Multi-walled carbon nanotube increases the excitability of hippocampal CA1 neurons through inhibition of potassium channels in rat's brain slices

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HIGHLIGHTS
- Investigating the neurotoxicity of multi-walled carbon nanotube (MWCNT).
- Whole cell patch-clamp technique was used.
- MWCNT produced a concentration-dependent inhibition in amplitudes of $I_h$ and $I_K$.
- Spike half-width and repetitive firing rate were significantly increased.
- MWCNT increases excitability of CA1 neurons by inhibiting voltage-gated potassium current.

ABSTRACT
This study was to investigate the neurotoxicity of multi-walled carbon nanotube (MWCNT) by measuring neuronal excitability in rat hippocampal neurons and exploring the underlying mechanism. Whole cell patch-clamp technique was used. Action potentials properties and the pattern of repetitive firing rate were assessed. Our data showed that spike half-width and repetitive firing rate were significantly increased in a concentration-dependent manner. Furthermore, voltage-activated potassium currents were recorded. It was found that MWCNT produced a concentration-dependent inhibition in amplitudes of $I_h$ and $I_K$. In addition, MWCNT had effect on the activation kinetics of $I_h$ and $I_K$ with $V_h$ being shifted to the negative potential at high concentration, while $I_h$ inactivation curve was considerably shifted to the hyperpolarize potential with $V_h$ being increased. However, no effect was found on the recovery from inactivation of $I_h$. The results suggest that MWCNT increases the excitability of hippocampal CA1 neurons by inhibiting voltage-gated potassium current.

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1. Introduction
Recently, nano-sized materials have been widely applied in various areas including medicine, biotechnology, environmental technology and energy (Liu et al., 2012). Moreover, great progresses and augment findings in global technological research on nanomaterials have been achieved. As a special nanomaterial, carbon nanotubes (CNTs) with seamless cylindrical structure of grapheme layers could be considered as one of the most widely studied allotrope of carbon in recent years. There are two kinds of nanotubes, which are single-walled nanotubes (SWNTs) and multi-walled nanotubes (MWNNTs). In due to the unique inherent physical and chemical characteristics endowed by the novel nano-structures, they have great potential applications in various aspects, especially in industry and medical science. The explicit applications of CNTs in the biomedical field are biosensors and enzyme detection (Jacobs et al., 2010), microelectrodes arrays (Yu et al., 2007), culture substrates (Voge and Stegemann, 2011), biomaterials (Lin et al., 2004) and drug molecules or vaccine delivery vehicles for therapies or diagnosis (Bianco et al., 2005). Furthermore, there are number of applications of MWNNTs including catalysis, composite materials with improved structural and electrical properties, fillers in composites for anti-static applications and components within rechargeable battery electrodes (Han et al., 2012). Thus, along with the increase of the applications, it has been more essential to examine the negative risks of MWCNT to human health (An et al., 2012).

Apparently, the toxicity and biocompatibility of CNT should be comprehensively investigated before they could be incorporated into novel and existing biomedical devices (Han et al., 2012). The assessments of the toxicity and biocompatibility of CNT both in vivo and in vitro were performed in a number of studies, which were mainly focused on the cytotoxicity, genotoxicity and toxicity
of gastrointestinal exposure (Simate et al., 2012). Nevertheless, diverse results were obtained from the studies. The toxicity of purified MWCNTs in normal human dermal fibroblast cells was examined by using cell viability, DNA damage and apoptosis as the toxicological endpoints, and it was suggested that there were considerable toxicities due to exposure to MWCNTs (Patilla et al., 2010a). However, a previous study showed that astrocytes preferentially adhered to and proliferated on the larger diameter and lowest surface energy CNT (McKenzie et al., 2004).

Obviously, much work needs to be done in establish the toxicity and biocompatibility of CNTs. Nanoparticles can pass through biological membranes. Furthermore, they can enter into the central nervous system (CNS) by sensory nerves or penetrating the blood brain barrier via olfactory nerve pathways and blood stream (Lockman et al., 2002; Oberdorster et al., 2004, 2009). In addition, in order to get better treatment of cerebral cancers, CNT may extensively be used as delivery vehicles or nano-fillers for transmitting macromolecule drugs (Koo et al., 2006; Ren et al., 2012; Yang, 2010). Nevertheless, several studies showed that MWCNTs possibly had neurotoxicity on nerve regeneration and neuronal network in vitro, a major concern for neurodegenerative (Lovat et al., 2005; Wu et al., 2012). It was reported that short MWCNTs were far more safety than SWCNT, consequently, small size MWCNTs had greater utility in medical application (Poland et al., 2008). However, there were few studies in which the direct effects of short MWCNTs were investigated on the alteration of hippocampal CA1 neuron excitability and the possible mechanisms involved these effects. Accordingly, the purpose of the present study was to investigate the impending neurotoxicity of MWCNTs by measuring evoked action potential and repetitive firing rate in rat hippocampal slices. Afterwards, the underlying mechanism was explored by recording voltage-activated potassium currents using whole cell patch-clamp technique.

2. Materials and methods

2.1. Drug application

The average length of MWCNTs is around 2 μm and the diameter is about 10–20 nm. The nanoparticles were suspended in the artificial cerebrospinal fluid (ACSF) with stock concentration of 10 g/L and thoroughly incorporated by a brief sonication for 20 min before each use. The final concentrations of MWCNTs used in the experiment were 50, 100 and 400 μg/mL. The characteristics and structure of MWCNTs used in this study could be found in our previous study (Han et al., 2012). ACSF contained (in mM): NaCl 125, NaHCO3 25, NaH2PO4 1.25, d-glucose 10, KCl 1.25, MgCl2 2.0, CaCl2 2.0, adjusted to pH 7.4 with NaOH. The standard pipette solution for recording (in mM): KCl 140, MgCl2·6H2O 2, HEPES 10, EGTA 10, ATP·Na2 2, buffered to pH 7.4 with KOH. Tetrodotoxin (TTX) was purchased from the Research Institute of the Aquatic Products of Hebei (China). 4-Aminopyridine (4-AP), tetraethyammonium chloride (TEA-Cl), CaCl2, EGTA, HEPES and ATP·Na2 were obtained from Sigma (USA) and other reagents were of A.R. grade.

2.2. Slice preparation

Male Wistar rats on postnatal days 10–14 were purchased from the Experimental Animal Center of Chinese Academy of Medical Science. The experiments were conducted in accordance with the guidelines of the Medicine Experimental Animal Administrative Committee of Nation, and all efforts were made to minimize the number of animals sacrificed and their suffering. Horizontal hippocampal slices included the entire hippocampus and subiculum were prepared with a vibratome (VT 1000S, Leica, Germany) with thickness 400 μm, and then incubated with ACSF (saturated with 95% O2 to 5% CO2) for at least 1 h before they were moved to the recording chamber (Yang et al., 2010b). All the experiments were performed at room temperature (22–24°C).

2.3. Patch clamp recording

The slices were promptly transferred in the recording chamber (1 ml) placed on the stage of a modified upright infrared DIC microscope equipped with Nomarski optics and continuously perfused with ACSF (95% O2 to 5% CO2). Then hippocampal CA1 neurons were visualized on a television monitor connected to a low light sensitive CCD camera (710M, DVC, USA) (Li et al., 2012). Patch electrodes were made of borosilicate glass with tip electrical resistance value of 3–8 MΩ by using a vertical electrode puller (PIPS, HEKA, Germany). Conventional patch-clamp techniques were used in the present investigation. After the formation of the whole-cell clamp configuration, the neurons were stabilized for 3–5 min before starting pulse protocols and then currents or potentials were recorded as the control group. Afterwards each concentration of MWCNTs was administered to the slices respectively to detect the effect on the evoked action potential and repetitive firing rate in current-clamp mode, and properties of Ik and Ia in voltage-clamp mode at each time point (5, 10, and 15 min). During the recording, signals were low-pass filtered at 3 kHz and digitized at 10 kHz and the series resistance was compensated at least 80%. Only one slice was used for any given experiment.

To record the outward potassium currents, TTX (1 μM) and CdCl2 (0.2 mM) were added to the bath solution to block voltage-gated sodium channels and calcium channels particularly, Ia and Ik were isolated by TEA-Cl (25 mM) and 4-AP (3 mM), which separately acted on the delayed rectifier potassium channels and transient outward potassium channels.

2.4. Data analysis

Data were obtained by using patch-clamp amplifier (HEKA, EPC 10, Germany) and analyzed by Clampfit 9.0, Origin 7.5 and SPSX 11.5. The data were represented as mean ± S.E.M. Statistical comparisons were made using one-way analysis of variance followed by the post hoc multiple tests by Turkey's comparison. The significant level was set at 0.05.

3. Results

3.1. Effects of MWCNTs on the evoked action potential properties

To demonstrate the effect of MWCNTs on the excitability of hippocampal pyramidal neurons, the evoked action potential properties were examined by using whole-cell current-clamp recordings at concentrations of 100 and 400 μg/mL, including single action potential (sAP), evoked by 5 ms brief depolarizing current pulses of 100 pA (Fig. 1A), and the effect of prolonged depolarizing current injection of 50 pA (Fig. 1B). The spike half-width of sAP and the frequency of repetitive firing were measured before and after drug applications. It was found that there were statistical differences of these two indices among different concentrations by one-way ANOVA analysis (spike half-width: F(2,26) = 10.401, P < 0.001; frequency of repetitive firing: F(2,24) = 5.059, P = 0.015). And then Turkey's post hoc multiple tests were further performed. It could be seen that there were significant differences of spike half-width between control and 100 μg/mL of MWCNTs groups (P = 0.003, Fig. 1C), as well as control and 400 μg/mL of MWCNTs groups (P = 0.001, Fig. 1C). Meanwhile, there were statistical differences of repetitive firing frequency between control and 100 μg/mL of MWCNTs groups (P = 0.028, Fig. 1D), as well as control and 400 μg/mL of MWCNTs groups (P = 0.035, Fig. 1D).

3.2. Time-dependent and concentration-dependent effects of MWCNTs on Ia and Ik

Experiments were performed at 5, 10, and 15 min respectively after MWCNTs exposed to the hippocampal CA1 neurons, in order to investigated the time effect of MWCNTs on Ia and Ik (Fig. 2A). The results showed that there were significant differences of Ik and Ik for Ia: F(3,24) = 19.970, P < 0.001; Ik: F(3,23) = 14.857, P < 0.001). When recording Ia, the normalized Ia amplitudes were decreased about 23.18 ± 3.97% (n = 7, P < 0.05), 41.64 ± 6.52% (n = 7, P < 0.001) and 50.50 ± 6.45% (n = 7, P < 0.001), respectively. Meanwhile, the normalized Ik amplitudes were reduced approximately 28.73 ± 6.06% (n = 9, P < 0.01), 45.27 ± 7.45% (n = 9, P < 0.001) and 52.59 ± 8.16% (n = 6, P < 0.001), respectively.

In addition, three kinds of concentrations (50, 100, and 400 μg/mL) were used to examine the effect of MWCNTs on Ia and Ik. It was showed that there were significant differences of Ia and Ik among these three different concentrations (for Ia: F(3,24) = 10.682, P < 0.001; Ik: F(3,20) = 10.726, P < 0.001). Moreover, the amplitudes of Ia were decreased about 28.21 ± 7.24% (n = 8, P < 0.05),
Fig. 1. Effect of MWCNTs of two concentrations on the evoked action potential properties. (A) Single action potentials were elicited using a 5 ms depolarizing current pulses before and after application of 100 µg/ml MWCNT. (B) To examine the effect of 100 µg/ml MWCNT on action potentials firing frequency, a long-term depolarizing current (500 ms, 50 µA) was given to the neurons. (C) Group data of firing frequency between control and MWCNTs with two concentrations of 100 µg/ml and 400 µg/ml. (D) Group data of spike half-width between control and MWCNTs with two concentrations of 100 µg/ml and 400 µg/ml. Data are presented as mean ± S.E.M. *P<0.05, **P<0.01 vs. controls (n ≥ 9).

41.64 ± 6.52% (n = 7, P < 0.001), 42.95 ± 6.74% (n = 6, P < 0.001), respectively. And the amplitudes of IK were reduced approximately 24.37 ± 10.75, 42.90 ± 4.18% (n = 10, P < 0.001), 45.27 ± 7.45% (n = 9, P < 0.001) respectively (Fig. 2B).

3.3. Effects of MWCNTs on I–V relationship of IA and IK

All tested neurons were held at −70 mV and the current traces were evoked by using 80 ms constant depolarizing pulse from −50 to +90 mV in increments of 10 mV during the recording IA (Fig. 3A). And IK was achieved using a 300 ms constant depolarizing pulse by a similar pulse protocol, while the holding potential was held at −50 mV (Fig. 3B). The application of different concentration produced obvious effect on IA and IK amplitudes. With the application of each concentration drugs, the IA and IK currents were reduced inordinately at different command potentials compared with that of control group, which were visible from I–V curves (Fig. 3C and D). It could be clearly seen that peak currents of both IA and IK channels were increased gradually with the incremental command potential, suggesting that these effects were in a voltage-dependent manner.

The statistical significances were found from command potential of 20 mV, 0 mV, and −20 mV for IA, while 80 mV, 10 mV, and 0 mV for IK respectively at 50, 100, 400 µg/ml of MWCNTs compared with that of control group.

3.4. Effects of MWCNTs on the steady-state activation kinetics of IA and IK

The steady-state activation curves of IA and IK under both control condition and after exposure to MWCNT were shown in Fig. 4. They were well fitted with the Boltzmann equation $G/G_{\text{max}} = 1/(1 + \exp[(V_m - V_h)/k])$. It was found that there were significant differences of $V_h$ among groups for both IA ($F_{(3,30)} = 3.945, P = 0.017$) and IK ($F_{(3,25)} = 3.271, P = 0.038$). However, it was only at the concentration of 400 µg/ml that MWCNTs had an effect on the activation curves of both IA and IK with significantly shifting the $V_h$ to the negative potential (n ≥ 7, P < 0.05), compared to that of the control group. There were no significant changes of the slope factor k at each concentration of MWCNTs. The effects of MWCNTs on the activation parameters of IA and IK were summarized in Table 1.
3.6. Effects of MWCNTs on the recovery from inactivation of \( I_A \)

In order to investigate the kinetics of recovery from the inactivated state, pyramidal cells were held at \(-70\) mV, and an 80 ms conditioning depolarizing pulse of +50 mV was fully applied to inactivate the transient outward potassium channels. Then an 80 ms test pulse of +50 mV was applied after a series of \(-80\) mV intervals varying from 10 to 265 ms (Fig. 6A). The peak value of \( I_A \) evoked by the conditioning pulse was designated as \( I_1 \), while the peak value of \( I_A \) evoked by the test pulse was designated as \( I_2 \). The ratio of \( I_2/I_1 \) was presented as the recovery of \( I_A \) from inactivation. The plot of \( I_2/I_1 \) vs. the duration of the \(-80\) mV intervals was well fitted with a monoexponential equation:

\[
\frac{I_2}{I_1} = A + B \exp(-t/\tau),
\]

where \( I_{\text{max}} \) was the maximal current amplitude, \( I \) the current after a recovery period of \( t \), \( A \) the amplitude coefficient, and \( \tau \) the time constant. There was no significant change of \( \tau \) value between control and multiple dose groups (Fig. 6B). Therefore, it was showed that MWCNTs had no effect on the recovery from inactivation of \( I_A \) (Fig. 6B).

4. Discussion

Previous studies reported that carbon influenced the electrophysiological properties of hippocampal neurons, for instance, the intracerebral injection of \( C_{60} \) increased the serotonin turnover rates of hippocampus (Yamada et al., 2008) and graphene substrates promoted neurite sprouting and outgrowth to the maximal extent in a mouse hippocampal culture model (Li et al., 2011). However, how multi-walled carbon nanotube (MWCNT), as an allotrope of carbon, acts on the hippocampal neuron has been still up in the air. A previous study on the nervous system suggested that MWCNTs coated with Pluronic F127 (PF127) surfactant could be injected in the mouse cerebral cortex without causing degeneration of the neurons surrounding the site of injection, and the presence of MWCNTs was able to avoid PF127-induced apoptosis (Bardi et al., 2009). Even so, it was shown that carboxyl-terminated MWCNTs suppressed the densities of delayed rectifier current, inward rectifier current and transient outward current in a time-dependent and irreversible manner in PC12 cells (Xu et al., 2009). In addition, it was reported that C6 rat glioma cell exposed to MWCNT resulted in cell
apoptosis with increasing the level of oxidative stress (Han et al., 2012). Accordingly, it was hypothesized that MWCNT had effect on the electrophysiological properties of hippocampus. In the present study, we found that MWCNT increased the excitability of CA1 pyramidal neurons in hippocampus by reducing the amplitudes of voltage-gated potassium current.

In our previous study, it was showed that MWCNT (average: length 2 μm, diameter: 10–20 nm) inhibited the viability of C6 rat glioma cells in the concentration range of 50–400 μg/ml, while induced oxidative stress if its concentration was higher than 100 μg/ml (Han et al., 2012). Moreover, there were other studies in which the toxicity of MWCNT were investigated and the concentration was within this range (Michaelis et al., 2006; Patolla et al., 2010b; Ye et al., 2009). Hence, MWCNT at concentrations 50, 100 and 400 μg/ml was employed in the present study.

As we know, action potential is a fundamental property of excitable cells in the mammalian CNS, and a dynamic reflection of changes of ion channels in the membrane. The results showed that high dose MWCNTs (100 and 400 μg/ml) increased the firing rate and spike half-width of pyramidal neurons. This suggested that there was alteration of excitability in the hippocampal CA1, which was in accordance with the fact that CNTs modified substrate enhanced the electrical excitability of rat hippocampal neurons (Khraiche et al., 2009). In the nervous system, ion channels in cell membrane play an essential role in the properties of neurons. Much damage on the CNS is caused by interrupting the function of ion channel, which is always the targets for many toxins and drugs (Calabresi et al., 1995; Du et al., 2008; Krnjevic and Leblond, 1989). It is well known that the voltage-gated potassium channels are crucial to regulate neuronal excitability by influencing the action potential (AP), the resting potential (RP) and the firing rate (Yang et al., 2010a). And they also make a difference to the releasing of neurotransmitters (Meir et al., 1999), hormones, and Ca2+-dependent synaptic plasticity (Fili et al., 2001; Muller and Bittner, 2002; Roeper and Pongs, 1996). Thus they are always concerned with the neuronal dysfunction. For instance, the alteration

Fig. 3. Effects of MWCNTs on both $I_h$ and $I_K$. $I_h$ and $I_K$ were obtained by 80 ms and 300 ms depolarizing pulses, respectively, from a command potential of −50 to +90 mV in increments of 10 mV, and the holding potentials were −70 mV and −50 mV, respectively (A and B). Two current–voltage curves showing effects of different concentration on $I_h$ (C, n ≥ 7) and $I_K$ (D, n ≥ 8). Data are presented as mean ± S.E.M.
of Kv subunits (Kv3.4, Kv4.2, Kv2.1 and KChIP2 et al.) of voltage-gated potassium channels in hippocampus was involved in the epilepsy and brain ischemia (Misonou et al., 2008; Monaghan et al., 2008; Pacheco Otalora et al., 2011). Reduction of $I_A$ induced neurons hyperexcitability and finally resulted in epilepsy (Schroder et al., 2000). All existing evidences suggest that MWCNTs are involved in some neurodegenerative disorders associated with alterations of neuronal action potential and voltage-gated potassium channels.

Therefore, a further investigation was performed to excavate the changes of voltage-gated potassium channels in the hippocampal CA1. It was found that MWCNTs suppressed the amplitudes of both $I_A$ and $I_K$ in time-dependent and concentration-dependent manners, which was in accordance with the similar results by Xu et al. (2009), Liu et al. (2009), and Shan et al. (2012). Inhibition of $I_A$ might be the direct reason for the alteration of excitability. In addition, it was only at the concentration of 400 $\mu$g/ml, in which the MWCNTs significantly shifted the activation curves of $I_A$ and $I_K$ to the hyperpolarize potential, as well as the inactivation curves of $I_A$. It suggested that high dose MWCNTs reduced the opening number of voltage-gated potassium channels in the hippocampal CA1 neurons. In addition, $K^+$ is the predominant cation in the cytosol. Maintenance of a high $[K^+]_c$ (140–150 mM) is essential for governing cell excitability, setting resting $E_m$, controlling cell volume and regulating apoptotic enzyme activity (Remillard and Yuan, 2004; Zhao et al., 2009). The intracellular accumulation of $K^+$ could be triggered by decreasing efflux of $I_A$ and $I_K$, resulting in the neuronal hyperexcitability and swell directly. Meanwhile, energy consumption and imbalance between intra and extra-cellular ions might lead to the dysfunction of Na$^+$–K$^+$-ATPase. Based on the fact that cellular energy expenditure did increase linearly with the frequency of action potential (Kadekaro et al., 1985), it might finally cause cellular energy compensation and dysfunction, which were associated with neuronal apoptosis.

![Fig. 4. Effects of MWCNTs on the steady-state activation curves of $I_A$ (A) and $I_K$ (B). Peak amplitudes of $I_A$ and $I_K$ were converted into conductance by using equation $G = I/(V_m - V_I)$, normalized conductance of potassium channels were plotted against the voltages of conditioning pulses, and fitted with Boltzmann function. Each point represents mean ± S.E.M. ($n \geq 7$).](image)

![Fig. 5. Effects of MWCNTs on the steady-state inactivation of $I_A$. (A) Currents were elicited with double pulse protocols before and after application of MWCNTs at 100 $\mu$g/ml ($n \geq 7$). (B) Normalized steady-state inactivation of $I_A$ with application of different concentration of MWCNTs was plotted against the voltages of conditioning pulses, and fitted with Boltzmann function. Data were presented as mean ± S.E.M. ($n \geq 7$).](image)
5. Conclusion

In the present study, it was found that MWCNT decreased the amplitudes of both $I_\text{A}$ and $I_\text{B}$ at time-dependent concentrations, and, accordingly, manipulated the AP events of CA1 pyramidal neurons. The inhibitory effect of MWCNT on hippocampal CA1 neurons probably induced abnormal alteration of neuronal action potential properties, namely, significantly increased both the firing frequency and spike half-width. It might be considered as an early characteristic of some neuronal diseases, possibly serving as a mechanism driving neuronal dysfunction (Liu et al., 2009; Shan et al., 2012). Therefore, the results suggested that MWCNT had neurotoxicity effects on the hippocampal pyramidal CA1 neurons of rats and provided further insight into the underlying mechanisms responsible for the effects of MWCNT on CNS.

Conflict of interest statement

The authors reported no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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References


Liu, Z., Ren, G., Zhang, T., Yang, Z., 2009. Action potential changes associated with the inhibitory effects on voltage-gated sodium current of hippocampal CA1 neurons by silver nanoparticles. Toxicology 264, 179–184.


Yang, J-Y., Tian, Y-T., Yang, Z., Zhang, T., 2010a. Effect of melamine on potassium currents in rat hippocampal CA1 neurons. Toxicology In Vitro 24, 397–403.


