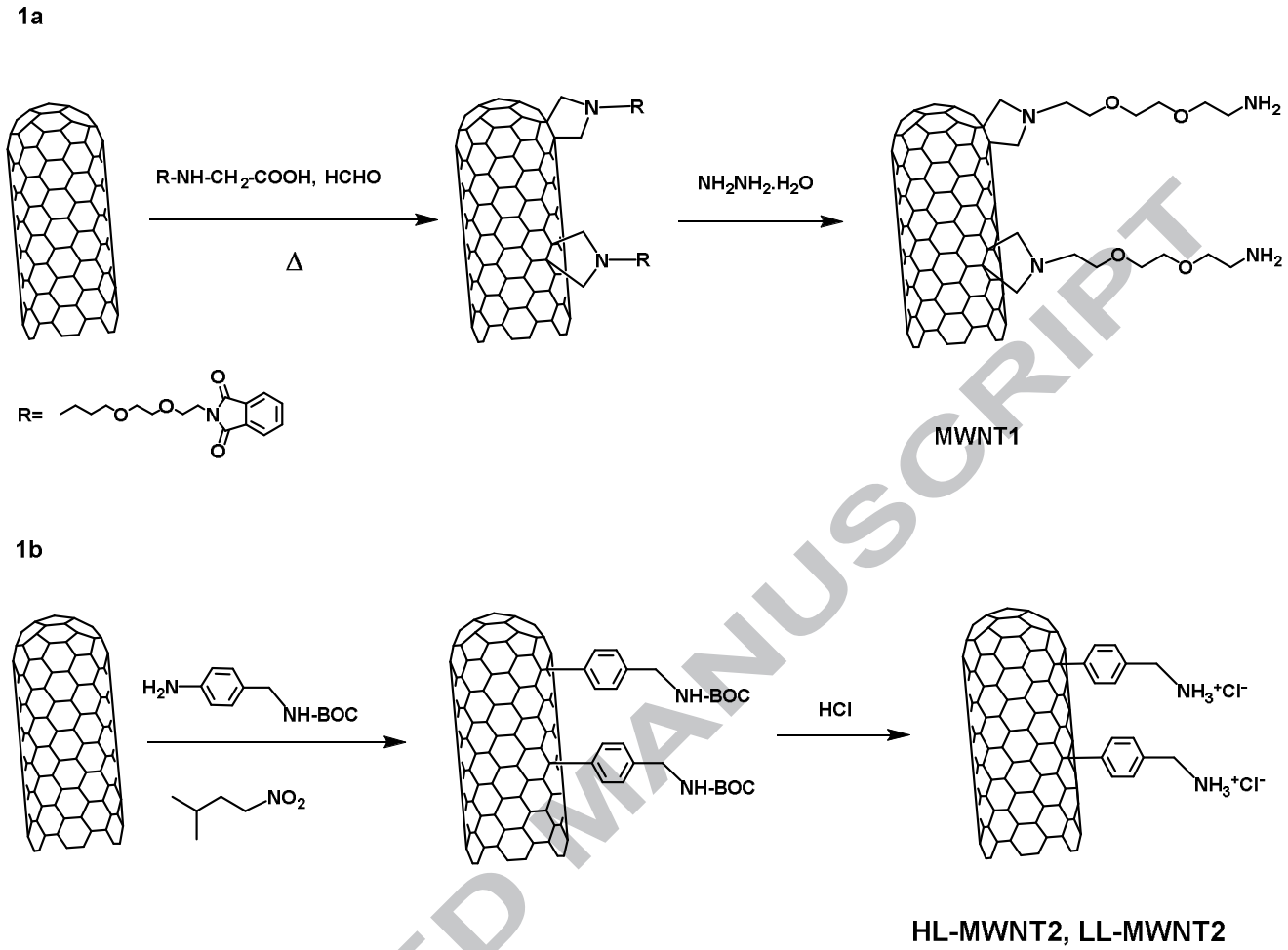

Fig. 1: a) 1,3-dipolar cycloaddition reaction route to provide MWNT1; b) diazonium salt arylation reaction route to provide MWNT2 (HL and LL)

All the products were characterized by TGA and the Kaiser test for primary amino groups.

Type of CNT	TGA (µmol/g)	Kaiser test (µmol/g)	Resistivity Ω * cm
pMWNT	-	-	0.16
MWNT1	170	140	0.32
HL-MWNT2	230	220	293
LL-MWNT2	120	110	171

Table 1. TGA (μmol of functionalizing groups per gram of material), Kaiser test and conductivity data for the MWNTs prepared in this work.

After the purification, the derivatives were dispersed at a concentration of 0.1 mg/ml in ethyl acetate and were deposited on glass coverslips by spray coating (see Supporting Information). The coated materials on the coverslips were observed by SEM (see Fig. 1 in Supplementary Data) and their sheet resistance has been evaluated by means of a 4-point probe conductimeter and have been compared to that of pMWNT. The resulting values are reported in Table 1.

The data reported in Table 1 indicate that the functionalization degree of the cycloaddition reaction (MWNT1) is lower than that of the arylation reaction (HL-MWNT2) [19]. However, the level of functionalization in LL-MWNT2 is even lower that MWNT1. In addition, it is interesting to note that the sheet resistance for MWNT1 is much lower than both HL- and LL-MWNT2. Therefore, this might indicate that not only the loading amount but also the chemical nature of the side chain may affect the electronic properties of the external shell of MWNT, that is known to possess metallic features [20].

In order to evaluate the effects of both the chemical functionalizations and the side chains on the synaptic activity of neuronal cells, dissociated hippocampal cultures [6, 7] were seeded on control glass, i.e. glass coverslips previously treated with (3-aminopropyl)trimethoxysilane to create amino group on their surface [21], and on functionalized CNTs.

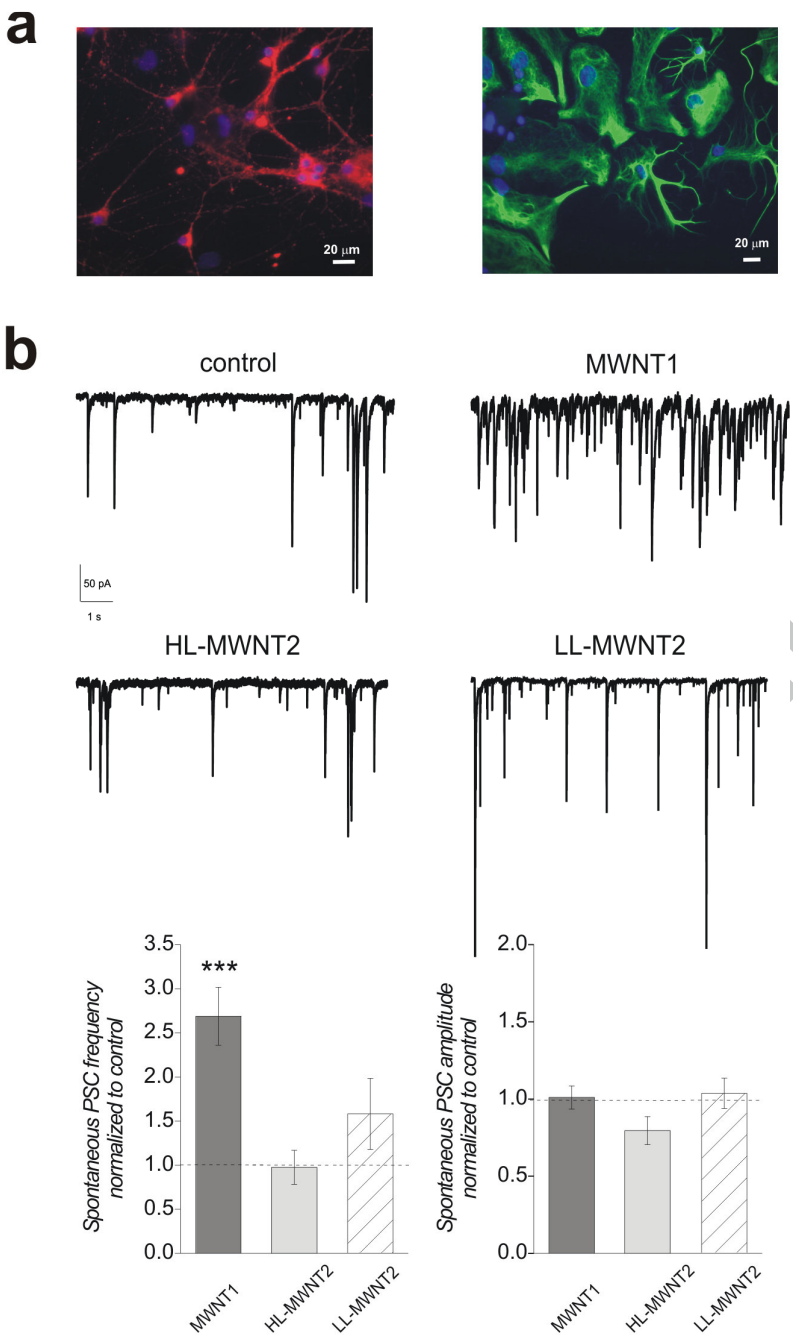
a
20 µm
20 µm
b
control
MWNT1
50 pA
1 s
HL-MWNT2
LL-MWNT2
Spontaneous PSC frequency normalized to control
***
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MWNT1
HL-MWNT2
LL-MWNT2
Spontaneous PSC amplitude normalized to control
2.0
1.5
1.0
0.5
0.0
MWNT1
HL-MWNT2
LL-MWNT2
c
presynaptic
postsynaptic
50 mV/pA
10 ms
Synaptic coupling / %
**
**
60
50
40
30
20
10
0
control
MWNT1
HL-MWNT2

Figure 2.

MWNT1 potentiate neuronal networks at the interface. a, immunofluorescence micrographs showing the typical appearance of hippocampal neurons (left, stained in red) and glial cells (right, stained in green) in culture on MWNT cultures. Cellular nuclei are marked in blue; b, top, representative traces of spontaneous postsynaptic currents (PSCs) recorded from neurons in control, MWNT1, HL-MWNT2 and LL-MWNT2. Bottom, PSC frequency is strongly increased in MWNT1, but unaltered in the other substrates (left), while PSC amplitude is similar for all substrates (right). ***: P<0.001; **c**, sketch for pair recordings. An action potential (top right) is evoked in the presynaptic neuron (top left). The presence of a unitary synaptic current (bottom right) in the postsynaptic neuron (bottom left) in response to the action potential indicates the presence of a unique coupling between the two neurons. The histogram shows the percentage of synaptically coupled pairs over the total number of pairs tested, which is increased by MWNT1. ***: P<0.001.

All the substrates used were biocompatible, in that they supported *in vitro* growth and development of both neurons and glial cells (Figure 2a). After 8 days *in vitro* (DIV) cell densities on functionalized MWNT were similar to controls (see “Experimental part”). Supporting the healthy cell condition, the neurons passive membrane properties displayed, for all the tested MWNT functionalizations, values in agreement with previous control assessments (see Supporting Information). Our main aim here was to verify whether MWNTs undergoing different chemical functionalization protocols, known to improve their biocompatibility, still retain the ability to interact at the interface with neurons and neuronal networks leading to increased neuronal synaptic connectivity [7,8]. To address this issue, in a first set of experiments we tested the main electrophysiological parameter known to be affected by pristine MWNTs i.e. the frequency of the spontaneous post synaptic currents (PSC) generated by the neuronal network activity [6, 7, 9, 22].

Figure 2b shows representative recordings of spontaneous synaptic activity (mixed PSCs are appearing as inward current deflections) from voltage clamped neurons in control, MWNT1, HL-MWNT2 and LL-MWNT2 substrates. We quantified synaptic activity as average frequency and amplitude of the detected PSCs [6], and normalized them to the value obtained on control substrate (frequency 2.04 ± 0.25 Hz; amplitude 52 ± 3 pA; n=103) for all the functionalized MWNT substrates tested. When compared to control substrate, MWNT1 maintained the ability to strongly increase PSC frequency (which was 269 ± 33 % of control; n=47; P<0.001) without altering PSC amplitude (101 ±8 %, n=47). Conversely, HL-MWNT2neurons were similar to controls in both PSC frequency (98 ± 19%; n=21) and amplitude (80± 9 %; n=21; Figure 2b). Notably, LL-MWNT2 showed a trend for an increase in PSC frequency (158 ± 40 % of control; n=19; P=0.08), while PSC amplitude was totally unaltered (103 ± 10 %; n=19). In a second set of experiments we investigated in more detail MWNT functionalization at the nanotube side (i.e. MWNT1 and HL-MWNT2) exploring two other electrophysiological parameters known to be modulated by pristine MWNTs: i) the probability of detecting monosynaptically coupled neurons, and ii) their short term synaptic plasticity [7]. We quantified synaptic coupling, an indirect measure of functional contacts formation [7], by simultaneous patch clamp of neurons' pairs. This allows investigating the presence of monosynaptic PSC in one neuron (postsynaptic neuron; sketch in Figure 2c, bottom) evoked by action potentials elicited in the other cell (presynaptic neuron; Figure 2c, top). The probability (expressed as %) of finding coupled pairs in MWNT1 (54%; 14 out of 26 pairs) was significantly higher than that detected in control pairs (15%, 5 out of 34 pairs; P<0.01) or in HL-MWNT2 (21%, 7 out of 34 pairs; Figure 2c, right). In the same set of experiments we tested the dynamic of neurotransmission when synapses are repeatedly activated on MWNT1 and HL-MWNT2 substrates. In each pair a train of 6 action potentials (20 Hz) was elicited in the presynaptic neuron and the amplitude of the evoked PSCs in the postsynaptic neuron evaluated (Figure 3). The depression of the PSC response was then quantified for each neuron pair as the ratio between the amplitudes of the sixth and first PSCs. Compared to controls (strongly depressing: $6^{th}/1^{st}$ PSC ratio

0.28 ± 0.07, n=7) neurons interfaced to MWNT1 showed lower depression ($6^{th}/1^{st}$ PSC ratio 0.46 ± 0.07, n=16), while on HL-MWNT2 substrates depression was similar to control ($6^{th}/1^{st}$ PSC ratio 0.18 ± 0.10, n=5; Figure 3).

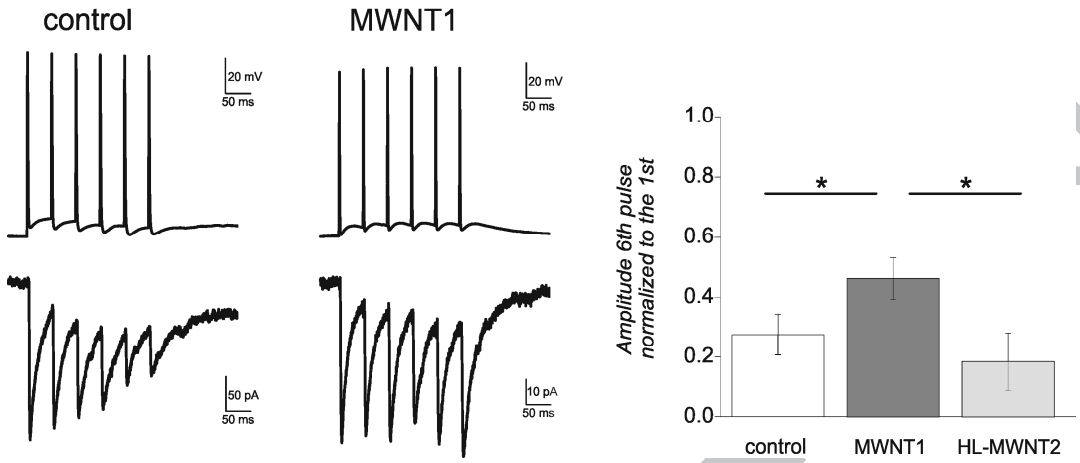

Figure 3.

MWNT1 modulate neuronal synaptic plasticity. Left, dual recordings in which a train of six action potentials at 20 Hz is evoked in the presynaptic neuron (top traces) and the response PSCs are recorded during the train in the postsynaptic neuron (bottom traces), from neurons grown on control substrate or on MWNT1. The repetitive synaptic activation induces a progressive decrease in PSCs amplitude during the train (depression), which is strong in controls in contrast to neurons grown on MWNT1. Right, plot quantifying depression as the ratio between the amplitudes of the sixth and first PSCs: compared to control, values were higher for neurons on MWNT1, while neurons on HL-MWNT2 are similar to controls. *: $P<0.05$.

From the biological data, it can be concluded that MWNT1 functionalization preserves MWNT physical-chemical properties involved in transforming neuronal networks at the interface, as described in our previous work [6, 7]. The electrophysiological results indicate that the type of

functionalization on MWNT is a key determinant of the effect on neuronal network activity, independently from its loading.

4. CONCLUSIONS

This study achieves a step forward in the study of CNTs as very promising materials for neural functional interfaces. The possibility of functionalization offers wide perspectives in the manipulation of these materials for the assembly of nanostructured devices able to support and stimulate neuronal activity, but the chemical nature of these materials has to be carefully tuned to optimize their stimulating effects.

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