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**Altered excitability of cultured chromaffin cells following exposure to multi-walled carbon nanotubes.**

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(Article begins on next page)



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13 **Altered excitability of cultured chromaffin cells following exposure to**  
14 **multi-walled carbon nanotubes**  
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**Abstract**

We studied the effects of multi-walled carbon nanotubes (MWCNTs) on the electrophysiological properties of cultured mouse chromaffin cells, a model of spontaneously firing cells (Marcantoni *al.* 2010). The exposure of chromaffin cells to MWCNTs at increasing concentrations (30 to 263  $\mu\text{g/ml}$ ) for 24 h reduced, in a dose-dependent way, both the cell membrane input resistance and the number of spontaneously active cells (from 80% to 52%). Active cells that survived from the toxic effects of MWCNTs exhibited more positive resting potentials, higher firing frequencies and unaltered voltage-gated  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$  and  $\text{K}^{+}$  current amplitudes. MWCNTs slowed down the inactivation kinetics of  $\text{Ca}^{2+}$ -dependent BK channels. These electrophysiological effects were accompanied by MWCNTs internalization, as confirmed by transmission electron microscopy, indicating that most of the toxic effects derive from a dose-dependent MWCNTs-cell interaction that damages the spontaneous cell activity.

**Keywords:** MWCNTs, voltage-gated  $\text{Na}^{+}$  and  $\text{K}^{+}$  channels,  $\text{Ca}^{2+}$  and BK channels, adrenal chromaffin cells, action potential firing, input resistance.

## Introduction

The interest in nanomaterials is rapidly increasing because of their potential application in biomedicine, cosmetics, electronics, sport goods, mechanics and other manufactured items (Nel et al. 2006), thus leading to a parallel rising of the social concern on their effects on human health. Carbon nanotubes (CNTs) are among the most widely studied nanomaterials because of their unique physical, chemical and mechanical properties, but they are also considered one of the most potentially harmful nanomaterials to humans (Kane and Hurt 2008; Lam et al. 2006). Carbon nanotubes (CNTs) are cylindrical structures formed by one (single-walled SWCNTs) or more (multi-walled MWCNTs) bent graphene layers of variable length, even above 5  $\mu\text{m}$ , but with diameter in the nanometer range. Depending upon the methods of production and purification, CNTs may largely differ in form, impurities (metals, amorphous carbon) and defects. CNTs have been the matter of debate as several *in vivo* and *in vitro* studies have shown that CNTs may trigger toxic effects, as reported in recent reviews (Donaldson et al. 2009; Lam et al. 2006; Lison et al. 2008; Shvedova et al. 2009). In some cases they have been reported to cause asbestos-like damage (Poland et al. 2008; Takagi et al. 2008).

The nature of their toxicity is still highly debated (Kane and Hurt 2008) and mostly focused on their potential lung toxicity, particularly on airways and mesothelium. Only few data are available on other organs and cell types, including toxic effects of CNTs on nervous tissues. Several types of nanoparticles have been reported to reach the central nervous system through sensory and olfactory nerve pathways (Elder et al. 2006; Oberdörster et al. 2001; Oberdörster et al. 2004), or translocate from the lung to the blood stream (Kreyling et al. 2002; Nemmar et al. 2001; Oberdörster et al. 2002). There is no clear evidence that CNTs may migrate in extra-pulmonary organs following intratracheal instillation (Al Faraj et al. 2009; Deng et al. 2007; Elgrabli et al. 2008). However, recently, Ryman-Rasmussen and

collaborators reported that CNTs may reach the subpleural tissue in mice, moving rapidly through the alveoli epithelium (Donaldson et al. 2009; Ryman-Rasmussen et al. 2009). So far, *in vitro* effects of single-walled (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) on neurons have not been thoroughly investigated. The few available data concern the toxic effects of SWCNTs on dorsal root ganglion (DRG) neurons (Belyanskaya et al. 2009), the blocking of neuronal  $\text{Ca}^{2+}$  channels by micromolar concentrations of yttrium released from SWCNTs (Jakubek et al. 2009), the direct blocking of various  $\text{K}^+$  channels by SWCNTs in chinese hamster ovary (CHO) cells (Park et al. 2003) and the suppression of  $\text{K}^+$  channel activity in PC12 cells, induced by carboxy-terminated MWCNTs (Xu et al. 2009).

Here we studied the dose-dependent effects of well characterized multi-walled carbon nanotubes on neuroendocrine cells. Our study focuses on the basic electrophysiological properties of adrenal chromaffin cells after MWCNTs exposure, representing a suitable model of neuronal-like excitable cells, with particular interest to their neuroendocrine function. We addressed this by means of electrophysiological trials and electron microscopy assays, to monitor MWCNTs-cell interaction, MWCNTs internalization and MWCNTs-induced alterations of ionic conductance mechanisms controlling chromaffin cell excitability. We found that increasing doses of MWCNTs reduce the number of spontaneously firing cells in a dose-dependent manner without altering the voltage-gated  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{K}^+$  channels. This is likely due to the MWCNTs-cell interactions occurring during the 24 h time exposure that lower the cell resistance and resting membrane potential and induce marked alterations to cell excitability.

## Materials and Methods

### *Preparation and physico-chemical characterization of CNTs*

Multi-walled CNTs were purchased by Mitsui Chemicals (Kawasaki-Shi, Japan). To improve

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3 the dispersibility in the culture media CNTs were ground in an oscillatory ball mill during 6  
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5 hours.  
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8 The dimension of CNTs has been evaluated directly by means of transmission  
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10 electron and scanning electron microscopies. The statistical evaluation of diameters and  
11  
12 lengths of the MWCNT fragments was obtained by considering a population of 213 data  
13  
14 **points for each sample**, from low resolution TEM images of the ground sample (4000X and  
15  
16 25000X as magnification, respectively). TEM images were collected by a JEOL 3010-UHR  
17  
18 TEM instrument operating at 300 kV.  
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20

21  
22 The surface area of CNTs has been measured by means of the B.E.T **(Brunauer, Emmett and**  
23  
24 **Teller)** method based on N<sub>2</sub> adsorption at -196°C (ASAP 2020 Micrometrics, Norcross, GA  
25  
26 30093-2901, U.S.A.). For the elemental analysis, the samples were heated at 700°C in a  
27  
28 furnace and the residue dissolved in 37% HCl. The concentration of iron in the solution was  
29  
30 quantified by atomic emission-ICP spectrometry (IRIS II Advantage/1000, Thermo Jarrel  
31  
32 Ash).  
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#### 39 *Evaluation of potentially bio-available iron*

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41 This test was performed to evaluate the amount of iron ions which may be exposed at the  
42  
43 surface of the MWCNTs. For this purpose, a strong chelator (ferrozine®) and a reducing  
44  
45 agent (ascorbic acid) were used to extract iron ions. 25 mg of the powders were incubated in  
46  
47 10 ml of a 3 mM solution of ascorbic acid and ferrozine. After 10 days an aliquot of the  
48  
49 supernatant was withdrawn after centrifugation and the concentration of iron evaluated by  
50  
51 measuring the absorbance at 562 nm (typical of the complex ferrozine-Fe(II)) with a UV/Vis  
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53 spectrophotometer (Uvikon, Kontron, spectrophotometer). The amount of extractable iron is  
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55 reported on Table 1.  
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### *Raman spectroscopy*

Micro-Raman spectra were acquired using an integrated micro/macro Raman system which includes a Horiba Jobin Yvon HR800 microspectrometer, an Olympus BX41 microscope and a CCD air-cooled detector. A polarized solid state Nd 80 mW laser operating at 532.11 nm was used as the excitation source. Calibration of the instruments was performed by measuring the Stokes and anti-Stokes bands and checking the position of the Si band at  $\pm 520.7$  cm<sup>-1</sup>. Each spectrum was acquired using a 100X objective, resulting in a laser beam size at the sample in the order of 2 mm. To optimize the signal to noise ratio, spectra were acquired using 10 scans of 10 seconds for each spectral region. In order to produce strong signals without inducing surface alteration due to the heat, a filter with optical density  $d = 0.6$  was used. The software LabSpec 5 (Horiba Jobin Yvon) was used to analyze the spectra. The ID/IG value is the ratios of the intensities of two bands designated as D (1340 cm<sup>-1</sup>) and G (1570 cm<sup>-1</sup>) which correspond respectively to structural defects and to the tangential in-plane stretching vibration of the carbon-carbon bonds within the graphene sheets.

### *Dispersion of MWCNTs in the culture media*

MWCNTs were suspended in the culture media at three different concentrations: 263  $\mu$ g/ml, 100  $\mu$ g/ml and 30  $\mu$ g/ml. The suspensions were sonicated two times for 2 minute with a probe sonicator (100 W, 20 kHz, Sonoplus, Bandelin, Berlin, Germany). Average hydrodynamic size of MWCNTs in the culture media has been evaluated by means of dynamic light scattering (DLS), (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, U.K.) for the sample at higher concentration (263  $\mu$ g/ml) . The measurement was repeated after 15 minutes to evaluate the stability of the suspension. Since in the DLS technique **aggregates** larger than 10  $\mu$ m are not detected, optical microscopy was also used to detect the presence of large aggregates.

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3 As a negative toxicity control we used a sample of tris(dicarboxymethylene)fullerene (Vinci-  
4 Biochem) dissolved in the culture medium at the concentration of 263 µg/ml.  
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8 To evaluate the effects of MWCNTs on the concentration of proteins in the cellular media,  
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10 MWCNTs were suspended in a 15% fetal bovine serum aqueous solution for 48 hours and  
11  
12 then removed by centrifugation. The total amount of proteins which remained in the  
13  
14 supernatant was measured spectrophotometrically by means of bicinchoninic acid (BCA)  
15  
16 assay and compared with the control solution (Smith et al. 1985).  
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### 19 20 21 22 *Isolation and culture of chromaffin cells*

23  
24 Mouse chromaffin cells (for electrophysiology): mouse chromaffin cells were obtained from  
25  
26 young C57BL/6J male mice, which were killed by cervical dislocation and cultured following  
27  
28 the method of Sørensen (Sørensen et al. 2003), with minimal modifications. After removal,  
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30 the adrenal glands were placed in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Locke's buffer containing (in  
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32 millimolar): 154 NaCl, 3.6 KCl, 5.6 NaHCO<sub>3</sub>, 5.6 glucose, and 10 HEPES, pH 7.3, at room  
33  
34 temperature. The glands were decapsulated, and the medullas were precisely separated from  
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36 the cortical tissue. Medulla digestion was achieved after 50 min at 37°C in the enzyme  
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38 solution (0.16 mM L-cysteine, 1 mM CaCl<sub>2</sub>, 0.5 mM EDTA, DMEM) containing 20 U/ml of  
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40 papain (Worthington Biochemical, Lakewood, NJ, USA) plus 0.1 mg/ml of DNase (Sigma).  
41  
42 The cell suspension was then washed twice with a washing solution (DMEM, 1 mM CaCl<sub>2</sub>,  
43  
44 10 mg/ml BSA), and resuspended in 2 ml DMEM supplemented with 15% fetal bovine serum  
45  
46 (FBS). Cells were plated in four-well plastic dishes treated with poly-L-ornithine (0.5 mg/ml)  
47  
48 and laminin (10 µg/ml in L-15 carbonate) by placing a drop of concentrated cell suspension  
49  
50 in the center of one well. After 1 h, 1.8 ml of DMEM supplemented with 15% FBS  
51  
52 (Invitrogen, Grand Island, NY, USA), 50 IU/ml penicillin, and 50 µg/ml streptomycin  
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54 (Lonza), 10 µM Cytosine b-D-arabino-furanoside-hydrochloride (Sigma), 10 µM 5-Fluoro-

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3 2'-deoxyuridine (Sigma) was added to the wells. Cells were then incubated at 37°C in a  
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5 water-saturated atmosphere with 5% CO<sub>2</sub> and used within 2–6 days after plating. All  
6  
7 experiments were performed in accordance with the guidelines established by the National  
8  
9 Council on Animal Care and were approved by the local Animal Care Committee of Turin  
10  
11 University.  
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14  
15 Rat chromaffin cells (for electron microscopy): the preparation was obtained as  
16  
17 explained before for mouse chromaffin cell culture, with the exception of papain, which was  
18  
19 substituted with a solution of liberase-blendizymes-3 at the concentration of 0.35 mg/ml  
20  
21 (Roche). Thus, 100 µl of lyberase solution were added for each ml of DMEM.  
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### 27 *Electrophysiology*

28  
29 Voltage-clamp and current-clamp experiments: Ca<sup>2+</sup> currents were recorded in perforated-  
30  
31 patch recording conditions using an EPC-9 patch-clamp amplifier (HEKA Elektronik,  
32  
33 Lambrecht, Germany). Patch pipettes were made of thin borosilicate glass (Kimax 51; Witz  
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35 Scientific, Holland, OH, USA) and filled with an internal solution containing (in millimolar):  
36  
37 135 CsMeSO<sub>3</sub>, 8 NaCl, 2 MgCl<sub>2</sub>, 20 HEPES, pH 7.3, with CsOH plus amphotericin B  
38  
39 (Sigma). The external bath contained (in millimolar): 128 NaCl, 10 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10  
40  
41 glucose, 10 HEPES, 4 KCl, 2 TEACl, pH 7.4, with NaOH. Amphotericin B was dissolved in  
42  
43 dimethyl sulfoxide stored at -20°C in stock aliquots of 50 mg/ml and used at a final  
44  
45 concentration of 500 µg/ml. To facilitate the sealing, the pipette was first dipped in a beaker  
46  
47 containing the internal solution and then back-filled with the same solution containing  
48  
49 amphotericin B. Pipettes with series resistance of 1–2 MΩ were used to form giga-seals.  
50  
51 Series resistance was compensated by 80% and monitored throughout the experiment. K<sup>+</sup>  
52  
53 currents were also recorded in perforated-patch conditions, using an internal solution  
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55 containing (mM): 135 KAsp, 8 NaCl, 20 Hepes, 2 MgCl<sub>2</sub> and 5 EGTA. As external solution  
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3 we used a normal physiological Tyrode's solution with the following composition (in mM):  
4 130 NaCl, 4KCl, 2CaCl<sub>2</sub>, 2MgCl<sub>2</sub>, 10 HEPES and 10 glucose adding 300 nM TTX in order  
5  
6 to block the sodium current. Also the current-clamp experiments were performed by means of  
7  
8 the perforated patch method. The intracellular solution contained in mM: 135 KAsp, 8 NaCl,  
9  
10 20 HEPES, 2 MgCl<sub>2</sub> and 5 EGTA. As an extracellular solution for the current clamp  
11  
12 experiments we used a normal physiological Tyrode's solution.  
13  
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15  
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17 Regarding the input resistance measurements we used the same solutions described above for  
18  
19 current clamp experiments.  
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### 22 23 24 *Electron microscopy*

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26 Chromaffin cells were cultured on Aclar Fluoropolymer film (Electron Microscopy Sciences,  
27  
28 USA) following the protocol explained before. After 24/48 h of treatment, cells were fixed  
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30 for 1 hour with 2% glutaraldehyde in PBS at room temperature, washed with cacodylate  
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32 buffer, post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 hour in ice and  
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34 dehydrated in gradient ethanol, followed by propylene oxide. Samples were then embedded  
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36 in Epon-Araldite. Ultrathin sections (80-100 nm) were cut with a diamond knife on an  
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38 ultramicrotome (Leica Microsystems, Germany) and collected on Pioloform-coated single  
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40 slot grids (Electron Microscopy Sciences, USA). Sections were stained with uranyl acetate  
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42 and lead citrate and examined in a JEM-1010 electron microscope (Jeol, Japan) equipped  
43  
44 with a side-mounted CCD camera (Mega View III; Soft Imaging System, Germany). To  
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46 determine whether the nanotubes could be present inside the cells, approximately 50  
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48 chromaffin cells treated with MWCNTs for 24 and 48 h were randomly examined. The  
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50 percentages of internalization were calculated considering the number of cells that  
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52 internalized MWCNTs as 100% for each group (24 h and 48 h).  
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## Results

### *Physico-chemical properties of MWCNTs*

One of the major flaws of the available data on MWCNTs toxicity is the poor physico-chemical characterization of the samples, which makes difficult any comparison, among different studies. Moreover the lack of correlation between physico-chemical properties and induced toxic effects deprives material chemists of the possible clues for the design of safer products (Fubini et al., 2010). In this study, a sample of commercial MWCNTs (Mitsui Chemicals, Kawasaki-Shi, Japan) having 0.27 % iron impurities has been ground in a ball mill in order to shorten the length of tubes and improve the dispersibility in physiological media. It is noteworthy that by moving from the commercial material to the ground one a rupture in the carbon nanotubes structure takes place with consequent increase in the specific surface area, likely following the opening of the internal pores and the formation of irregular terminations (Table 1).

Ground MWCNTs consist of bundles of short straight tubes having a mean diameter of  $67 \pm 2$  nm and a mean length of  $1.12 \pm 0.5$   $\mu\text{m}$  (Fig. 1, Table 1). From this image a few rounded agglomerates of carbon particles, together with more dark regions at the borders of the nanotubes are observed. These thickness differences can be explained with interlayer spacing defects, i.e. stacking faults, plausibly arising from the ground process. The opening of some isolated fragments is also imaged, as evidenced from the enlarged view shown in the inset of figure 1. The grinding process also introduces reactive defects following the cleavage of C-C bonds. Raman spectroscopy provides a useful method to broadly evaluate the extent of defects present in CNT (Jorio et al. 2004; Osswald et al. 2007; Fenoglio et al. 2008). In the Raman spectra of the pristine and ground MWCNTs (Fig. 2) two main bands are visible: the band designated as D ( $1340\text{ cm}^{-1}$ ) is associated to structural defects while the bands G ( $1570\text{ cm}^{-1}$ ) is due to the tangential in-plane stretching vibration of the carbon-carbon bonds

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2  
3 within the graphene sheets. The ratios of the relative intensities of the D and G bands ( $I_D/I_G$ )  
4 may be confidently used to estimate variations in the concentration of defects. The pristine  
5 samples exhibited a very low  $I_D/I_G$  value (Table 1) which corresponds to a high degree of  
6 crystallinity. As expected, the  $I_D/I_G$  value increased upon grinding, confirming the formation  
7 of defects under mechanical stress. In a previous study such defects have been shown to  
8 affect the inflammogenic and genotoxic potential of MWCNTs (Fenoglio et al. 2008; Muller  
9 et al. 2008) but not their cancerogenicity (Muller et al. 2009). The sample contains trace  
10 levels of iron as impurity which is almost totally exposed to the solvent, thus bioavailable  
11 (Table 1) (Guo et al. 2007; Liu et al. 2008). The presence of rare iron particles, whose  
12 diameters range in the  $0.2 \div 2 \mu\text{m}$  interval, is also confirmed from TEM and SEM images,  
13 not reported here for sake of brevity.

14  
15 Being highly hydrophobic, CNTs are not well dispersed in aqueous media, therefore  
16 different protocols for the preparation of stable suspension have been proposed (Elgrabli et al.  
17 2007; Yu et al. 2007). Here, we dispersed CNTs directly in the cellular media ( $263 \mu\text{g/ml}$ )  
18 supplemented with 15% fetal bovine serum (FBS) as detailed explained in Materials and  
19 Methods. The presence of proteins is known to improve the dispersion of MWCNTs (Elgrabli  
20 et al. 2007) most probably acting as surfactants. The degree of dispersion and the stability of  
21 the suspensions were evaluated by means of dynamic light scattering technique (DLS) (Fig.  
22 3a). The single peak corresponds to particles having an equivalent hydrodynamic diameter of  
23 300 nm that could be due to both, single CNTs or small agglomerates. Since this technique  
24 has an upper limit resolution of  $1 \mu\text{m}$ , the presence of larger aggregates was confirmed by  
25 optical microscopy (Fig. 3b). CNTs appear uniformly distributed in the suspension with some  
26 aggregates having diameters of  $1\text{-}5 \mu\text{m}$ . The stability of the suspension was followed for 15  
27 min, which is sufficient for a correct dosage.

28  
29 Note that the addition of MWCNTs did not significantly modify the concentration of

total proteins in the media (see Materials and Methods).

### *MWCNTs are internalized in chromaffin cells*

A first set of electron microscopy trials has been performed with the aim of assessing whether nanotubes could be internalized in chromaffin cells, as shown in human epidermal keratinocytes (Monteiro-Riviere et al. 2005) and other works focused on the cellular uptake of CNTs (Raffa et al. 2010). In order to simplify the experimental procedure, for these experiments we used rat chromaffin cells (RCCs) instead of mouse (Fig. 4a-d). RCCs were exposed to 263  $\mu\text{g/ml}$  MWCNTs dispersed in the culture medium as previously described, for 24 and 48 h and then cut in ultrathin sections of 80-100 nm thickness. In both groups of cells (24 and 48 h treatment) MWCNTs crossed the plasma membrane and were clearly visible inside the cells (Fig. 4b-d). The nanomaterial was present in 78% of cells after 24 h and 80% after 48 h exposure at 263  $\mu\text{g/ml}$  concentration (Fig. 4e). In particular, single nanotubes (white square in panel b and arrows in panels d) and aggregates (dashed white lines in panels b and c) were already visible inside the cells after 24 h, while after 48 h there was a predominance of aggregates. Among those cells exhibiting internalization after 48 h exposure to MWCNTs, 78% contained nanotubes as agglomerates and just 22% contained single nanotubes (Fig. 4f), suggesting that percentage of aggregation increased with time. These findings demonstrate that MWCNTs are able to cross the plasma membrane and reach both the cytoplasm (Fig. 4c and d) and the nuclear membrane (Fig. 4). Especially after prolonged exposure (48 h) they can disrupt the internal organization of the cell (Fig. 4c). In this case, organelles are no longer visible and the cytoplasm appears rich of MWCNTs aggregates (dashed white square). These results are in agreement with previous reports (Kostarelos et al. 2007), suggesting that CNTs probably penetrate the cells due to their long and thin shape, crossing the plasma membrane as a “nanosyringe”. Interestingly, we found that the

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3 internalization process is not limited to the plasma membrane but proceeds up to the nucleus  
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5 (N) (Fig. 4b), with possible adverse effects at the genomic level.  
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#### 10 *MWCNTs reduce the number of spontaneously active chromaffin cells*

11 Isolated chromaffin cells from the adrenal gland are spontaneously firing (Marcantoni et al.  
12 2010). The goal of our study was to understand whether the exposure to MWCNTs for 24  
13 hours could interfere with the generation of action potentials (Marcantoni et al. 2009). By  
14 means of current-clamp perforated-patch recordings, we compared the spontaneous activity  
15 of MCCs in control conditions and after exposure to increasing concentrations of MWCNTs  
16 (from 30  $\mu\text{g/ml}$  to 263  $\mu\text{g/ml}$ ). Control MCCs exhibited spontaneous firing activity in 80% of  
17 cases (n=33). On the contrary, exposure to MWCNTs for 24 h reduced the number of  
18 spontaneously firing MCCs in a dose-dependent manner. The lower dose of MWCNTs (30  
19  $\mu\text{g/ml}$ ) reduced the number of firing cells to 67% (n=21) while higher concentrations (100  
20  $\mu\text{g/ml}$  and 263  $\mu\text{g/ml}$ ), reduced the number of firing cells to 48% (n=25) and 52% (n=29),  
21 respectively (Fig. 5a). This clearly indicates that MWCNTs affect MCCs functioning. To  
22 clarify if the observed effect was specific for MWCNTs, cells were exposed to 263  $\mu\text{g/ml}$  of  
23 a carboxymethylene derivative of fullerene, which shares with CNTs the same chemical  
24 nature but not their fibrous shape. This material has been previously reported to be non-toxic  
25 toward neuronal cells (Dugan et al. 1997; Lin et al. 1999). In this case the percentage of  
26 active chromaffin cells (80%, n=14) was not significantly different from controls.  
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#### 51 *MWCNTs reduce the membrane input resistance of chromaffin cells*

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53 Since the loss of function of chromaffin cell activity could be due to a MWCNTs-cell  
54 interaction damaging the cell membrane integrity, we next monitored the membrane input  
55 resistance. We quantified this parameter by applying a small hyperpolarizing current pulse (-  
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3 10 pA) of 100 ms duration from resting potential and then measuring the induced voltage  
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5 change ( $\Delta V$ ; Fig. 5b). On average, the cell input resistance of MCCs after exposure to  
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7 MWCNTs was smaller if compared to controls (grey vs. black trace in Fig. 5b): mean input  
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9 resistance was  $2.1 \pm 0.3 \text{ G}\Omega$  (either with 100 or 263  $\mu\text{g/ml}$  MWCNTs) versus  $3.3 \pm 0.1 \text{ G}\Omega$  in  
10  
11 control ( $p < 0.01$ ). On the contrary, neither the treatment for 24 h with fullerenes ( $2.9 \pm 0.4$   
12  
13  $\text{G}\Omega$ ;  $n=4$ ) nor with MWCNTs 30  $\mu\text{g/ml}$  ( $2.9 \pm 0.2 \text{ G}\Omega$ ;  $n=7$ ), induced a significant reduction  
14  
15 of membrane resistance as compared to controls ( $p > 0.05$ ). The cell input resistance  
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17 decreased in a dose-dependent manner with an  $\text{IC}_{50}$  of  $37.0 \pm 0.5 \mu\text{g/ml}$  and a Hill slope of  
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19  $3.5 \pm 0.2$  (fig. 5c).

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21 We also found that the percentage of firing cells was linearly correlated with the cell input  
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23 resistance values (Fig. 5d,  $R = 0.99$ ), suggesting that exposure to increasing MWCNTs  
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25 concentrations reduces the membrane integrity (increases background leakage currents) and  
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27 affects cell excitability.

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37 *When spontaneous firing is not abolished by MWCNTs, the firing frequency increases*

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39 Next we monitored the firing frequency of those cells whose spontaneous firing activity was  
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41 not suppressed by MWCNTs treatment. Figure 6a shows the spontaneous activity of of  
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43 representative control cell, after treatment with fullerenes (263  $\mu\text{g/ml}$ ) and with MWCNTs at  
44  
45 the maximal concentration (263  $\mu\text{g/ml}$ ).

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47 In the three groups of MWCNTs-treated cells (using 30, 100 and 263  $\mu\text{g/ml}$ ), the spiking  
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49 frequency showed a tendency to increase with increasing MWCNTs concentration. The  
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51 highest discharge rate ( $1.00 \pm 0.18 \text{ Hz}$ ) was observed when applying the maximal MWCNTs  
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53 concentration (263  $\mu\text{g/ml}$ ,  $n = 7$ ,  $p < 0.05$ ) while at lower concentrations there was no  
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55 significant difference compared to controls. Firing frequency decreased to  $0.8 \pm 0.2 \text{ Hz}$  ( $n = 7$ )  
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57 or  $0.60 \pm 0.18 \text{ Hz}$  ( $n = 9$ ) for 100  $\mu\text{g/ml}$  and 30  $\mu\text{g/ml}$  MWCNT, respectively. Controls and  
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3 cells treated with fullerenes fired at  $0.63 \pm 0.07$  Hz (n= 26) and  $0.69 \pm 0.12$  Hz (n= 8),  
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5 respectively (Fig. 6a). Increasing doses of MWCNTs caused also a reduction of the action  
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7 potential (AP) amplitude that reached minimal values at 100  $\mu\text{g/ml}$  MWCNTs ( $70 \pm 2.2$  mV;  
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9 n=6,  $p < 0.05$ ), whereas was indistinguishable from control values ( $76.9 \pm 1.2$  mV; n= 25) with  
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11 fullerenes (Fig. 6b).  
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15 We next examined how the interspike (resting) potential changed after exposure to different  
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17 MWCNTs concentrations. The results revealed more depolarized values in the presence of  
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19 carbon nanotubes in the medium. In control MCCs and with fullerenes, interspike potential  
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21 values were statistically indistinguishable ( $-47.2 \pm 0.7$  mV; n= 26 and  $-46.9 \pm 0.8$  mV; n= 9).  
22  
23 On the contrary, MCCs incubation with MWCNTs induced a shift of the resting potential to  
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25 more positive values, proportional to the rise of MWCNTs concentration. Cells treated with  
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27 30  $\mu\text{g/ml}$  had membrane resting potentials comparable to controls ( $-47.4 \pm 1.5$  mV; n= 9),  
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29 while MCCs exposed to 100  $\mu\text{g/ml}$  had significantly lower values:  $-42.5 \pm 0.6$  mV (n= 7;  $p <$   
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31 0.05). This trend persisted with 263  $\mu\text{g/ml}$  CNTs. Fig. 6c shows the linear relationship  
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33 existing between firing frequency (Hz) and membrane resting potential (interspike value)  
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35 (mV), in control and at different MWCNTs concentrations (R= 0.74), while fig. 6d shows  
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37 that firing frequency increases with increasing MWCNT concentrations, to reach nearly twice  
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39 the control value at 263  $\mu\text{g/ml}$  MWCNTs ( $0.63 \pm 0.07$  vs.  $1.00 \pm 0.18$  Hz;  $p < 0.05$ ). In  
40  
41 conclusion, MWCNTs act on MCCs by lowering the resting membrane potential, the action  
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43 potential amplitude and increasing the rate of firing.  
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53 *MWCNTs do not alter  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and voltage-gated  $\text{K}^+$  currents and slow-down BK currents*  
54  
55 *inactivation*  
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58 The changes of firing frequency, AP amplitude and resting potential could be due to a  
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60 selective action of MWCNTs on one or more of the ion channel conductances controlling the

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3 resting potential and spontaneous activity of MCCs. For this reason, we first tested whether  
4 the effects of 24 h exposure to MWCNTs were on voltage-gated  $\text{Na}^+$  and  $\text{Ca}^{2+}$  currents by  
5 using the *voltage-clamp* technique (Fig. 7a). Steady-state  $\text{Ca}^{2+}$  current amplitudes (Fig. 7b)  
6 and quantity of  $\text{Ca}^{2+}$  charges entering the cell during 100 ms depolarizing pulses (Fig. 7c)  
7 were measured from  $-50$  to  $+40$  mV and appeared unaffected by MWCNTs-treatment. A lack  
8 of effects was also observed on the peak  $\text{Na}^+$  currents, which was of  $-0.9 \pm 0.2$  nA in control  
9 conditions and  $-1.2 \pm 0.2$  nA at 0 mV with MWCNTs (Fig. 7d). In good agreement with the  
10 observation that during spontaneous firing the AP overshoot was preserved after MWCNTs  
11 exposure. Taken together these findings suggest that MWCNTs had no evident effects on the  
12 size of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  currents which are responsible for pacemaking the firing and shaping the  
13 fast AP depolarization in MCCs (Marcantoni et al. 2009; Marcantoni et al. 2010).  
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Next we measured the effects of MWCNTs on  $\text{K}^+$  channel conductances. MCCs express at least two types of  $\text{K}^+$  channels which contribute to shape and affect the AP frequency: the voltage-gated ( $\text{K}_v$ ) and the  $\text{Ca}^{2+}$ -activated Big- $\text{K}^+$  (BK) channels (Marcantoni et al. 2010). Ion currents flowing through these open channels are mostly evident using an external solution containing TTX (300 nM) to block inward  $\text{Na}^+$  currents and double-pulse stimulation protocols, consisting of a long step depolarization to  $+80$  mV followed, after 10 s interval, by a second pulse to  $+80$  mV, preceded by a short pre-step of 20 ms to 0 mV. The first pulse allows estimating the size and time course of voltage-gated  $\text{K}^+$  currents, while the second pulse induces sufficient intracellular  $\text{Ca}^{2+}$  loading to activate a large fraction of the  $\text{Ca}^{2+}$ -dependent BK currents (Fig. 8a). Separation of  $\text{K}_v$  and BK currents was confirmed using paxilline (a selective BK channel blocker; Marcantoni et al. 2010), which abolishes the exceeding  $\text{K}^+$  current activated during the short pre-step (recordings in panel b and c, bottom).

We found that in control MCCs, BK channels undergo rapid and rather complete inactivation

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3 (black trace in Fig. 8b-top) while in cell pre-treatment with MWCNTs, inactivation was  
4 slower and largely incomplete (black trace in panel c-top). The peak value of BK currents  
5 (BK<sub>p</sub>) remained nearly unchanged ( $3.5 \pm 0.4$  nA in control vs  $3.0 \pm 0.3$  nA with MWCNTs;  
6 black boxes in panel b and c) while the steady-state value of the BK current measured at the  
7 end of the pulse (BK<sub>ss</sub>) increased by nearly a factor 8: from  $0.3 \pm 0.1$  nA to  $2.2 \pm 0.4$  nA  
8 (white boxes in panel b and c). As shown to the left, the size of K<sub>v</sub> currents was unaffected by  
9 MWCNTs-treatment, indicating that the main effect of MWCNTs was a slowing down of BK  
10 channel inactivation. This effect could be ascribed to an increased intracellular Ca<sup>2+</sup> charge  
11 associated to the damaged cell membrane after MWCNTs exposure (decreased membrane  
12 resistance and increased background leakage current). In conclusion, the voltage-clamp data  
13 suggests that MWCNTs do not significantly alter the main ionic conductances that are  
14 responsible for AP generation and spontaneous firing of MCCs (Marcantoni et al. 2010;  
15 Vandael et al. 2010): the voltage gated Na<sup>+</sup>, Ca<sup>2+</sup> and K<sup>+</sup> and the BK channels, except for a  
16 prolongation of BK channel inactivation. This implies that most of the toxic effects of  
17 MWCNTs after 24 h exposure to concentrations above 30 µg/ml derive from the decreased  
18 cell membrane resistance (increased leakage) and more depolarized resting potentials, which  
19 drives the MCCs either to stop their activity or remain active at higher firing frequencies.  
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## 45 Discussion

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49 The main novelty of the present findings is that the toxic effects of long-term exposure to  
50 different doses of MWCNTs on excitable cells may not simply derive from a depressive  
51 action on Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> currents, as previously reported (Jakubek et al. 2009; Park et al.  
52 2003; Xu et al. 2009; Belyanskaya et al., 2009), but could be the consequence of a damage  
53 involving the cell membrane. After 24 h incubation, MWCNTs enter chromaffin cells as  
54 shown by the electron microscopy images and indicated by the reduced input resistance of the  
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3 plasmamembrane, without any preferential cytoplasmic localization. This effect led us to  
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5 suppose that most likely MWCNTs are taken up by chromaffin cells by creating small pores  
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7 into the lipid bilayer, allowing the passage of unspecific ion currents. However, it is worth to  
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9 be mentioned that the process of nanoparticles internalization may occur through different  
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11 pathways, depending on cell type, size and bundling of carbon nanotubes. Functionalized  
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13 carbon nanotubes can cross the membrane through phagocytosis, or may penetrate the plasma  
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15 membrane and be transported towards the perinuclear region, even under endocytosis-  
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17 inhibiting conditions (Kostaleros et al. 2007). Also, nanoparticles can form receptor-bound  
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19 complexes and be endocytosed (Jin et al. 2009), as demonstrated by tracking their intrinsic  
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21 photoluminescence.  
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28 The presence of cellular damage, after MWCNTs exposure, is also suggested by the reduced  
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30 number of MCCs with spontaneous firing activity. The reduction of the input resistance  
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32 values after MWCNTs incubation could be due to the presence of more unspecific open  
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34 channels (background leakage), compared to controls, or to the presence of pores induced by  
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36 the entrance of the nanomaterial. In order to solve this question we measured the amplitude of  
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38 voltage-gated  $\text{Na}^+$   $\text{Ca}^{2+}$  and  $\text{K}_v^+$  channels, and we found that MWCNTs exposure did not  
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40 induce any change in the conductance of any of these channels. The only conductance that  
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42 was substantially changed was the paxilline sensitive  $\text{Ca}^{2+}$  activated BK current, which is  
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44 more slowly inactivating. Since no changes in the total  $\text{Ca}^{2+}$  channel conductance have been  
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46 observed in MWCNTs treated cells, we hypothesized that this BK current might be affected  
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48 by calcium entering the cell through non-selective ionic pathways (MWCNTs induced pores).  
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50 Overall, this supports our hypothesis, suggesting that exposure to MWCNTs induces cell  
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52 damage, proved by the reduced membrane integrity and by the smaller number of MCCs  
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54 displaying spontaneous activity. Moreover, the small group of cells that maintained firing  
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56 activity presents an increased discharge rate and a smaller AP amplitude, both related to the  
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3 lowering of resting potentials. A more positive resting potential reduces the interspike  
4 interval required to reach the threshold of AP firing between consecutive spikes and partially  
5 inactivates the voltage-gated  $\text{Na}^+$  channels that support the AP upstroke, lowering the AP  
6 amplitude.  
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13 Given that the mechanism of spontaneous firing of MCCs relies on calcium influx through  
14 subthreshold L-type  $\text{Ca}^{2+}$  channels (Marcantoni et al. 2010; Vandael et al. 2010), an increased  
15 firing rate might reflect increased cytosolic calcium levels. Increased cytosolic calcium levels  
16 are related to cell death and it might be that the faster firing rate after MWCNTs treatment  
17 becomes indicative of a pre-apoptotic/necrotic state of the cell. Notice that,  $\text{Ca}^{2+}$ -dependent  
18 pacemaker mechanisms have been associated with the progress of neurodegenerative  
19 diseases, such as Parkinson, in dopaminergic neurons of the substantia nigra pars compacta  
20 (Chan et al. 2007). Paradoxically, a slower  $\text{K}^+$  current carried by BK channels, theoretically,  
21 should induce more profound hyperpolarizations, leading to decreased firing frequencies. In  
22 the literature, however, one can find that the acute block of BK channels by paxilline has  
23 opposite effects compared to the chronic block or knock out of BK channels in Purkinje  
24 neurons (Sausbier et al. 2004), suggesting that any change to BK channel conductance may  
25 lead to different effects on cell firing, depending on the tight coupling with voltage-gated  
26  $\text{Ca}^{2+}$  channels and levels of intracellular  $\text{Ca}^{2+}$  regulated by a variety of intracellular pathways.  
27 Since our cellular preparations were chronically incubated with MWCNTs, we assume that  
28 the potentiating effects on BK channels persisted for prolonged periods. Sustained BK  
29 currents could help the cells that maintained their spontaneous firing in AP repolarization and  
30 a faster recovery of voltage-gated  $\text{Ca}^{2+}$  channels that are necessary to restart the AP upstroke.  
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32 Given the reduced input resistance, it does not necessarily mean that bigger currents lead to  
33 more profound membrane voltage changes. We hypothesize that the effects of the MWCNTs-  
34 induced membrane damage prevail on the MWCNTs-induced effects on BK currents.  
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3 The cytotoxic effect of MWCNTs, highlighted by our work, is in agreement with other papers  
4 where they demonstrate that MWCNTs induce a significant dose-dependent decrease of cell  
5 viability either in human fibroblasts or NIH 3T3 mouse fibroblasts, block of cell cycle and  
6 impaired DNA synthesis (Zhang et al. 2010). Moreover, these authors reported destruction of  
7 actin fibrils and decreased stress fibers after treatment with MWCNTs, pointing out an  
8 important damage at the level of the cytoskeleton, in line with our results.  
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11 Our findings suggests that health hazard of nanomaterials may develop through different  
12 molecular pathways, which not necessarily lead to a reduced functionality of membrane ion  
13 channel conductances. Here we demonstrate that carbon nanotubes induce damage at the  
14 level of primary chromaffin cell cultures through a dose-dependent process, more evident at  
15 higher concentrations (100 and 263 µg/ml). We also observed that MWCNTs can cross the  
16 plasma membrane after 24 h incubation, producing disrupting effects at the cytoplasmic level,  
17 especially after 48 h. Internalization of MWCNTs leads to an irritation response, which  
18 causes the release of pro-inflammatory cytokines as interleukin 8 in human epidermal  
19 keratinocytes (Monteiro-Riviere et al. 2005). This could occur also in MCCs but there is no  
20 present evidence to support it. An inflammatory response together with the reduced  
21 membrane integrity might explain the increased number of non-firing cells after MWCNTs-  
22 treatment, but this needs to be tested in future investigations. All these effects need to be  
23 carefully considered when planning the diagnostic or therapeutic use of CNTs, highlighting  
24 the need of more detailed studies focused on the safer use and production of this material.  
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## References

- Al Faraj A, Cieslar K, Lacroix G, Gaillard S, Canet-Soulas E, Crémillieux Y. 2009. In vivo imaging of carbon nanotube biodistribution using magnetic resonance imaging. *Nano Lett.* 9(3):1023-7.
- Belyanskaya L, Weigel S, Hirsch C, Tobler U, Krug HF, Wick P. 2009. Effects of carbon nanotubes on primary neurons and glial cells. *Neurotoxicology* 30(4):702-11.
- Chan CS, Guzman JN, Llijic E, Mercer JN, Rick C, Tkatch T, Meredith GE, Surmeier DJ. 2007. Rejuvenation“ protects neurons in mouse models of Parkinson’s disease. *Nature* 447(7148):1081-6.
- Deng X, Jia G, Wang H, Sun H, Wang X, Yang S, Wang T, Liu Y. 2007. Translocation and fate of multi-walled carbon nanotubes in vivo. *Carbon* 45:1419-1424.
- Donaldson K, Aitken R, Tran L, Stone V, Duffin R, Forrest G, Alexander A. 2006. Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety. *Toxicol. Sci.* 92(1):5-22.
- Donaldson K, Poland CA. 2009. Nanotoxicology: new insights into nanotubes. *Nat. Nanotechnol.* 4(11):708-10.
- Dugan LL, Turetsky DM, Du C, Lobner D, Wheeler M, Almlı CR, Shen CK, Luh TY, Choi DW, Lin TS. 1997. Carboxyfullerenes as neuroprotective agents. *Proc Natl Acad Sci U S A.* 94(17):9434-9.
- Elder A, Gelein R, Silva V, Feikert T, Opanashuk L, Carter J, Potter R, Maynard A, Ito Y, Finkelstein J, Oberdoster G. 2006. Translocation of inhaled ultrafine manganese oxide particles to the central nervous system. *Environ. Health Persp.* 114(8):1172-8.
- Elgrabli D, Abella-Gallart S, Robidel F, Rogerieux F, Boczkowski J, Lacroix G. 2008. Biodistribution and clearance of instilled carbon nanotubes in rat lung. *Toxicol.* 253(1-3):131-6.

- 1  
2  
3 Elgrabli D, Abella-Gallart S, Aguerre-Chariol O, Robidel F, Rogerieux F, Boczkowski J,  
4  
5 Lacroix G. 2007. Effect of BSA on carbon nanotube dispersion for in vivo and in vitro  
6  
7 studies. *Nanotoxicology*. 1(4):266-278.  
8  
9
- 10 Fenoglio I, Greco G, Tomatis M, Muller J, Raymundo-Pinero E, Beguin F, Fonseca A, Nagy  
11  
12 JB, Lison D, Fubini B. 2008. Structural defects play a major role in the acute lung toxicity  
13  
14 of multi-wall carbon nanotubes: physico-chemical aspects. *Chem. Res. Toxicol.* 21:1690-  
15  
16 1697.  
17  
18
- 19 Fubini B, Ghiazza M, & Fenoglio I. 2010. Physico-Chemical Features of Engineered  
20  
21 Nanoparticles Relevant to Their Toxicity. *Nanotoxicology*. 4(4): 347–363  
22  
23
- 24 Guo L, Morris DG, Liu XY, Vaslet C, Hurt RH, Kane AB. 2007. Iron bioavailability and  
25  
26 redox activity in diverse carbon nanotube samples. *Chem. Mater.* 19(14):3472-3478.  
27  
28
- 29 Jakubek LM, Marangoudakis S, Raingo J, Liu X, Lipscombe D, Hurt RH. 2009. The  
30  
31 inhibition of neuronal calcium ion channels by trace levels of yttrium released from carbon  
32  
33 nanotubes. *Biomaterials*. 30(31):6351-7.  
34  
35
- 36 Jin H, Heller DA, Strano M. 2009. Size-dependent cellular uptake and expulsion of single-  
37  
38 walled carbon nanotubes: single particle tracking and a generic uptake model for  
39  
40 nanoparticles. *ACS Nano*, 3(1):149-158.  
41  
42
- 43 Jorio A, Saito R., Dresselhaus G, Dresselhaus MS. 2004. Determination of nanotubes  
44  
45 properties by Raman spectroscopy *Philosoph. Trans. Royal Soc. A-Mat. Phys. Engin.*  
46  
47 *Sci.*, 362: 2311-2336.  
48  
49
- 50  
51 Kane AB, Hurt RH. 2008. Nanotoxicology: the asbestos analogy revisited. *Nat. Nanotechnol.*  
52  
53 3(7):378-9.  
54  
55
- 56 Kostarelos K, Lacerda L, Pastorin G, Wy W, Wieckowski S, Luangsivilay J, Godefroy S,  
57  
58 Pantarotto D, Briand JP, Muller S et al. 2007. Cellular uptake of functionalized carbon  
59  
60 nanotubes is independent of functional group and cell type. *Nat. Nanotechnol. Lett.*

- 1  
2  
3 2(2):108-112.  
4  
5  
6 Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, Oberdörster G, Ziesenis  
7  
8 A. 2002. Translocation of ultrafine insoluble iridium particles from lung epithelium to  
9  
10 extrapulmonary organs is size dependent but very low. *J. Toxicol. Environ. Health A.*  
11  
12 65(20):1513-30.  
13  
14  
15 Lam CW, James JT, McCluskey R, Arepalli S, Hunter RL. 2006. A review of carbon  
16  
17 nanotube toxicity and assessment of potential occupational and environmental health risks.  
18  
19 *Crit. Rev. Toxicol.* 36(3):189-217.  
20  
21  
22 Lin AMY, Chyi BY, Wang SD, Yu HH, Kanakamma PP, Luh TY, Chou CK and Ho L T.  
23  
24 1999. Carboxyfullerene Prevents Iron-Induced Oxidative Stress in Rat Brain. *J.*  
25  
26 *Neurochem* 72(4):1634–1640.  
27  
28  
29 Lison D and Muller J. 2008. Lung and systemic responses to carbon nanotubes (CNT) in  
30  
31 mice. *Toxicol. Sci.* 101(1):179-80.  
32  
33  
34 Liu X, Guo L, Morris D, Kane AB, Hurt RH. 2008. Targeted Removal of Bioavailable Metal  
35  
36 as a Detoxification Strategy for Carbon Nanotubes. *Carbon.* 46(3):489-500.  
37  
38  
39 Marcantoni A, Carabelli V, Vandael DH, Comunanza V, Carbone E. 2009. PDE type-4  
40  
41 inhibition increases L-type  $Ca^{2+}$  currents, action potential firing, and quantal size of  
42  
43 exocytosis in mouse chromaffin cells. *Pflügers Archiv Eur J Physiol.* 457(5):1093-1110.  
44  
45  
46 Marcantoni A, Vandael DH, Mahapatra S, Carabelli V, Sinnegger-Brauns MJ, Striessnig J,  
47  
48 Carbone E. 2010. Loss of Cav1.3 channels reveals the critical role of L-type and BK-  
49  
50 channel coupling in pacemaking mouse adrenal chromaffin cells. *J. Neurosci.* 30(2):491–  
51  
52 504.  
53  
54  
55 Monteiro-Riviere NA, Nemanich RJ, Inman AO, Wang YY, Riviere JE. 2005. Multiwalled  
56  
57 carbon nanotube interactions with human epidermal keratinocytes. *Toxicol. Lett.*  
58  
59 155(3):377-384.  
60

- 1  
2  
3 Muller J, Huaux F, Fonseca A, Nagy JB, Moreau N, Delos M, Raymundo-Piñero E, Béguin  
4  
5 F, Kirsch-Volders M, Fenoglio I, Fubini B, Lison D. 2008. Structural defects play a major  
6  
7 role in the acute lung toxicity of multi-wall carbon nanotubes : toxicological aspects.  
8  
9 Chem. Res. Toxicol. 21(9):1698-1705.  
10  
11  
12 Muller J, Delos M, Panin N, Rabolli V, Huaux F and Lison D. 2009. Absence of carcinogenic  
13  
14 response to multiwall carbon nanotubes in a 2-year bioassay in the peritoneal cavity of the  
15  
16 rat. Toxicol. Sci. 110(2):442-448.  
17  
18  
19 Nel A, Xia T, Mädler L, Li N. 2006. Toxic potential of materials at the nanolevel. Science.  
20  
21 311(5761):622-627.  
22  
23  
24 Nemmar A, Vanbilloen H, Hoylaerts MF. 2001. Passage of intratracheally instilled ultrafine  
25  
26 particles from the lung into the systemic circulation in hamster. Am. J. Respir. Crit. Care  
27  
28 Med. 164(9):1665-8.  
29  
30  
31 Oberdörster, G. 2001. Pulmonary effects of inhaled ultrafine particles. Int. Arch. Occup.  
32  
33 Environ. Health. 74(1):1-8.  
34  
35  
36 Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, Kreyling W, Cox C. 2002.  
37  
38 Extrapulmonary translocation of ultrafine carbon particles following whole-body  
39  
40 inhalation exposure of rats. J. Toxicol. Environ. Health A. 65(20):1531-43.  
41  
42  
43 Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, Cox C. 2004.  
44  
45 Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol. 16(6-7):437-45.  
46  
47  
48 Osswald S., Havel M., Gogotsi, Y. 2007. Monitoring oxidation of multiwalled carbon  
49  
50 nanotubes by Raman spectroscopy J. Raman Spectr. 38:728-736.  
51  
52  
53 Park KH, Chhowalla M, Iqbal Z and Sesti F. 2003. Single-walled carbon nanotubes are a new  
54  
55 class of ion channel blockers. JBC. 278:50212-50216.  
56  
57  
58 Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WA, Seaton A. 2008. Carbon  
59  
60 nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity

- 1  
2  
3 in a pilot study. *Nat. Nanotechnol.* 3(7):423-8.  
4  
5  
6 Raffa V, Ciofani G, Vittorio O, Riggio C, Cuschieri A. 2010. Physicochemical properties  
7  
8 affecting cellular uptake of carbon nanotubes. *Nanomedicine.* 5(1), 89-97.  
9  
10 Ryman-Rasmussen JP, Mark F, Cesta MF, Brody AR, Shipley-Phillips JK, Everitt JJ,  
11  
12 Tewksbury EW, Moss OR, Wong BA, Dodd DE et al. 2009. Inhaled carbon nanotubes  
13  
14 reach the subpleural tissue in mice. *Nat. Nanotechnol.* 4(11):747-51.  
15  
16  
17 Sausbier M, Hu H, Arntz C, Feil S, Kamm S, Adelsberger H, Sausbier U, Sailer CA, Feil R,  
18  
19 Ofmann F et al. 2004. Cerebellar ataxia and purkinje cell dysfunction caused by  $Ca^{2+}$ -  
20  
21 activated  $K^+$  channel deficiency. *PNAS.* 101(25):9474-9478.  
22  
23  
24 Shvedova AA, Kisin ER, Porter D, Schulte P, Kagan VE, Fadeel B, Castranova V. 2009.  
25  
26 Mechanisms of pulmonary toxicity and medical applications of carbon nanotubes: Two  
27  
28 faces of Janus? *Pharmacol. Ther.* 121(2):192-204.  
29  
30  
31  
32 **Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto**  
33  
34 **EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic**  
35  
36 **acid. *Anal Biochem* 150:76-85.**  
37  
38  
39 Sørensen JB, Nagy G, Varoquaux F, Nehring RB, Brose N, Wilson MC, Neher E. 2003.  
40  
41 Differential control of the releasable vesicle pools by SNAP-25 splice variants and  
42  
43 SNAP-23. *Cell* 114(1):75-86.  
44  
45  
46 Takagi A, Hirose A, Nishimura T, Fukumori N, Ogata A, Ohashi N, Kitajima S, Kanno J.  
47  
48 2008. Induction of mesothelioma in p53 $\pm$  mouse by intraperitoneal application of multi-  
49  
50 wall carbon nanotube. *J. Toxicol. Sci.* 33(1):105-16.  
51  
52  
53 Xu H, Bai J, Meng J, Hao W, Xu H, Cao JM. 2009. Multiwalled carbon nanotubes suppress  
54  
55 potassium channel activities in PC12 cells. *Nanotechnology* 20(28):285102-285111.  
56  
57  
58 Yu JR, Grossiord N, Koning CE, Loos J. 2007. Characterization of conductive multiwall  
59  
60 carbon nanotube/polystyrene composites prepared by latex technology. *Carbon*

1  
2  
3 45(15):618-623.  
4

5 Vandael D, Marcantoni A, Mahapatra S, Caro A, Ruth P, Zuccotti A, Knipper M, Carbone E.  
6

7  
8 2010. Cav1.3 and BK channel coupling for timing and regulating cell firing. *Molecular*  
9

10 *Neurobiology* 42:185-198.  
11

12 Zhang Y, Wang B, Meng X, Sun G, Gao C. 2010. Influences of Acid-treated Multiwalled  
13

14 carbon nanotubes on fibroblasts: proliferation, adhesion, migration, and wound healing.  
15

16 *Ann Biomed Eng.* DOI: 10.1007/s10439-010-0151-y.  
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**Figure captions**

**Fig. 1** – *Transmission Electron Microscopy (TEM) image of ground MWCNTs.* In the inset enlarged view of a single broken nanotube.

**Fig. 2** – *Raman spectra of MWCNTs.* Full line pristine MWCNTs, dotted line ground MWCNTs. The positions of the bands are indicated in the upper part of the figure. The band centred at  $1340\text{ cm}^{-1}$ , designated as D, is associated to structural defects, while the band at  $1570\text{ cm}^{-1}$ , designated as G, corresponds to the tangential in-plane stretching vibration of the carbon-carbon bonds within the graphene sheets. The observed decrease of the relative intensity of the G band indicates the increase of the number of defects during the grinding process.

**Fig. 3** – *Dispersion of MWCNTs in culture media.* MWCNTs  $263\text{ }\mu\text{g/ml}$  dispersed in DMEM +15% FBS. a) Equivalent hydrodynamic diameter of single CNTs or agglomerates evaluated by dynamic light scattering (DLS). The curves correspond to five measurements made during 15 minutes. b) Optical micrograph of the suspension.

**Fig. 4** – *Electron microscopy photographs of cultured RCCs.* a) Image of the cytoplasm of a control chromaffin cell. The black spots highlighted by the white arrow represent the chromaffin granules. b) High magnification of the nuclear (N) and perinuclear region of a chromaffin cell exposed for 24 h to MWCNTs. To the top-left is visible an aggregate outside the nucleus (dashed white lines). The rectangle on the bottom left (white lines) clearly shows a single nanotube crossing the nuclear membrane. c) Image of a chromaffin cell after 48 h of exposure to MWCNTs. The cytoplasm is full of aggregates (one is indicated by the dashed square) and the internal organization of the cell is damaged. d) Image of the cytoplasm of a

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3 chromaffin cell exposed for 48 h to MWCNTs. A couple of single nanotubes is indicated by  
4 the black arrows. e) Percentage of internalization of MWCNTs after 24 h (78%) and 48 h  
5 (80%) of exposure. f) After 24 h (left panel) the nanotubes inside the cells were either single  
6 or in form of aggregates at similar percentages (51% and 49%, respectively). After 48 h (right  
7 panel), the percentage of aggregates increased (78%) while decreased that containing single  
8 MWCNTs (22%). These percentages were calculated considering the number of cells that  
9 internalized MWCNTs as 100% for each group (24 h and 48 h).

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22 **Fig. 5** – *MWCNTs reduce the percentage of MCCs with spontaneous activity.* a) Percentage  
23 of cells showing spontaneous firing activity in control and treated groups (30, 100 and 263  
24  $\mu\text{g/ml}$ ). b) Quantification of membrane input resistance by applying a 100 ms hyperpolarizing  
25 current pulse (-10 pA) from resting potential and then measuring the induced voltage change  
26 ( $\Delta V$ ), as indicated. Traces were averaged over  $n= 3$  control cells (black) and  $n= 5$  cells  
27 treated with 263  $\mu\text{g/ml}$  MWCNTs (grey) obtained from the same culture. c) Membrane input  
28 resistance versus MWCNTs concentration. Data are fitted with a dose-response curve with  
29  $\text{IC}_{50}= 37.0 \pm 0.5 \mu\text{g/ml}$  and Hill slope=  $3.5 \pm 0.2$ . Differences are statistically significant  
30 between control and higher concentrations: 100 and 263  $\mu\text{g/ml}$  (Student's t-test,  $p<0.01$  \*\*).  
31 d) Linear correlation between the percentage of spontaneously firing MCCs and the input  
32 resistance ( $\text{G}\Omega$ ), in control and at different MWCNTs concentrations ( $R= 0.99$ ).

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51 **Fig. 6** – *MWCNTs increase the firing frequency of spontaneously active MCCs.* a)  
52 Spontaneous action potentials (APs) recorded in current-clamp mode from a control,  
53 fullerenes-treated (263  $\mu\text{g/ml}$ ) and a 24 h MWCNTs-treated MCCs (263  $\mu\text{g/ml}$ ). b) Effects of  
54 MWCNTs and fullerenes on the AP amplitude measured as indicated in the top panel. The  
55 box diagram shows the mean AP amplitudes at control, with fullerenes and with MWCNTs at  
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3 the indicated concentrations. Mean values were:  $76.9 \pm 1.2$  mV, n= 25 (control);  $75.6 \pm 2.8$   
4 mV, n= 8 (fullerenes);  $71.8 \pm 2.1$  mV, n= 7 (MWCNT 30  $\mu\text{g/ml}$ );  $70.0 \pm 2.2$  mV, n= 6  
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6 (MWCNT 100  $\mu\text{g/ml}$ );  $70.6 \pm 3.6$  mV, n= 5 (MWCNT 263  $\mu\text{g/ml}$ ). c) Linear correlation  
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8 between baseline values (mV) and firing frequency (Hz) in controls and MWCNTs-treated  
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10 cells (R= 0.74). d) Firing frequency (Hz) measured at different MWCNTs concentrations  
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12 ( $\mu\text{g/ml}$ ) and in controls. At the highest concentration of MWCNTs, data are significantly  
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14 different from controls ( $p < 0.05$ ).  
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23 **Fig. 7** – Voltage-gated  $\text{Ca}^{2+}$  and  $\text{Na}^+$  currents are not affected by MWCNTs. a)  $\text{Na}^+$  and  $\text{Ca}^{2+}$   
24 currents recordings in control and MWCNTs-treated cells following sequential  
25 depolarizations of increasing voltages from  $-50$  to  $+40$  mV by 10 mV steps. b) Steady-state  
26  $\text{Ca}^{2+}$  current amplitudes measured at the end of the pulse, in control MCCs (black squares)  
27 and after MWCNTs-treatment (grey circles). c) Quantity of  $\text{Ca}^{2+}$  charges (pC) at different  
28 voltages in control and MWCNTs-treated cells. The amount of  $\text{Ca}^{2+}$  charge entering the cell  
29 was estimated by integrating each current trace for the duration of the pulse, excluding the  
30 first 5 ms in which the currents were mainly carried by  $\text{Na}^+$  channels. Data were not  
31 statistically different ( $p < 0.2$ ). d) Peak  $\text{Na}^+$  current amplitudes recorded in control conditions  
32 and after 24 h MWCNTs treatment. At each voltage, data are not statistically different.  
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49 **Fig. 8** – Exposure to MWCNTs promotes a switch from fast-inactivating to noninactivating  
50 BK currents. a) Voltage pulse protocol used to record Kv and BK currents. The first voltage  
51 pulse was a step depolarization from  $-70$  mV to  $+80$  mV lasting 400 ms and return to  $-70$   
52 mV. The second pulse was delivered after 10 s and consisted of a pre-step of 20 ms at 0 mV  
53 before the test pulse to  $+80$  mV (filled dot). b) Voltage-clamp recordings in control MCCs.  
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55 The grey trace represents the voltage-gated Kv current recorded during the first pulse while  
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3 the black trace is the BK + Kv current recorded during the second pulse. To the right are the  
4 mean amplitudes (nA) of Kv current (grey bar) and BK currents measured at the peak (BKp;  
5 black bar) and at the steady-state (BKss; white bar) as indicated in panel c. To the bottom are  
6 shown current recordings in the presence of 1  $\mu$ M paxilline in which is evident the full block  
7 of transient BK currents. c) Voltage-clamp recordings in MWCNTs-treated mouse  
8 chromaffin cells (24 h). Grey and black traces have the same meaning of those reported in  
9 panel b, with the exception that BK currents appear less inactivating. To the bottom are  
10 shown current recordings in the presence of 1  $\mu$ M paxilline in which is evident the full block  
11 of transient BK currents. To the right are reported the mean amplitudes of Kv, BKp and BKss  
12 as determined in panel b. Only the BKss values were statistically different (\*\*\*)  $p < 0.001$ ;  
13 control vs. MWCNTs using Student's t-test).  
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## Tables

**Table 1.** Physico-chemical properties of multi-walled carbon nanotubes

samples	Mean diameter (nm)	Mean length ( $\mu\text{m}$ )	SSA ( $\text{m}^2/\text{g}$ )	Elemental analysis (% oxides) <sup>c</sup>	Iron potentially bioavailable <sup>d</sup> (% $\text{Fe}_2\text{O}_3$ )	Amount of defects <sup>e</sup> $I_D/I_G$
CNT	50÷100 <sup>a</sup>	>10 $\mu\text{m}$ <sup>a</sup>	35.0 $\pm$ 0.1	Fe 0.61 $\pm$ 0.01 Al 0.04 $\pm$ 0.05 Co < d.l. Ni 0.003 $\pm$ 0.001	0.43 $\pm$ 0.01	0.12 $\pm$ 0.03
ground CNT	67 $\pm$ 2 <sup>b</sup>	1.12 $\pm$ 0.05 <sup>b</sup>	60.3 $\pm$ 0.2	Fe 0.50 $\pm$ 0.01 Al 0.06 $\pm$ 0.03 Co < d.l. Ni < d.l.	0.42 $\pm$ 0.01	0.34 $\pm$ 0.08

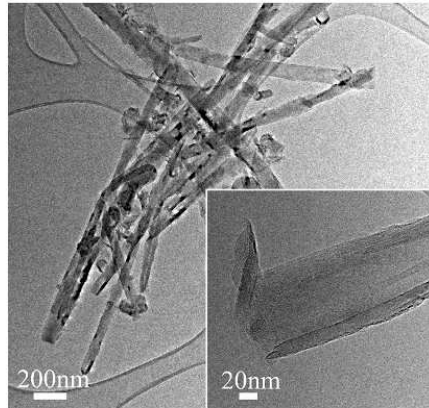
<sup>a</sup> roughly evaluated by SEM

<sup>b</sup> evaluated by TEM

<sup>c</sup> evaluated by means of AE-ICP spectrometry

<sup>d</sup> evaluated by measuring spectrophotometrically the amount of iron ions extracted by ferrozine/ascorbic acid system

<sup>e</sup> evaluated by means of Raman spectroscopy



**Figure 1**

Transmission Electron Microscopy (TEM) image of ground MWCNTs.  
254x190mm (96 x 96 DPI)

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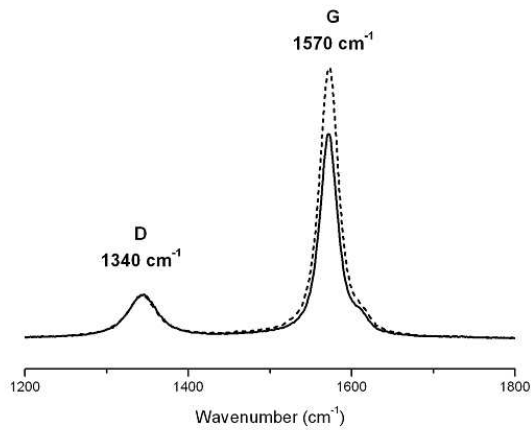


Figure 2

Raman spectra of MWCNTs.  
254x190mm (96 x 96 DPI)

View Only

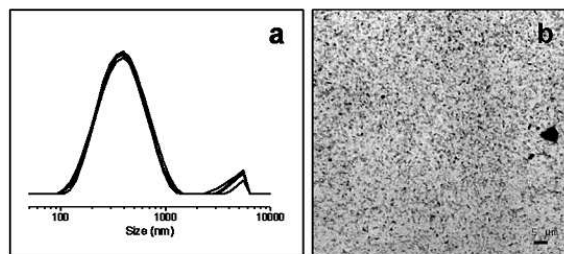
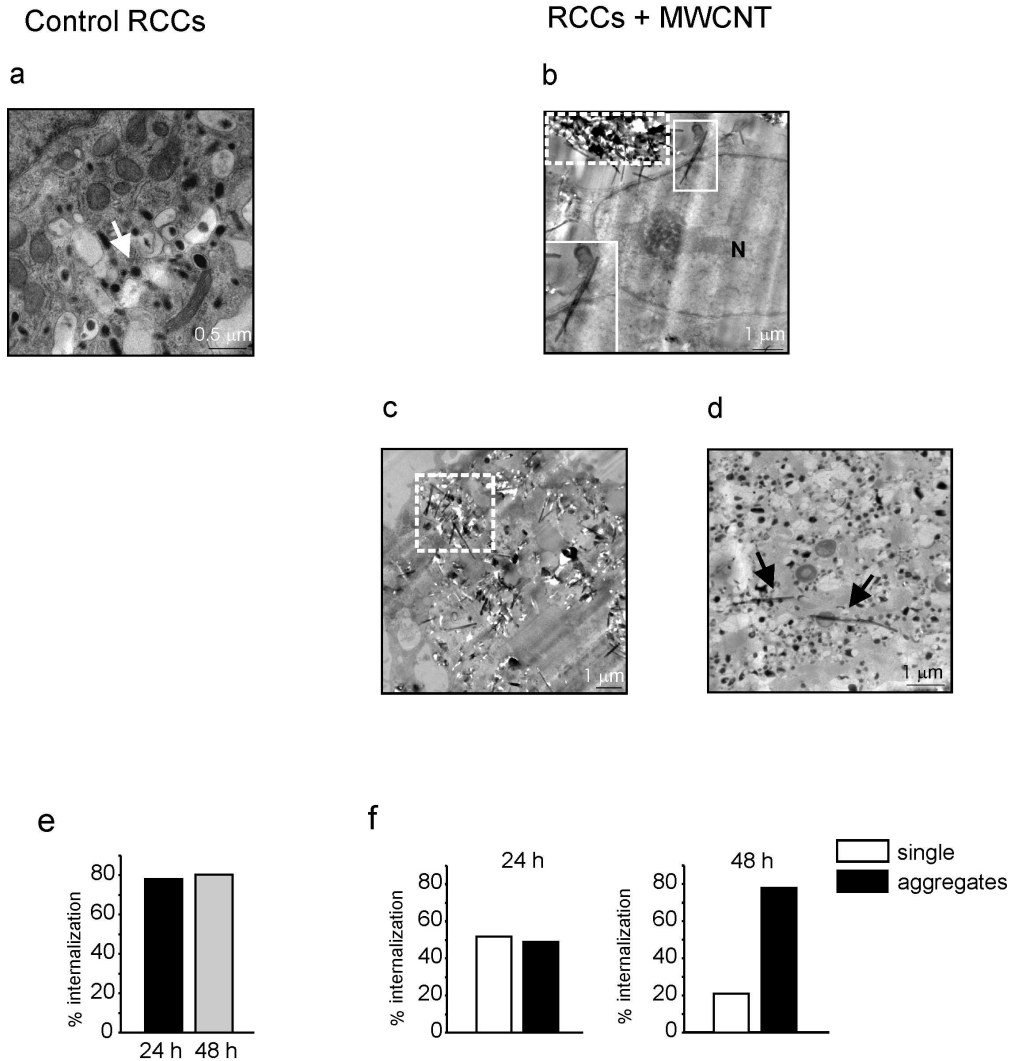
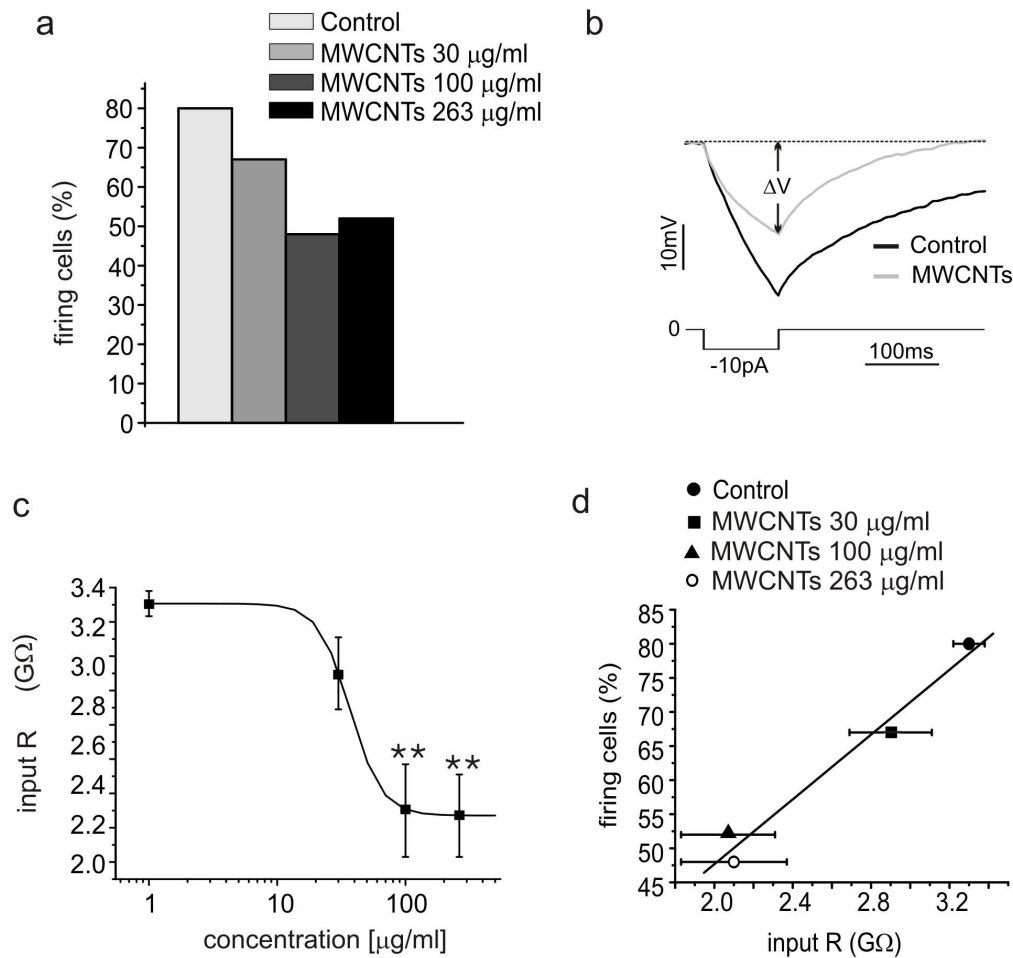


Figure 3

Dispersion of MWCNTs in culture media.  
254x190mm (96 x 96 DPI)

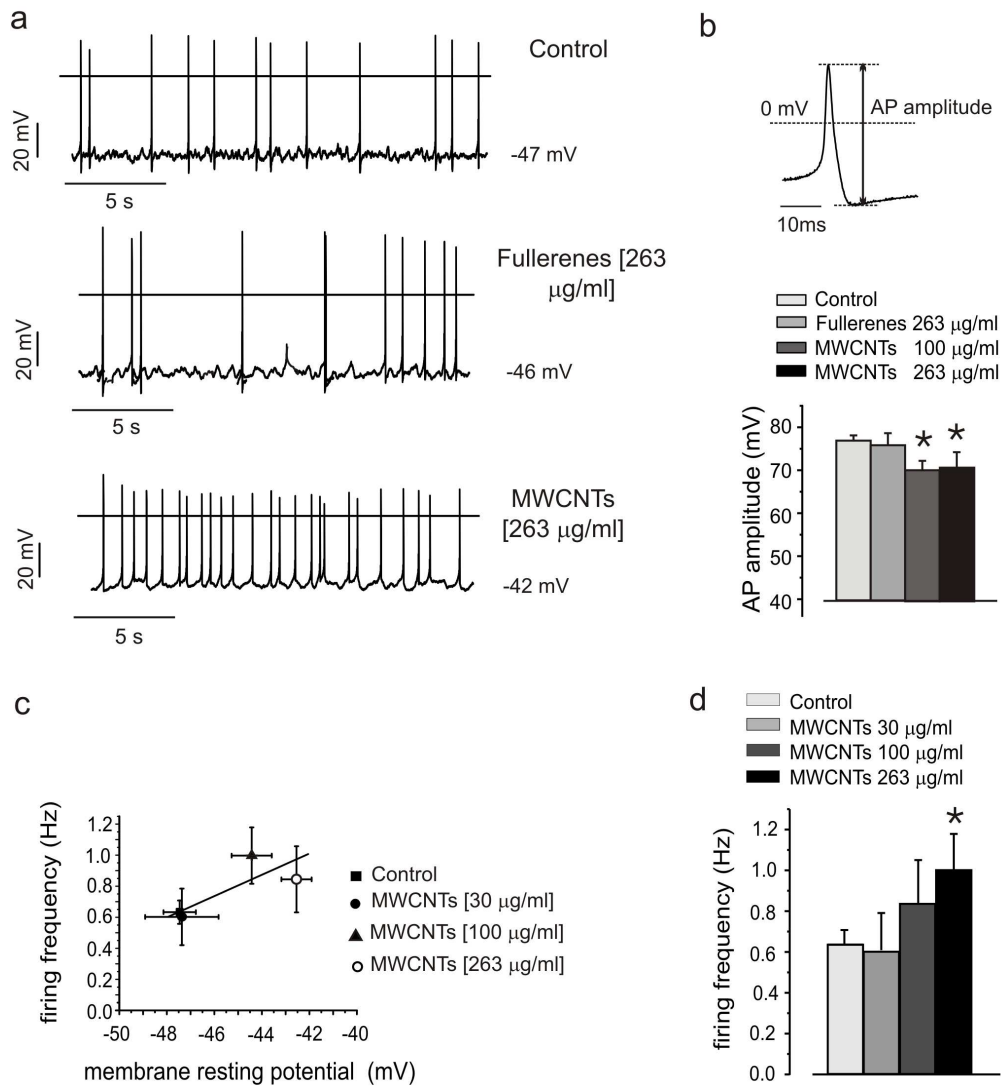
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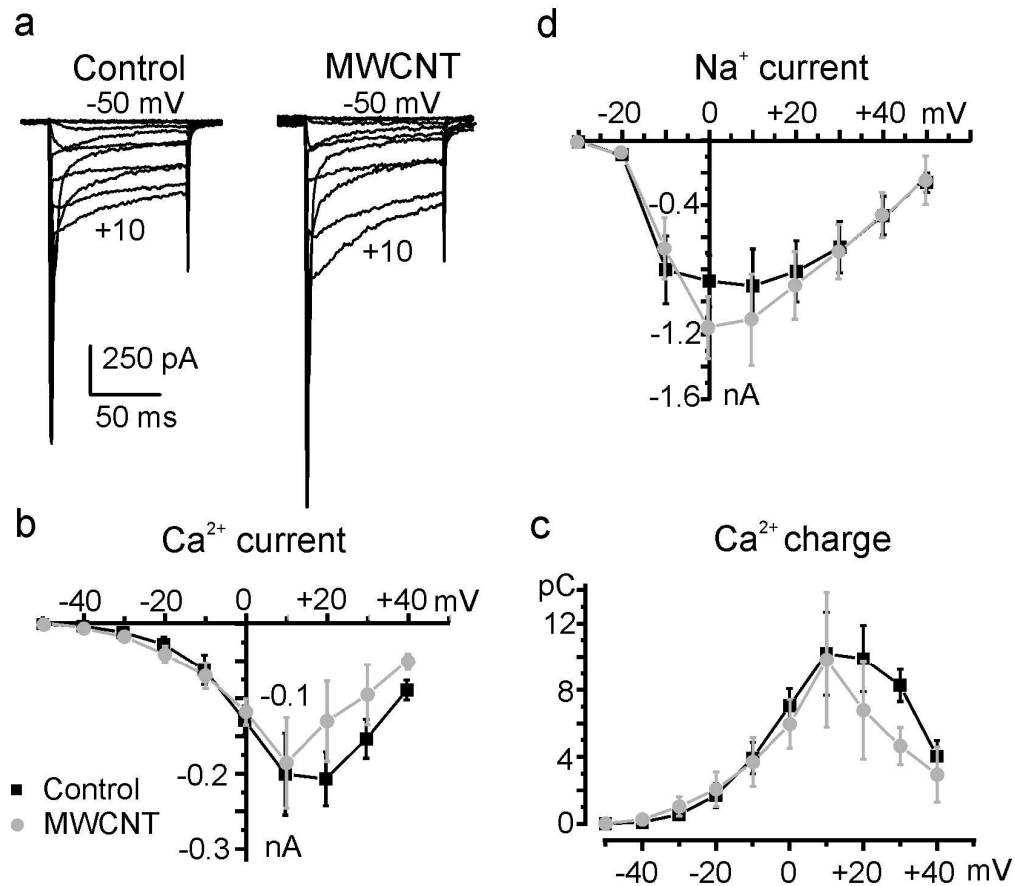
**Figure 5**

MWCNTs reduce the percentage of MCCs with spontaneous activity.  
161x168mm (300 x 300 DPI)



**Figure 6**

MWCNTs increase the firing frequency of spontaneously active MCCs.  
171x199mm (300 x 300 DPI)



**Figure 7**

Voltage-gated Ca<sup>2+</sup> and Na<sup>+</sup> currents are not affected by MWCNTs.  
139x139mm (300 x 300 DPI)

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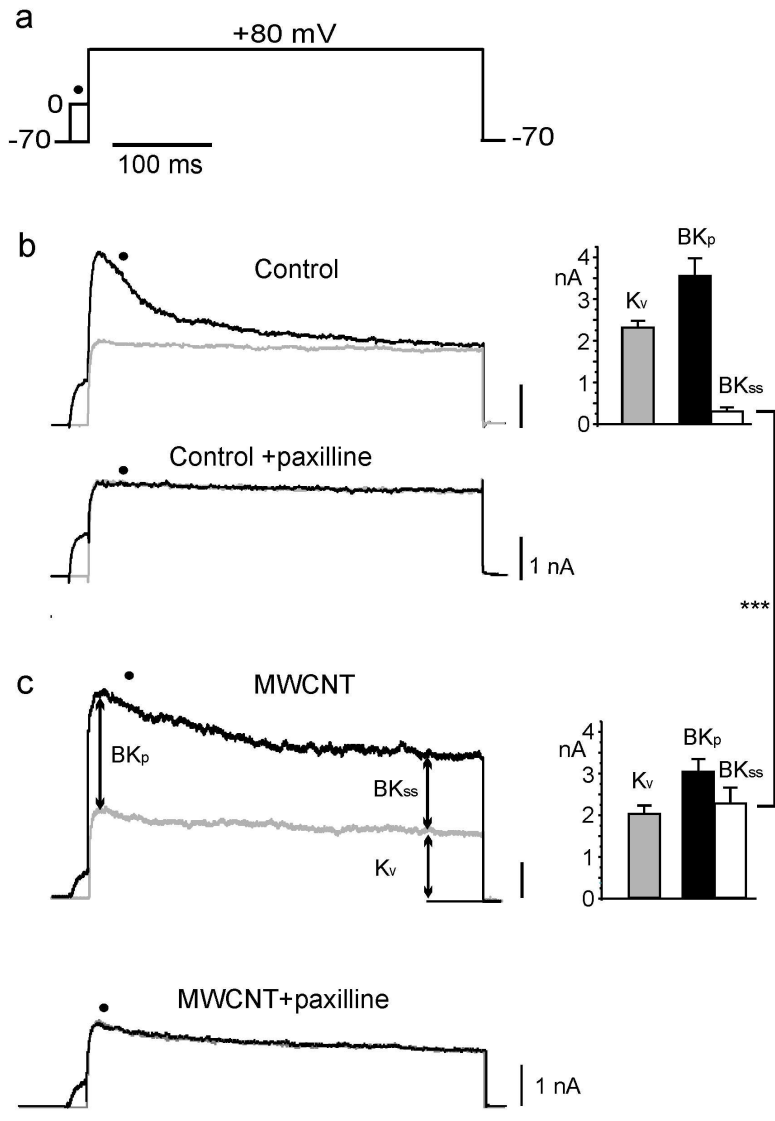


Figure 8

Exposure to MWCNTs promotes a switch from fast-inactivating to noninactivating BK currents.  
143x224mm (300 x 300 DPI)