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Water Soluble Single-Walled Carbon Nanotubes Inhibit Stimulated Endocytosis in Neurons

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Abstract

We report the use of chemically-functionalized water soluble single-walled carbon nanotube (SWNT) graft copolymers to inhibit endocytosis. The graft copolymers were prepared by the functionalization of SWNTs with poly-ethylene glycol. When added to the culturing medium, these functionalized water soluble SWNTs were able to increase the length of various neuronal processes, neurites, as previously reported. Here we have determined that SWNTs are able to block stimulated membrane endocytosis in neurons, which could then explain the previously noted extended neurite length.

The numerous electrical, mechanical and chemical properties that carbon nanotubes possess make them an intriguing material with great promise for application in neuroscience. Single-walled and multi-walled carbon nanotubes are being increasingly investigated for use as scaffolds for neuronal growth and for use in interfaces with neurons (reviewed in ref. 1). Their size, strength and flexibility make them well suited for use as substrates and scaffolds, and they can be made conductive enabling them to detect neuronal electrical activity as well as deliver electrical stimulation to cells in contact with them.

Previously we reported that water soluble SWNTs applied to the culture medium used for neuronal growth resulted in altered growth and morphology of neurons, notably the extension of neurite length and reduced number of neurites 2. We found that these water soluble SWNTs affected Ca^{2+} dynamics in neurons, reducing the depolarization-dependent influx of Ca^{2+} during cell stimulation. It has been implicated that plasma membrane/vesicular recycling plays a role in the rate of neurite elongation 3. This process of vesicular recycling can be regulated by an increase in intracellular Ca^{2+} levels due to depolarization of neurons which leads to an influx of this ion from the extracellular space. Thus, to further elucidate the mechanisms responsible for the effects of SWNTs on neurite outgrowth we examined how SWNTs could affect membrane recycling.

SWNTs were chemically functionalized in order to make them soluble in water as previously described 4. Briefly, we modified commercially available, purified SWNTs with carboxylic acid groups at their ends (Carbon Solutions, Inc., Riverside, CA) to make an acyl chloride intermediate, SWNT-COCl, which was then reacted with glycol (PEG) to form SWNT-PEG

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graft copolymers. These functionalized SWNTs were reconstituted in distilled water for a 5.0 mg/mL stock solution.

Next, we treated neuronal cultures with these water soluble SWNTs. Hippocampal neuronal cultures were prepared from 0- to 2- day-old Sprague-Dawley rats using previously described procedures 2,5-7. Briefly, following treatment with papain and trituration of hippocampal tissue, the cell suspension was applied onto poly-ethylene imine (PEI; 1 mg/mL) -coated coverslips that were inlaid into culture dishes. After 3 hours of incubation to allow for neuronal adhesion, fresh culture medium (pH=7.35), consisting of minimum essential medium supplemented with fetal bovine serum (10% v/v; Hyclone), Mito+ serum extender (0.1% v/v, Collaborative Biomedical Products), D-glucose (20 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL) and sodium bicarbonate (14 mM), was applied to the dishes. At that juncture, we applied SWNT-PEG to the medium at dilutions yielding final concentrations of 0.1, 0.5, 1.0 or 5.0 µg/mL. In control dishes we applied the SWNT's vehicle, 1 µL of distilled water, instead of nanotubes. Cultured cells were then maintained in a humidified 5% CO₂/95% air incubator at 37°C for 3 days until used in experiments.

The living neurons were studied at room temperature using light microscopy. In these experiments, the coverslips containing cultured neurons were mounted into a recording chamber filled with normal external solution composed of (in mM): NaCl (140), KCl (5), CaCl₂ (2), MgCl₂ (2), glucose (5), and Hepes (10) (pH=7.4). We examined neurons at room temperature (20–24° C) by using a microscope (Nikon TE300) equipped with differential interference contrast (DIC) and fluorescence illumination (Xenon arc lamp; 100 W). The cells were visualized using a standard FITC/fluorescein filter set (Chroma Technology Corp.) and a 20× Plan Fluor objective, although in some experiments we also utilized a 60× Plan Apo objective. We used a CoolSNAP®-HQ cooled, charge-coupled device (CCD) camera (Roper Scientific Inc.) driven by V++ imaging software (Digital Optics Ltd., Auckland, New Zealand) to acquire images. To reduce photo-bleaching we inserted neutral density filters and an electronic shutter (Vincent Associates, Rochester, NY) that was controlled by software in the excitation pathway.

Neurons were identified based on their morphological features using DIC. We confirmed their identification by labeling with neuron-specific antibodies. After 3 days in culture, the neurons on coverslips were identified through immunocytochemistry using a modification of a previously described procedure 8. All agents used for immunocytochemistry experiments were prepared in phosphate buffered saline (PBS) containing (in mM): NaCl (137), KCl (2.7), Na₂HPO₄•7H₂O (10), and KH₂PO₄ (1.8) (pH=7.3). The cellular proteins were fixed in paraformaldehyde (4% w/v; Fisher Scientific) for 30 min, followed by cell membrane permeabilization with Triton-X (0.25% v/v; Fisher Scientific). After triple wash with PBS, the fixed cells were then incubated with a blocking agent (Blokhen™; AvesLabs, Tigard, OR) and one of two primary chicken antibodies (Aves Labs), either anti-neuron-specific enolase (NSE; dilution 1:50), or anti-β-tubulin III (dilution 1:100), for ~ 18 h at 4°C. Following a triple wash with PBS, cells were incubated with the secondary, FITC-conjugated goat anti-chicken antibody (AvesLabs), for 1.5 h at room temperature. In control experiments, the primary antibodies were omitted from the procedure to account for non-specific fluorescence. As shown in Figure 1, neurons identified based on their DIC morphological criteria and treated with the primary antibodies exhibited much greater fluorescence than surrounding glial cells (Figure 1, Left). Control cells, where primary antibodies were omitted from the procedure, show low background fluorescence (Figure 1, Right). These data indicate that morphologically identified cells in our experiments express well known neuronal markers NSE 9,10 and β-tubulin III, also referred to as TuJ1 11,12. Therefore, in all subsequent experiments we used a morphological identification to study live neurons.

In an attempt to determine the mechanism responsible for the effects of water soluble SWNT-PEG on enhancement of selected neurite outgrowth seen previously 2, we examined whether SWNTs affect depolarization-dependent plasma membrane/vesicular recycling. We exposed neurons to a fluorescent dye *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM1-43; 10 μ M, 5 min) 13 to monitor membrane recycling (reviewed in ref. 14). Since FM1-43 does not passively diffuse across cell membranes, the dye is taken up by endocytosis (Figure 2A). The extent of FM1-43 cellular load (plasma membrane and vesicles) was assessed measuring the fluorescence intensity peak value taken at the end of exposure to the dye, while the steady state fluorescence after extensive washing, reported on the vesicular dye load (Figure 2B). All data were background subtracted and expressed as dF/F_0 (percentage), where F_0 represents the (auto)fluorescence level before cell stimulation, and dF represents the change in fluorescence. Since hippocampal neurons at this stage in culture express synaptic proteins that are distributed mainly within the somata 15, we measured FM1-43 fluorescence originating from neuronal bodies. When we applied FM1-43 and monitored constitutive vesicular recycling, which occurs in unstimulated cells, we found that SWNT-PEG did not affect the endocytotic loading of the recycling dye. The amount of dye uptake that occurred during loading and the exponential rate of decay after rinsing were similar in unstimulated neurons exposed to the various concentrations of SWNTs and those not exposed to SWNT (one-way ANOVA; $P(4, 257)=0.986$ and 0.068 , at peak and steady-state, respectively). An exponential fit to the fluorescence decay showed correlation coefficients of $r = -0.985$ to -0.998 for these various conditions. The decay time constant in control cells was 100s, while neurons exposed to 5 μ g/mL of SWNT-PEG displayed a time constant of 97s.

In addition to constitutive recycling, exocytosis can occur in neurons in response to depolarizing stimuli that lead to intracellular Ca^{2+} elevations. This Ca^{2+} -dependent, regulated exocytosis is the main mechanism for transmitter release in synaptic neurotransmission. To induce regulated exocytosis we stimulated neurons by depolarizing these cells using a modified external solution (HiK^+) in which KCl was increased to 50 mM with a compensatory reduction in NaCl to 95 mM. In this case SWNT-PEG caused a concentration-dependent reduction in the total cellular load of FM1-43 (peak dF/F_0 ; Figure 3A,B), without affecting the rate of its exponential fluorescence decay ($r = -0.976$ to -0.998) after the extensive rinsing of FM1-43 loaded cells with normal external solution (Figure 3B). For example, the decay time constants in depolarized control neurons and of those neurons exposed to 5 μ g/mL of SWNT-PEG were 65 s and 66 s, respectively. As indicated earlier, the steady-state level of FM 1-43 dye retention reports on dye trapped in vesicular compartments. Exposing neurons to SWNT-PEG reduced the initial amount of dye taken up and the steady-state dye retention in stimulated neurons (Figure 3C). Thus, SWNT-PEGs affect regulated/stimulated, but not constitutive, plasma membrane/vesicular recycling.

The total load of recycling dye results from a complex interplay between exocytotic and endocytotic processes. To discern whether SWNT-PEG during our experimental procedure could preferentially affect one of these related processes, we applied a “paired-pulse” stimulation (destaining) protocol 13. After the first stimulus with HiK^+ in the presence of FM1-43 followed by washout to reach a steady-state level of fluorescence as done before, we then stimulated with HiK^+ again, but for shorter time (200 s) and without adding recycling dye to the external solution (Figure 4, Left). The second stimulus would depolarize neurons and lead to the exocytosis of previously loaded vesicular FM1-43. To study the amount and rate of exocytosis we normalized fluorescence decay during the second stimulus to the steady state level just prior to the second stimulus (Figure 4, Right). When neurons were tested in the presence of 5 μ g/mL SWNT-PEG they showed more extensive destaining of vesicular FM1-43 than that of the time-matched control neurons (Student t-test, $P<0.01$). This destaining was also faster in neurons treated with SWNT-PEG (decay time constant of 24 s) than in control neurons (decay time constant of 32s). This accelerated and more effective destaining of

vesicular FM1-43 during the second depolarizing stimulus, combined with the reduction of endocytotic loading of FM1-43 during the first stimulation points to a preferential action of SWNT-PEG on regulated endocytosis (as opposed to exocytosis) by inhibiting the endocytotic component of vesicular recycling. The faster rate of destaining may also be due to an increase in exocytosis but this seems unlikely since, as shown previously 2, application of SWNT-PEG results in impaired cytoplasmic Ca^{2+} elevation during stimulus which would hinder exocytosis since it is a Ca^{2+} dependent process. Consequently, the exocytotic incorporation of vesicles into the plasma membrane is not balanced by the endocytotic retrieval in the presence of SWNTs, which could effectively cause the increase in neurite length observed elsewhere 2; SWNTs' effect on the reduction of the number of neurites 2 could be then a compensatory mechanism to keep the cell surface/volume relatively constant.

We tested the mechanism of action by which SWNT-PEG lead to enhancement of selected neurite outgrowth 2. Our results point to an inhibitory action of SWNT-PEG on regulated endocytosis (Scheme 1). An increase in total membrane area during neurite outgrowth occurs by incorporating new membrane by vesicle fusion or exocytosis. Membrane can also be retrieved by the reverse process of endocytosis. When cells are exposed to soluble carbon nanotubes stimulated endocytosis is impaired. This could be due to the nanotubes directly affecting the endocytotic vesicle. It should be noted that clear synaptic vesicles are on the order of 50 nm in diameter. The sizes of SWNT-PEG we used have been previously characterized; their diameter and length range from 2–11 nm and 0.1–2.2 μm , respectively 2. Thus, the small diameter of the SWNT could allow it to easily fit into the opening of a vesicle that has transiently fused with the membrane or that is being internalized. The relatively long length of the nanotube could prevent the vesicle from closing and pinching off from the membrane, trapping the vesicle and preventing endocytosis. Under stimulation, the vesicle fusion pore may have a longer dwell time, increased frequency of reopening and an increased diameter than that of spontaneous events (reviewed in ref. 16). Since spontaneous events can have much shorter pore open times and sub-nanometer diameters, the probability of nanotubes entering the vesicle would be greatly reduced. Also during stimulation the total amount of membrane that is turned over is increased, further increasing the chance of vesicles taking up nanotubes. Thus, stimulated exocytosis would be preferentially affected by the application of soluble nanotubes. Also, this configuration of nanotubes physically sticking in vesicles would not prevent exocytosis, as the nanotube would simply be expelled as the vesicle collapses into the membrane during full fusions. Many groups have shown that peptide, DNA and RNA conjugates to nanotubes can be introduced into cells 17-20. It is not clear how these compounds get into the cytosol, whether by directly crossing the plasma membrane or by crossing the vesicular membrane while they are being endocytosed. Therefore, although unlikely, it is possible that SWNT-PEG could be also exerting its influence on endocytosis from inside the cell, perhaps interacting with proteins involved in stabilizing the fusion pore.

Acknowledgments

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References

1. Malarkey EB, Parpura V. *Neurodegener Dis* 2007;4(4):292–9. [PubMed: 17627132]
2. Ni Y, Hu H, Malarkey EB, Zhao B, Montana V, Haddon RC, Parpura V. *J Nanosci Nanotechnol* 2005;5(10):1707–12. [PubMed: 16245532]
3. Zakharenko S, Popov S. *Neuroscience* 2000;97(1):185–94. [PubMed: 10771350]
4. Zhao B, Hu H, Haddon RC. *Adv. Func. Mater* 2004;14:71–76.

5. Hu H, Ni Y, Mandal SK, Montana V, Zhao B, Haddon RC, Parpura V. *J Phys Chem B* 2005;109(10):4285–9. [PubMed: 16851493]
6. Hu H, Ni YC, Montana V, Haddon RC, Parpura V. *Nano Letters* 2004;4(3):507–511.
7. Parpura V, Haydon PG. *Proc. Natl. Acad. Sci. USA* 2000;97:8629–8634. [PubMed: 10900020]
8. Montana V, Ni Y, Sunjara V, Hua X, Parpura V. *J Neurosci* 2004;24(11):2633–42. [PubMed: 15028755]
9. Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH. *Nat Med* 1998;4(11):1313–7. [PubMed: 9809557]
10. Lu B, Yokoyama M, Dreyfus CF, Black IB. *Proc Natl Acad Sci U S A* 1991;88(14):6289–92. [PubMed: 1712487]
11. Braun H, Schafer K, Holtt V. *J Neurotrauma* 2002;19(8):975–83. [PubMed: 12225657]
12. Roskams AJ, Cai X, Ronnett GV. *Neuroscience* 1998;83(1):191–200. [PubMed: 9466409]
13. Betz WJ, Bewick GS. *Science* 1992;255(5041):200–3. [PubMed: 1553547]
14. Cochilla AJ, Angleson JK, Betz WJ. *Annu Rev Neurosci* 1999;22:1–10. [PubMed: 10202529]
15. Basarsky TA, Parpura V, Haydon PG. *J Neurosci* 1994;14(11 Pt 1):6402–11. [PubMed: 7965045]
16. Vardjan N, Stenovec M, Jorgacevski J, Kreft M, Zorec R. *J Physiol* 2007;585(Pt 3):655–61. [PubMed: 17556387]
17. Dumortier H, Lacotte S, Pastorin G, Marega R, Wu W, Bonifazi D, Briand JP, Prato M, Muller S, Bianco A. *Nano Lett* 2006;6(7):1522–8. [PubMed: 16834443]
18. Pantarotto D, Briand JP, Prato M, Bianco A. *Chem Commun (Camb)* 2004;(1):16–7. [PubMed: 14737310]
19. Kam NW, Liu Z, Dai H. *J Am Chem Soc* 2005;127(36):12492–3. [PubMed: 16144388]
20. Krajcik R, Jung A, Hirsch A, Neuhuber W, Zolk O. *Biochem Biophys Res Commun* 2008;369(2):595–602. [PubMed: 18298946]

Figure 1.

Neuron-specific enolase (NSE) and β -tubulin III (also referred to as TuJ1) immunoreactivity in hippocampal cultures. Cells were incubated with chicken antibodies against either NSE or β -tubulin III (Left). Neurons show strong immunoreactivity, as expected from neuron-specific markers, while astrocytes surrounding neurons display faint fluorescence emission. Cells submitted to the immunocytochemistry procedure, but with the omission of the primary antibodies, serve as control for background fluorescence (Right). Scale bar, 10 μ m.

Figure 2.

Water soluble graft co-polymer SWNT-PEG does not affect the endocytotic load of the recycling dye FM1-43 during constitutive recycling. A) The application of FM1-43 labels membranes (load), internalized membrane remains labeled after washout of the dye (rinse). In the loading phase, the dye is taken up by endocytosis. B) Time course of the experiment outlined in (A). The arrow indicates the beginning of the 5 minute FM1-43 application (broken abscissa). The peak fluorescence signal is an indication of the extent of FM1-43 endocytotic load. The signal decays during rinsing phase reaching a steady-state level, which reports on the fluorescence of FM1-43 loaded in vesicular compartments. Treatment of neurons with different concentrations of SWNT-PEG, for 3 days in culture and throughout the entire course of experiment, did not affect the endocytotic load of FM1-43 (grey/black squares) when compared to untreated neurons (control; open squares). Images show DIC of a neuron, its fluorescence before (0s), immediately after addition of FM1-43 (400s) and at steady-state (900s). Numbers in parenthesis indicate the number of neurons studied in each condition. Points

and bars represent means $\pm$ standard error of the mean. Concentrations of SWNT-PEG are given in $\mu\text{g/mL}$. Scale bar, $10\mu\text{m}$.

Figure 3. SWNT-PEG reduces endocytotic load of the recycling dye FM1-43 in stimulated neurons. A) Time course of the experiment using HiK^+ solution to stimulate exocytosis. The arrow indicates the beginning of the 5 minute FM1-43/ HiK^+ application (broken abscissa). Treatment of neurons with SWNT-PEG, for 3 days in culture and throughout the entire course of experiment, reduced the stimulated endocytotic load of FM1-43 (black squares) when compared to untreated neurons (control; open squares). B) This effect of SWNT-PEG on the endocytotic load (plasma membrane and vesicular compartment; peak response) is dose-dependent (grey bars) and is not seen in unstimulated cells (black bars). C) The steady-state level of FM 1-43 dye retention reports on vesicular compartments. SWNT-PEG treatment reduced steady-state

dye retention in stimulated (grey bars), but not in unstimulated (black bars) neurons. Points and bars represent means $\pm$ standard error of the mean. Concentrations of SWNT-PEG are given in $\mu\text{g/mL}$. Numbers in parenthesis indicate the number of neurons studied in each condition; the number of cells in C are the same as in B. Asterisks indicate a significant difference in measurement when compared to control (one-way ANOVA followed by Fisher's LSD test; * $p < 0.05$, ** $p < 0.01$).

Figure 4. Soluble SWNTs preferably affect endocytosis over exocytosis. (Left) Peak FM1-43 fluorescence indicates the extent of plasma membrane and vesicular load. Using HiK⁺ solution to stimulate neurons in the presence of FM1-43 (FM1-43/HiK⁺) shows a large difference in the cellular load between control (open squares) and SWNT-PEG treated cells (black squares). The second application of HiK⁺ solution at the end of the experiment stimulates exocytosis of FM1-43 loaded in vesicles. The arrows (from left to right) indicate the beginning of the 5 minute FM1-43/HiK⁺ application (broken abscissa) and the second application of HiK⁺ alone lasting 200 s (horizontal line). (Right) The rate of FM1-43 de-staining during second application of HiK⁺ is faster and significantly more pronounced in SWNT-PEG treated neurons when compared to their controls. Note that FM1-43 fluorescence emission was normalized to the steady-state level just prior to the application of HiK⁺. Points and bars represent means $\pm$ standard error of the mean. Asterisks indicate a significant difference in measurement when compared to time-matched control (Student t-test; ** $p < 0.01$).

Scheme 1.

Possible mechanism of soluble SWNTs action on endocytosis. An increase in total membrane area during neurite outgrowth occurs by incorporating new membrane by vesicle fusion or exocytosis. Membrane can also be retrieved by the reverse process of endocytosis. When neurons are exposed to soluble carbon nanotubes endocytosis is impaired effectively allowing for the net insertion of plasma membrane.