Physiology and Pharmacology

Physiol Pharmacol 19 (2015) 167-176 www.phypha.ir/ppj

Original Article

Carbon nanotubes prolong the regulatory action of nerve growth factor on the endocannabinoid signaling

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Abstract

Introduction: Carbon nanotubes (CNTs) have shown enormous potential in neuroscience. Nerve growth factor (NGF)-CNTs complex promotes the neuronal growth, however, the underlying mechanism(s) have remained elusive. Based on the interplay between NGF and the endocannabinoid system, involvement of the neuroprotective endocannabinoid, 2-arachidonoyl glycerol (2-AG), was investigated in the mechanism of action of NGF.

Materials and Methods: Multi-walled CNTs (MWCNTs)-NGF complex was prepared using amino-functionalized COOH-MWCNTs. MWCNTs were characterized by Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM). *In vitro* cytotoxicity was evaluated by MTT assay. Following three times daily intracerebroventricular injections of NGF solution (2, 5, and 10 µg), and 5, 10, and 20 µg of acid- or amine-modified MWCNTs, or MWCNTs-NGF complex for either one or 7 days, 2-AG contents were quantified in the frontal cortex and hippocampus of rats by isotope-dilution liquid chromatography/mass spectrometry.

Results: FTIR confirmed the amino-functionalization of COOH-MWCNTs and NGF immobilization on the aminated MWCNTs. Aminated MWCNTs and MWCNTs-NGF complex showed less cytotoxicity than COOH-MWCNTs (P<0.05, P<0.01, and P<0.01). Chronic, but not acute, administration of MWCNTs-NGF complex and NGF solution at the highest dose tested led to the elevation of 2-AG at 1 h from the last injection (P<0.01 and P<0.001). 2-AG enhancement induced by MWCNTs-NGF complex lasted for up to 5 and 12 h post-injection (P<0.01 and P<0.001). 2-AG contents remained at the baseline level in the sham and groups receiving vehicle, acid- or amine-modified MWCNTs (P>0.05).

Conclusion: Functionalized MWCNTs-NGF complex induces a long-lasting increase of brain 2-AG content indicating the efficiency of this nanostructure to provide a sustained concentration of NGF. Implication of 2-AG in the mechanism of action of NGF might be of great therapeutic significance in the neurological disorders.

Keywords:
Carbon nanotubes;
Nerve growth factor;
Endocannabinoid signaling

Received:
31 May 2015
Accepted:
22 Oct 2015

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Introduction

Treatment of the neurological disorders has remained as a major challenge in medicine and pharmacological agents or medical devices have shown limited efficacies. In this context, development of novel treatment modalities would be of great clinical significance. Growth factors, the endogenous polypeptides which initiate intracellular signals to regulate the cellular activities such as proliferation, migration and differentiation, have been represented as the leading therapeutic candidates in the neural tissue

engineering (Cottrell, 2006; Lindsay, 1994). Despite the therapeutic significance of growth factors, their short half-lives, slow tissue penetration, or vulnerability to a variety of environmental factors (Lindsay, 1994; Pfister et al., 2007) usually limit their effectiveness. During the last decades, outstanding breakthroughs in the emerging field of nanotechnology have resulted to the development of novel theranostic strategies. In order to provide a better efficacy profile and patient compliance, nanoparticulate delivery systems were designed for the delivery of growth factors, however, several disadvantages prevented the effective delivery (Zhang and Uludağ, 2009) leading to the development of more efficient technologies including the advanced nanovectors for the delivery of compounds with short half-life or poor solubility. In this context, carbon nanotubes (CNTs) with immense potential in various scientific fields including the nanomedicine, have attracted a considerable interest for the protection or targeted delivery of a wide variety of compounds (Foldvari et al., 2008). These nanosystems with high thermoelectrical conductivities, superior mechanical properties, improved biocompatibility and solubility, are becoming increasingly attractive for the application in theranostic settings (Cellot et al., 2009; Kam and Dai, 2005). Besides the application for high-resolution and non-invasive imaging, biosensing, tissue engineering and regenerative medicine (Mohammadi et al., 2009; Fabbro et al., 2012), CNTs may be used as the nanoreserviors for the controlled release of drugs or growth factors (Son et al., 2006; Bhirde et al., 2009) that might be of great therapeutic significance. Moreover, CNTs promote neurite outgrowths and modulate the synaptic plasticity (Cellot et al., 2011). Meanwhile, functionalization of CNTs is necessary to improve their solubility, bioactivity, and biocompatibility (Ya-Ping et al., 2002). In a rat model of ischemic brain injury, functionalized CNTs showed greater protective capacity and less adverse effects. These nanomaterials reduced the neuronal apoptotic markers and post-ischemic inflammation and improved the behavioural functions (Al-Jamal et al., 2011). In the spinal cord injury (SCI), post-treatment with polyethylene glycol-functionalized single-walled CNTs has been shown to reduce the lesion volume, promote the axonal regeneration, and improve the hind-limb locomotor recovery (Roman et al., 2011) indicating their effectiveness against SCI. The therapeutic potential of functionalized CNTs against stroke and glioblastoma have also been reported (Lee et al., 2011; Zhao et al., 2011). The ability of neurotrophin-coated MWCNTs to promote the neurite outgrowth has been previously reported (Matsumoto et al., 2007). The prototypical neurotrophin, nerve growth factor (NGF), which plays a pivotal role in the survival and maintenance of neurons in the peripheral and central nervous systems, is trophic for the cholinergic neurons which are critically involved in the cognitive processes. Furthermore, NGF has shown therapeutic potential in the neurological disorders such as SCI and Alzheimer's disease (Huang et al., 2006; Lad et al., 2003). In this respect, MWCNTs-NGF complex has been designed to promote the neurite outgrowth (Chen et al., 2014; Meng et al., 2013), however, the underlying mechanism(s) have remained elusive. In recent years, the interaction between NGF and the endocannabinoid system has been well-documented (Calatozzolo et al., 2007; Hassanzadeh and Rahimpour, 2011; Hassanzadeh and Hassanzadeh, 2011; Hassanzadeh and Hassanzadeh, 2012; Hassanzadeh and Hassanzadeh, 2013). For instance, endocannabinoids have been shown to activate transient receptor potential vanilloid subfamily type 1 (TRPV1) channels (Zhang et al., 2005) and NGF modulates the functions of TRPV1 channel and endocannabinoid signaling (Keimpema et al., 2014).

Contribution of endocannabinoid signaling to the formation of neuronal networks and neuroprotective processes has been the focus of intense research. This ubiquitous signaling system is implicated in the survival signaling pathways and plays a pivotal role against the neuronal insult and excitotoxic damage. The endogenous or exogenous cannabinoids have shown neuroprotective effects in a variety of *in vivo* and *in vitro* models of neuronal injury (Hassanzadeh, 2014; van der Stelt and Di Marzo, 2005). The therapeutic potential of the endocannabinoid system in neurological diseases has also been well documented (van der Stelt and Di Marzo, 2003; Centonze et al., 2007). The endocannabinoid, 2-arachidonylglycerol (2-AG), due to its involvement in the neuroprotective processes, has attracted a considerable interest. Indeed, the endocannabinoid-induced axonal growth and guidance

majorly depend on the integrity of 2-AG signaling networks (Panikashvili et al., 2001; Keimpema et al., 2010). Based on this background, we aimed to investigate; i) the suitability of functionalized MWCNTs-NGF complex for providing longer-lasting effect of NGF, ii) involvement of 2-AG in the central mechanism of action of NGF.

Materials and methods

Preparation of MWCNTs-NGF complex

Amino functionalization of CNTs improve their dispersibility and reduce the toxicity (Lee et al., 2011; Chen et al., 2014). Meanwhile, we used COOH-MWCNTs instead of the direct aminization of MWCNTs as the carboxylation of CNTs before aminization has been shown to enhance the reactivity of CNTs and improve further aminization (Hamdi et al., 2015). Amine-modified MWCNTs-NGF complexes were prepared as previously described in detail (Freitas et al., 2014; Hamdi et al., 2015; Matsumoto et al., 2010) with some modifications. In brief, 500 mg of COOH-functionalized MWCNTs (Plasmachem GmbH, Berlin, Germany) and 50 ml of 98% $SOCl_2$ (Sigma Aldrich, Germany) were sonicated using ultrasonic system (Tecna 6, Tecno-Gaz, Italy) at 70% amplitude for 40 min and stirred using a magnetic stirrer (IKA, Germany) at 25 °C for 48 h. The suspension was filtered with 0.45 µm pore-sized microporous membrane (Sartorius, Germany), washed with tetrahydrofuran for 5 times to remove the excess $SOCl_2$, and vacuumed for 25 min at 25 °C. The residue was reacted with 50 ml of ethylenediamine (EDA) (Sigma Aldrich, Germany) and stirred for 10 h. Afterwards, the suspension was filtered, washed with tetrahydrofuran for 5 times, vacuumed for 25 min, dialyzed in the deionized distilled water using a dialysis bag (MW cut-off 14 KD) for 72 h, and vacuumed to obtain the aminated MWCNTs. In order to prepare amine-modified MWCNTs-NGF complex, rat nerve growth factor (Sigma Aldrich, Germany) in phosphate-buffered saline (PBS) (30 µg/ml) was added to the mixture of aminated MWCNTs and PBS (0.25% w/v), stirred for 24 h at 25 °C, and centrifuged using the sigma-3k30 centrifuge (Sigma, Germany) at 10,000 rpm for 15 min. Following the supernatant removal, the sample was washed with PBS, re-centrifuged (10,000 rpm for 15 min), and dispersed in 10 ml of PBS.

Characterization of the MWCNTs

Fourier transform infrared (FTIR) spectroscopy, a powerful tool for the comprehensive characterization of the chemical structures of MWCNTs, was performed using the FTIR spectrophotometer (Shimadzu, Japan). The surface morphology and dispersion of MWCNTs were evaluated by the *scanning electron microscopy (SEM*, KYKY-EM3200, KYKY Technology Development Ltd., Beijing, China).

Cytotoxicity of MWCNTs

Cell viability was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma Aldrich, Germany) colorimetric assay (Mosmann, 1983). Briefly, rat pheochromocytoma PC_{12} cells (Pasteur Institute, Tehran, Iran) in the phase of exponential growth were seeded in 96-well plates (Nunc, Denmark) at a density of 10^4 cells/well and incubated in a 5% CO_2 incubator for 24 h at 37°C. The viability of cells exposed to the serial dilutions of MWCNTs in PBS (10, 20, 30, 50, 100, and 200 µg/ml) was evaluated at 1st, 3rd, and 7th day of incubation in 5% CO_2 incubator at 37°C. In this respect, MTT was dissolved in PBS to provide a stock solution (5 mg/ml) and 20 µl of it was added to each well and incubated for 4 h in a 5% CO_2 incubator at 37 °C. Afterwards, the culture medium was carefully aspirated and 100 µl of dimethyl sulfoxide was added to each well to dissolve the formazan crystals. In order to completely dissolve the crystals, the plate was subjected to low-speed oscillation for 10 min and the absorbance (Abs) was measured at 570 nm using a microplate reader (Anthos 2020, Anthos Labtec Instruments, Austria). Other groups including a blank experimental group (MWCNTs without PC_{12} cells), a control group (non-exposed cells), and a blank control group (culture medium) were also considered for the calculation of cell viability as Formula 1.

The results were reported as the mean±SE of six independent experiments (n=6).

Formula 1.

$$\text{Cell viability (\%)} = \frac{\text{Abs of treated group - Abs of blank experimental group}}{\text{Abs of control group - Abs of blank control group}} \times 100$$

Animals

Male Wistar rats weighing 250-280 g (Pasteur Institute, Tehran, Iran) were housed in pairs under the standard laboratory conditions (temperature: 22 ± 2 °C, humidity: 55–65%) on a 12-h light/dark cycle and food pellets and water were available *ad libitum*. The experiments began after 1 week of habituation to the housing conditions and the procedures were carried out in accordance with the National Institutes of Health guidelines for the humane use of laboratory animals and approved by the local Ethics Committee.

Surgical procedures and treatments

In order to prepare the randomly assigned animals for intracerebroventricular (i.c.v.) injection, rats were deeply anaesthetized by the intraperitoneal injections of 2.5 mg/kg of acepromazine (Sigma Aldrich, Germany), 10 mg/kg of xylazine (Sigma Aldrich, Germany), and 80 mg/kg of ketamine (Sigma Aldrich, Germany) at 10-min intervals. Each anaesthetized animal was mounted on a stereotaxic frame (Stoelting, Wood Lane, IL, USA) with lambda and bregma in the same horizontal plane. The shaved scalp was swabbed with 70% ethanol and 0.25% bupivacaine (Sigma Aldrich, Germany) and a midline incision was made on the skull skin. Following the removal of tissue debris, a burr hole was drilled through the skull and a 4-cm cannula (PE-20 tubing, BD Intramedic, Clay Adams, Parsippany, NJ) which had been placed in 70% ethanol overnight, was inserted through the hole according to the stereotaxic coordinates for the cannulation into the right lateral ventricle as follows; 1.1 mm posterior to the bregma, 1.7 mm lateral to the midline, and 4.0 mm below the skull surface (Paxinos and Watson, 2007). A stylet was inserted into the guide cannula to keep it open and the cannula was fixed onto the skull by three sterile stainless steel screws and the acrylic dental cement was applied over the screws and cannula. The scalp incision was closed with 3-0 silk sutures (Mersilk, Ethicon, UK) and treated with local antiseptic. Following the removal from the stereotaxic unit, animals were oxygenated and heated using a blanket containing a thermostat to ensure 37 °C rectal temperature. After the recovery from anesthesia, an additional 5-day recovery period was considered for the individually housed animals under the postoperative care including the subcutaneous injections of 50 µg/kg of buprenorphine (Sigma Aldrich, Germany) and 2 ml of 0.9% saline solution to reduce pain and dehydration, respectively. Animals exhibiting postsurgical motor deficit were excluded from the study. In order to verify the correct insertion of cannula, 3 randomly chosen animals in each group received 10 µl i.c.v. injection of methylene blue and were decapitated following the pentobarbital overdose. The brains were removed, kept in 10% formaldehyde solution for 24 h, and then sliced into 1-mm thick sections. The site of the cannula tip and the ventricular distribution of methylene blue were evaluated.

Upon the injections, the stylet was removed from the cannula and animals received three times a day injection of 2, 5, and 10 µg of NGF (Sigma Aldrich, Germany) (Cho and Borgens, 2013, Hassanzadeh and Hassanzadeh, 2010) or 5, 10, and 20 µg of COOH-MWCNTs, aminated MWCNTs, or EDA-MWCNTs-NGF complex (all dissolved in PBS) for 1 or 7 consecutive days (Matsumoto et al., 2007, Chen et al., 2014, Meng et al., 2013) using a 10 µl Hamilton syringe (Hamilton Company, Reno, NV) followed by 10 µl PBS to flush the cannula (n=12/group). Injections were performed during 1 min and the volume of injection was 10 µl. Animals in sham group were subjected only to the cannulation and the control group received an equal volume of the vehicle (n=12/group).

Endocannabinoid extraction and analysis

1, 5, and 12 hr after the last injection (Hassanzadeh and Rahimpour, 2011; Hassanzadeh and Hassanzadeh, 2011; Hassanzadeh and Hassanzadeh, 2012; Hassanzadeh and Hassanzadeh, 2013), rats were decapitated without anaesthesia and the brain of each animal was quickly and carefully removed from the skull. Brain regions including the frontal cortex and hippocampus were dissected on a frozen pad taken from a -80 °C freezer using the atlas for morphological orientation (Paxinos and Watson, 2007) and were immediately frozen at -80 °C until the lipid extraction and analysis of 2-AG. Upon the extraction, glycerol was used to prepare the deuterated standards. Each frozen brain region was homogenized in 50 mM chloroform/methanol/Tris-HCl (2:1:1) containing 1 nmol of d8-2-AG. Then, the homogenates were centrifuged at 13000 ×g and 4 °C for 15 min. The aqueous phase

and debris were re-extracted with 1 vol of chloroform. The organic solvents were evaporated using a rotating evaporator and the samples were lyophilized and stored at -80 °C. Upon 2-AG analysis, the lyophilized extracts were re-suspended in chloroform/methanol (9:1) and the solutions were purified by open bed chromatography on silica, fraction containing 2-AG was collected, and the excess solvent was evaporated. Normal phase high-pressure liquid chromatography using a semipreparative silica column (Spherisorb S5W, Phase Sep, Queensferry, CLWYD, UK) and gas chromatography/mass spectrometry were carried out for the separation of 1(3)-, and 2-acyl-glycerols and monoacylglycerols. 2-AG content (nanomole per gram of wet tissue extracted) was calculated based on its area ratio considering the internal deuterated standard signal areas that has been previously described in detail (Bisogno et al., 1997; Devane et al., 1992; Patel et al., 2003). The measurements were performed in duplicate and analyzed by an investigator blind to the experimental set-up.

Statistical analysis

The Kolmogorov-Smirnov test was used to verify the normal distribution of the experimental data. Three-way ANOVA followed by Tukey's post hoc test was used for the analysis of brain regional levels of 2-AG and cytotoxicity data. Data are presented as mean ± SEM. The level of significance was set at $P<0.05$.

Results

Characterization of MWCNTs:

Regarding the acid-functionalized MWCNTs, FTIR spectroscopy demonstrated the carboxyl groups at 3286 and 1733 cm^{-1} and C-H stretching at 2914 and 2893 cm^{-1} (Fig. 1, curve A). The peaks at 1629 and 1218 cm^{-1} corresponded to C=O and C-O stretching, respectively (Fig. 1, curve A). Following EDA treatment, two peaks at 3378 and 3193 cm^{-1} were observed which corresponded to N-H stretching (Fig. 1, curve B). The peaks at 2947 and 2826 cm^{-1} attributed to C-H stretching and peaks at 1729 and 1618 cm^{-1} corresponded to C=O stretching because of the formation of amide linkage (Fig. 1, curve B). The peak at 1173 cm^{-1} attributed to C-N stretching of amide group (Fig. 1, curve B). NGF conjugation on amine-modified MWCNTs has been demonstrated in the curve C (Fig. 1) with a characteristic peak at 1621 cm^{-1}. Other peaks were also observed in the curve C including those at 3413 and 3177 cm^{-1} (N-H stretching), 2969 and 2832 cm^{-1} (C-H stretching), 1768 and 1643 cm^{-1} (C=O stretching), and 1192 cm^{-1} (C-N stretching).

The Morphology of MWCNTs

SEM showed a well-dispersed EDA-MWCNTs-NGF complex (Fig. 2C) as compared to acid- or amine-modified MWCNTs (Figs 2A and 2B, respectively).

Cytotoxicity of MWCNTs

The most cytotoxic effects were observed following the application of increasing concentrations of COOH-MWCNTs (Fig. 3, $P<0.01$, and $P<0.001$ *vs*. control). Aminated MWCNTs and MWCNTs-NGF complex showed less cytotoxicity than COOH-MWCNTs (Fig. 3, $P<0.05$, $P<0.01$, and $P<0.001$). Furthermore, MWCNTs-NGF complex exhibited less toxicity than EDA-MWCNTs (Fig. 3, $P<0.05$, $P<0.01$, and $P<0.001$). Regarding the incubation time, the toxicity of COOH-MWCNTs was significantly increased on 3rd day of incubation (Fig. 3, $P<0.01$), however, the cell viability on 7rd day of incubation did not significantly differ from that of day 1 (Fig. 3, $P>0.05$). The toxicity of aminated MWCNTs or MWCNTs-NGF complex was not affected by the incubation time (Fig. 3, $P>0.05$).

The effects of the various types of MWCNTs and NGF solution on the brain 2-AG contents

2-AG contents in the sham and groups receiving vehicle, acid- or amine-modified MWCNTs (Table 2) remained at the baseline level (Table 1, $P>0.05$). Acute treatment with MWCNTs-NGF complex or NGF solution at any dose tested did not significantly alter brain 2-AG contents (data not shown). Chronic administration of MWCNTs-NGF complex (20 µg/rat) or NGF solution (10 µg/rat) led to the significant elevation of 2-AG contents in the frontal cortex and hippocampus at 1 h from the last injection (Table 2, $P<0.01$ and $P<0.001$). 2-AG enhancement lasted for up to 5 and 12 h post-injection using MWCNTs-NGF complex (Table 2, $P<0.01$ and $P<0.001$), while, NGF solution failed to induce 2-AG enhancement at these time points (Table 2, $P>0.05$ as compared to the sham or groups receiving

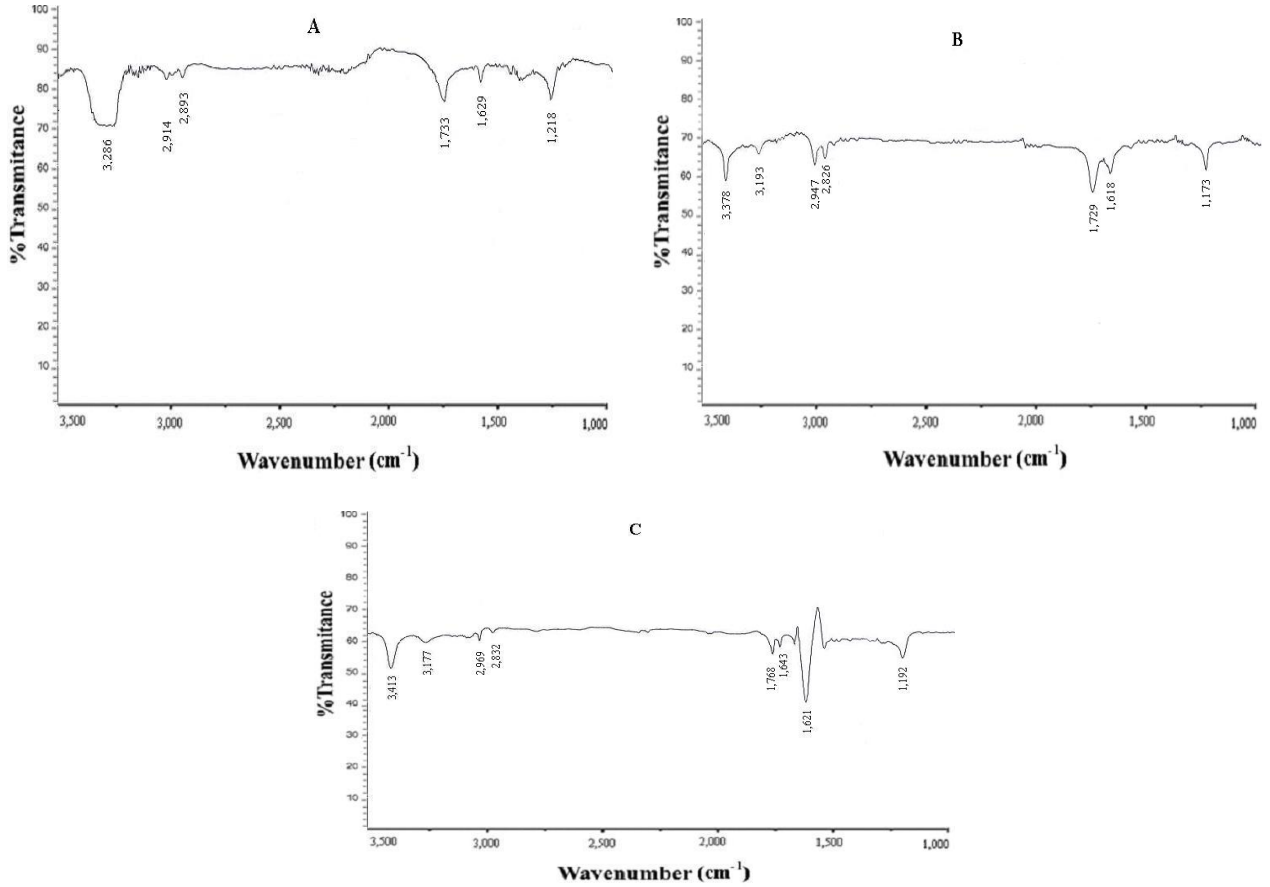

Fig.1. FTIR spectra of A: COOH-MWCNTs, B: EDA-MWCNTs, and C: EDA-MWCNTs-NGF complex. (MWCNTs: multi-walled carbon nanotubes, EDA: ethylenediamine, NGF: nerve growth factor)

Table 1: Brain regional contents of 2-AG at baseline

Brain region	2-AG (nmol/ g tissue)
Frontal cortex Hippocampus	5.17± 0.37 8.62± 0.64

Data are expressed as mean ± SEM of n=7/group. (2-AG: 2-Arachidonoyl glycerol)

Table 2: The effects of chronic exposure to the various types of MWCNTs (20 µg/rat) and NGF (10 µg/rat) on 2-AG contents in rat brain regions

2-AG (nmol/ g tissue)	Brain regions	Sham	Vehicle	COOH-MWCNTs	EDA-MWCNTs	EDA-MWCNT-NGF complex	NGF solution
A	Frontal cortex	5.23± 0.47	4.82± 0.53	5.62± 0.48	4.79± 0.52	13.85± 1.03***	14.06 ± 1.16***
	Hippocampus	7.94± 0.63	8.11± 0.66	8.12± 0.94	8.28± 0.71	18.32± 1.79**	17.83± 1.21***
B	Frontal cortex	5.41± 0.38	4.69± 0.45	4.48± 0.52	5.76 ± 0.63	11.74± 0.97***	6.17 ± 0.72
	Hippocampus	8.27± 0.51	8.41± 0.72	9.08± 0.93	7.94± 0.61	15.25± 0.73**	9.36 ± 0.85
C	Frontal cortex	4.93± 0.44	5.33± 0.65	4.49± 0.35	5.13± 0.62	8.86± 0.51**	5.41 ± 0.58
	Hippocampus	8.76± 0.83	7.88± 0.92	7.83± 0.69	8.48± 0.94	14.45± 0.92**	7.69 ± 0.83

Data were obtained at 1 h (A), 5 h (B), or 12 h (C) after the last injection in 7-day three times daily administration of COOH-MWCNTs, EDA-MWCNTs, EDA-MWCNTs-NGF complex, NGF solution, or the vehicle. Data are presented as mean ± SEM (n=7/group).
** $P<0.01$ and *** $P<0.001$ vs. the sham and groups receiving vehicle, acid- or amine-modified MWCNTs.

vehicle, acid- or amine-modified MWCNTs). Chronic treatment with MWCNTs-NGF complex or NGF solution at lower doses did not significantly alter 2-AG levels (data not shown).

Discussion

NGF, a protein which plays a critical role in the neuronal growth and differentiation, protects neurons

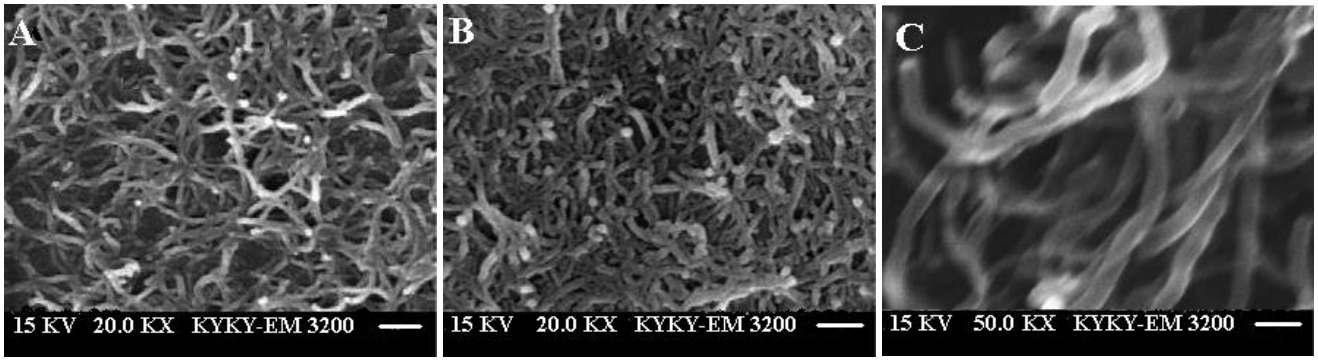

Fig 2. SEM images of MWCNTs. A: COOH-MWCNTs, B: EDA-MWCNTs, and C: EDA-MWCNTs-NGF complex (scale bares: 50 and 100 nm, respectively).

against the injuries. This neurotrophin regulates the synaptic connectivity of the cholinergic neurons which are involved in the learning and memory (Hefti and Will, 1987; Pfister et al., 2007). Furthermore, NGF prevents apoptosis and exhibits therapeutic potential in the neurodegenerative disorders (Lad et al., 2003; Lindsay, 1994). Since NGF is not sufficiently produced in the injured tissues (Boyd and Gordon, 2003; Lad et al., 2003), therefore, exposure to the exogenous NGF is required for the neuroregeneration and functional recovery. However, rapid inactivation in the aqueous solutions as well as the vulnerability to a variety of factors including pH and temperature, negatively affect the efficiency of NGF (Hassanzadeh and Hassanzadeh, 2010; Huang et al., 2006; Lad et al., 2003; Pfister et al., 2007). In this respect, application of the appropriate NGF carrier is required for providing prolonged NGF activity. In recent years, functionalized CNTs have been used as the carriers for controlled-release of drugs or macromolecular proteins including the growth factors (Bhirde et al., 2009; Cho and Borgens, 2013; Son et al., 2006). Amino-functionalized MWCNTs with low toxicity, high reactivity and solubility, are able to improve the dispersibility of CNTs and promote their biocompatibility with neurons leading to the neuronal growth (Lee et al., 2011). In PC_{12} cells and dorsal root ganglion, aminated MWCNTs have been shown to promote cell differentiation (Yen et al., 2011). Based on the favourable characteristics of amino-functionalized MWCNTs, we have investigated their suitability as the carriers of NGF for further evaluation of the implication of the endocannabnoid system with well-characterized regulatory effects in the neuroprotective processes (Hassanzadeh and Rahimpour, 2011; Hassanzadeh and Hassanzadeh, 2011; Hassanzadeh and Hassanzadeh, 2012; Hassanzadeh and Hassanzadeh, 2013; Calatozzolo et al., 2007; van der Stelt and Di Marzo, 2003; Centonze et al., 2007) in the mechanism of action of NGF. Following the confirmation of amino-functionalization of COOH-MWCNTs by FTIR (the presence of N-H and C-N bands; Fig. 1, curve B) and successful NGF grafting on the amine-modified MWCNTs (the presence of NGF peak; Fig. 1, curve C), we found that carboxylated MWCNTs induce toxicity towards the PC_{12} cells in concentration- and time-dependent manner (Fig. 3). EDA-MWCNTs and NGF-MWCNTs complex showed less cytotoxicity than COOH-MWCNTs (Fig. 3) suggesting that amine modification as well as addition of NGF enhance the cell viability and improve the biocompatibility of MWCNTs. This is in accordance with previous reports indicating the neuroprotective effects of amine-modified CNTs or neurotrophin-coated CNTs (Lee et al., 2011; Matsumoto et al., 2010; Matsumoto et al., 2007; Meng et al., 2013). Despite the increased toxicity of COOH-MWCNTs on 3rd day of incubation, their cytotoxicity on day 7 did not significantly differ from that of day 1 that may be due to the cell recovery. Chronic exposure to the highest dose of MWCNTs-NGF complex led to a significant and durable elevation of 2-AG contents in the frontal cortex and hippocampus, the brain regions which are critically involved in the neuroptotective processes (Cottrell, 2006; Keimpema et al., 2010; Panikashvili et al., 2001; Patel et al., 2003). Following the repeated injections of NGF solution, 2-AG enhancement was observed only at 1 h after the final injection and did not lasted up to 5 or 12 h (Table 2) that might be due to the short half-life and/or rapid degradation of free NGF. As aforementioned, slow tissue penetration, short half-

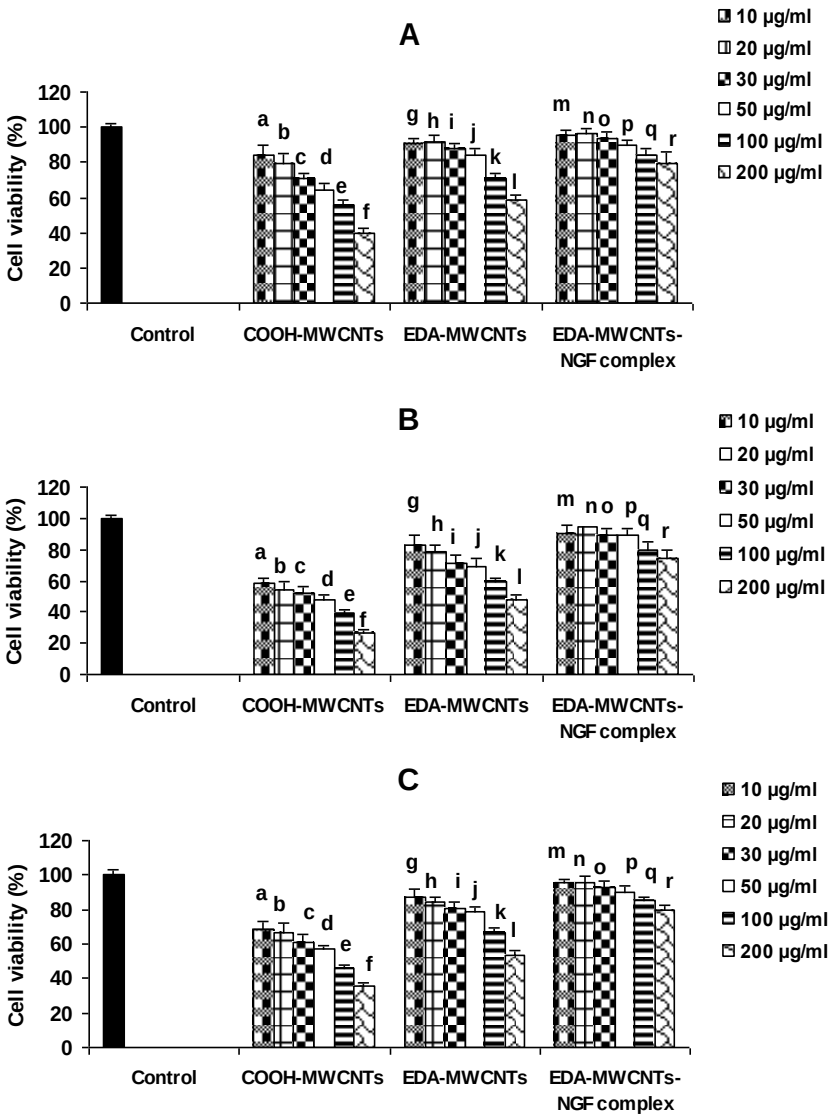

Fig. 3. Cytotoxicity assay at 1st (A), 3rd (B), and 7th (C) day of PC_{12} cells exposure to the different concentrations of MWCNTs. EDA-MWCNTs and EDA-MWCNTs-NGF complex have shown less cytotoxicity than COOH-MWCNTs. Data are presented as mean ± SEM (n=6).

(A): b, c, d, e, and f: $^{**}P$<0.01, $^{***}P$<0.001, $^{***}P$<0.001, $^{***}P$<0.001 and $^{***}P$<0.001 vs. control, respectively; j, k, and l: $^{*}P$<0.05, $^{*}P$<0.05, and $^{***}P$<0.001 vs. control, respectively; b vs. n: $^{*}P$<0.05; c vs. i: $^{**}P$<0.01; c vs. o: $^{***}P$<0.001; d vs j and p: $^{**}P$<0.01 and $^{***}P$<0.001; e vs. k and q: $^{*}P$<0.05 and $^{***}P$<0.001; f vs. l and r: $^{***}P$<0.001; r vs. control and l: $^{***}P$<0.001 and $^{*}P$<0.05.
{a vs. e and f: $^{**}P$<0.01 and $^{***}P$<0.001; b vs. e and f: $^{*}P$<0.05 and $^{***}P$<0.001; c vs. f: $^{***}P$<0.001; d vs. f: $^{**}P$<0.01; g and h vs. l: $^{***}P$<0.001; i and j vs. l: $^{**}P$<0.01}.

(B): a, b, c, d, e, and f: $^{***}P$<0.001 vs. control; j, k, and l: $^{**}P$<0.01, $^{***}P$<0.001, and $^{***}P$<0.001 vs. control, respectively; q and r vs. control: $^{*}P$<0.05 and $^{***}P$<0.001, a vs. g and m: $^{**}P$<0.01 and $^{***}P$<0.001, b vs. h and n: $^{**}P$<0.01 and $^{***}P$<0.001, c vs. i and o: $^{*}P$<0.05 and $^{***}P$<0.001, d vs. j and p: $^{**}P$<0.01 and $^{***}P$<0.001, e vs. k and q: $^{**}P$<0.01 and $^{***}P$<0.001, f vs. l and r: $^{***}P$<0.001; r vs. control and l: $^{***}P$<0.001 and $^{***}P$<0.001.
{a vs. e and f: $^{*}P$<0.05 and $^{***}P$<0.001; b, c, and d vs. f: $^{***}P$<0.001, $^{**}P$<0.01, and $^{*}P$<0.05 , g vs. k and l: $^{*}P$<0.05 and $^{***}P$<0.001; h vs. l: $^{**}P$<0.01, i and j vs. l: $^{*}P$<0.05}.

(C): a, b, c, d, e, and f: $^{***}P$<0.001 vs. control; g, h, i, j, k, and l: $^{*}P$<0.0, $^{*}P$<0.05, $^{*}P$<0.05, $^{***}P$<0.001, $^{***}P$<0.001, and $^{***}P$<0.001 vs. control, respectively; q and r vs. control: $^{**}P$<0.01 and $^{***}P$<0.001; q and r vs. k and l: $^{***}P$<0.001; a vs. g and m: $^{***}P$<0.001; b vs. h and n: $^{*}P$<0.05 and $^{***}P$<0.001; c vs. i. and o: $^{**}P$<0.01 and $^{***}P$<0.001; d vs. j and p: $^{***}P$<0.001; e vs. k and q: $^{***}P$<0.001; f vs. l and r: $^{**}P$<0.01 and $^{***}P$<0.001.
{a vs. e and f: $^{**}P$<0.01 and $^{***}P$<0.001; b vs. e and f: $^{**}P$<0.01 and $^{***}P$<0.001, c and d vs. f: $^{**}P$<0.01 and $^{*}P$<0.05; m and n vs. r: $^{*}P$<0.05}.
[a_{day1} vs. a_{day3}: $^{**}P$<0.01, a_{day1} vs. a_{day7}: P>0.05, b_{day1} vs. b_{day3} : $^{*}P$<0.05, b_{day1} vs. b_{day7}: P>0.05, c_{day1} vs. c_{day3} : $^{*}P$<0.05, c_{day1} vs. c_{day7}: P>0.05, d_{day1} vs. d_{day3} : $^{*}P$<0.05, d_{day1} vs. d_{day7}: P>0.05, e_{day1} vs. e_{day3} : $^{**}P$<0.01, e_{day1} vs. e_{day7}: P>0.05, f_{day1} vs. f_{day3} : $^{**}P$<0.01, f_{day1} vs. f_{day7}: P>0.05; g, h, i, j, k, and l_{day1} vs. those of days 3 and 7: P>0.05; m, n, o, p, q, and r_{day1} vs. those of days 3 and 7: P>0.05].

lives, or vulnerability of growth factors to the environmental factors may negatively affect their therapeutic effects (Lindsay, 1994; Pfister et al., 2007). Prolonged enhancement of 2-AG due to the application of MWCNTs-NGF complex demonstrates the ability of this nanostructure to provide a sustained concentration of NGF in the brain. Furthermore, our findings indicate the implication of 2-AG, at least in part, in the mechanism of action of NGF. In fact, among the mechanism(s) through which NGF may exert its neuroprotective effects, the implication of endocannabinoid system appears of critical importance because of its pivotal modulatory role in the neuroprotective processes (Hassanzadeh, 2014; van der Stelt and Di Marzo, 2005). Regarding the regulatory action of NGF on endocannabinoid signaling (Keimpema et al., 2014), the major putative pathways for cellular 2-AG synthesis including; i) the conversion of diacylglycerol (DAG) to 2-AG by DAG lipase, and ii) hydrolysis of phosphatidic acid (van der Stelt and Di Marzo, 2005) or the activity of degrading enzymes of endocannabinoids may be modulated by NGF in order to stimulate 2-AG production. Meanwhile, involvement of other signaling pathway(s) merits further investigation. Altogether, based on the neuroprotective activities of both NGF and endocannabinoid signaling systems (Lindsay, 1994; Pfister et al., 2007; Huang et al., 2006; Lad et al., 2003; Calatozzolo et al., 2007; van der Stelt et al., 2003; Centonze et al., 2007), the regulatory effect of MWCNTs-NGF complex on 2-AG signaling might be of great therapeutic significance in the neurological disorders. Indeed, the coordinated action of endocannabinoid and NGF by inducing the survival signaling may result in dramatic advances in the regenerative medicine. As aforementioned, 2-AG is an endocannabinoid with well-documented neuroptotective effects (Panikashvili et al., 2001; Keimpema et al., 2010; van der Stelt and Di Marzo, 2003). Following the brain injury in mice, 2-AG through the suppression of reactive oxygen species (ROS) and tumor necrosis factor-α (TNF-α) formation has shown neuroprotective effects including the reduction of hippocampal cell death, infarct volume, brain edema, and excitotoxicity leading to the improved functional recovery (Gallily et al., 2000). Furthermore, 2-AG has protected rat cerebral neurons against the ischemia, glucose deprivation, and hypoxia (Sinor et al., 2000). Based on the regulatory effects of 2-AG on the synaptic plasticity (Stella et al., 1997; Yoshida et al., 2006), the ability of CNT scaffolds to re-establish the synaptic contacts and modulate the synaptic plasticity (Cellot et al., 2011) may be mediated, at least in part, by this valuable endocannabinoid.

In conclusion, amine-modified MWCNTs may be used as the promising carriers of NGF which provide a sustained concentration of this neurotrophin leading to the longer-lasting effects that might be of great therapeutic significance in the neurological disorders. Furthermore, stimulation of 2-AG production by MWCNTs-NGF complex indicate the implication of 2-AG, at least in part, in the central mechanism of action of NGF.

Acknowledgments

This work was funded by a grant from Tehran University of Medical Sciences. Authors wish to thank Dr. Mehdi Khoobi, Department of Medicinal Chemistry, Tehran University of Medical Sciences, for fruitful discussion.

Conflict of interest

None of the authors has any conflict of interest to disclose.

References

Al-Jamal KT, Gherardini L, Bardi G, Nunes A, Guo C, Bussy C, et al. Functional motor recovery from brain ischemic insult by carbon nanotube-mediated siRNA silencing. Proc Natl Acad Sci USA 2011; 108: 10952–97.

Bhirde AA, Patel V, Gavard J, Zhang GF, Sousa AA, Masedunskas A, et al. Targeted killing of cancer cells in vivo and in vitro with EGF-directed carbon nanotube-based drug delivery. Acs Nano 2009; 3: 307–16.

Bisogno T, Sepe N, Melck D, Maurelli S, De Petrocellis L, Di Marzo V. Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoyl-glycerol in mouse neuroblastoma cells. Biochem J 1997; 322: 671–77.

Boyd JG, Gordon T. Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. Mol Neurobiol 2003; 27: 277–324.

Calatozzolo C, Salmaggi A, Pollo B, Sciacca FL, Lorenzetti M, Franzini A, Boiardi A, Broggi G, Marras C. Expression of cannabinoid receptors and neurotrophins in human gliomas. Neurol Sci 2007; 28: 304–10.

Cellot G, Toma FM, Varley ZK, Laishram J, Villari A, Quintana M, et al. Carbon nanotube scaffolds tune synaptic strength in cultured neural circuits: novel frontiers in nanomaterial-tissue interactions. J

Neurosci 2011; 31: 12945–53.
Centonze D, Rossi S, Finazzi-Agrò A, Bernardi G, Maccarrone M. The endocannabinoid system in multiple sclerosis and amyotrophic lateral sclerosis. Int Rev Neurobiol 2007; 82: 171-86.
Chen W, Xiong Q, Ren Q, Guo Y, Li G. Can amino-functionalized carbon nanotubes carry functional nerve growth factor? Neural Reg Res 2014; 9: 285-92.
Cottrell GW. New life for neural networks. Science 2006; 313: 454–55.
Cho Y, Borgens RB. Electrically controlled release of the nerve growth factor from a collagen–carbon nanotube composite for supporting neuronal growth. J Mater Chem 2013; 1: 4166–4170.
Devane WA, Hanus L, Bruer A, Pertwee RG, Stevenson LA, Griffin G, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 1992; 258: 1946–49.
Fabbro A, Bosi S, Ballerini L, Prato M. Carbon nanotubes: artificial nanomaterials to engineer single neurons and neuronal networks. ACS Chem Neurosci 2012; 3: 611−18.
Freitas TA, Mattos AB, Silva BVM, Dutra RF. Amino-functionalization of carbon nanotubes by using a factorial design: human cardiac troponin T immunosensing application. BioMed Res Int 2014 (2014) 929786 (9pp).
Gallily R, Breuer A, Mechoulam R. 2-Arachidonylglycerol, an endogenous cannabinoid, inhibits tumor necrosis factor α production in murine macrophages, and in mice. Eur J Pharmacol 2000; 406: 5-7.
Hamdi H, De La Torre-Roche R, Hawthorne J, White JC. Impact of non-functionalized and amino-functionalized multiwall carbon nanotubes on pesticide uptake by lettuce (Lactuca sativa L.). Nanotoxicology 2015; 9: 172-180.
Hassanzadeh P, Rahimpour S. The cannabinergic system is implicated in the upregulation of central NGF protein by psychotropic drugs. Psychopharmacology 2011; 215: 129–41.
Hassanzadeh P, Hassanzadeh A. Involvement of the neurotrophin and cannabinoid systems in the mechanisms of action of neurokinin receptor antagonists. Eur Neuropsychopharmcol 2011; 21: 905–17.
Hassanzadeh P, Hassanzadeh A. Implication of NGF and endocannabinoid signaling in the mechanism of action of sesamol: a multi-target natural compound with therapeutic potential. Psychopharmacology 2013; 229: 571–78.
Hassanzadeh P, Hassanzadeh A. The CB_1 Receptor-Mediated Endocannabinoid Signaling and NGF: The Novel Targets of Curcumin. Neurochem Res 2012; 37: 1112-20.
Hassanzadeh P, Hassanzadeh A. Effects of different psychotropic agents on the central nerve growth factor protein. Iran J Basic Med Sci 2010; 13: 202-9.
Hefti F, Will B. Nerve growth factor is a neurotrophic factor for forebrain cholinergic neurons; implications for Alzheimer's disease. J Neural Transm Suppl 1987; 24: 309–15.
Huang F, Wang J, Chen A. Effects of co-grafts mesenchymal stem cells and nerve growth factor suspension in the repair of spinal cord injury. J Huazhong Univ Sci Technolog Med Sci 2006; 26: 206-10.
Keimpema E, Barabas K, Morozov YM, Tortoriello G, Torii M, Cameron G, et al. Differential subcellular recruitment of monoacylglycerol lipase generates spatial specificity of 2-arachidonoyl glycerol signaling during axonal path finding. J Neurosci 2010; 30: 13992–14007.
Lad SP, Neet KE, Mufson EJ. Nerve growth factor: structure, function and therapeutic implications for Alzheimer's disease. Curr Drug Targets CNS Neurol Disord 2003; 2: 315–34.
Lee HJ, Park J, Yoon OJ, Kim HW, Lee do Y, Kim do H, et al. Amine-modified single-walled carbon nanotubes protect neurons from injury in a rat stroke model. Nat Nanotechnol 2011; 6: 121–5.
Lindsay RM. Neurotrophic growth factors and neurodegenerative diseases: therapeutic potential of the neurotrophins and ciliary neurotrophic factor. Neurobiol Aging 1994; 15: 249-51.
Matsumoto K, Sato C, Naka Y, Kitazawa A, Whitby RLD, Shimizu N. Neurite outgrowths of neurons with neurotrophin-coated carbon nanotubes. J Biosci Bioeng 2007; 103: 216–20.
Matsumoto K, Sato1 C, Naka Y, Whitby R, Shimizu N. Stimulation of neuronal neurite outgrowth using functionalized carbon nanotubes. Nanotechnology 2010; 21: 115101.
Meng L, Chen R, Jiang A, Wang L, Wang P, Li C, Bai R, et al. Short multiwall carbon nanotubes promote neuronal differentiation of PC12 cells via up-regulation of the neurotrophin signaling pathway. Small 2013; 9: 1786–98.
Mohammadi A, Moghaddam AB, Dinarvand R, Rezaei-Zarchi S. Direct electron transfer of polyphenol oxidase on carbon nanotube surfaces: Application in Biosensing. Int J Electrochem Sci 2009; 4: 895-905.
Panikashvili D, Simeonidou C, Ben-Shabat S, HanusÏ L, Breuer A, Mechoulam R, et al. An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. Nature 2001; 413: 527-31.
Patel S, Rademacher DJ, Hillard CJ. Differential regulation of the endocannabinoids anandamide and 2-arachidonylglycerol within the limbic forebrain by dopamine receptor activity. J Pharmacol Exp Ther 2003; 306: 880–88.
Paxinos G, Watson C.The rat brain in stereotaxic coordinates. San Diego: Academic press; 2007.
Pfister LA, Papaloïzos M, Merkle HP, Gander B. Nerve conduits and growth factor delivery in peripheral nerve repair. J Peripher Nerv Syst 2007; 12: 65-82.
Roman JA, Niedzielko TL, Haddon RC, Parpura V, Floy CL. Single walled carbon nanotubes chemically functionalized with polyethylene glycol promote tissue repair in a rat model of spinal cord injury. J Neurotrauma 2011; 28: 2349–62.
Sinor AD, Irvin SM, Greenberg DA. Endocannabinoids protect cerebral cortical neurons from in vitro ischemia in rats. Neurosci Lett 2000; 278: 157-60.
Son SJ, Bai X, Nan A, Ghandehari H, Lee SB, Template synthesis of multifunctional nanotubes for controlled release. J Control Release 114 (2006) 143-52.
Stella N, Schweitzer P, Piomelli D. A second endogenous cannabinoid that modulates long-term potentiation. Nature (Lond) 1997; 388: 773–78.
van der Stelt M, Di Marzo V. The endocannabinoid system in the basal ganglia and in the mesolimbic reward system: implications for neurological and psychiatric disorders. Eur J Pharmacol 2003; 480: 133–50.
Ya-Ping S, Kefu F, Yi L, Weijie H. Functionalized carbon nanotubes: properties and applications. Acc Chem Res 2002; 35: 1096–1104.
Yen SJ, Hsu WL, Chen YC, Su HC, Chang YC, Chen H, et al. The enhancement of neural growth by amino-functionalization on carbon nanotubes as a neural electrode. Biosens Bioelectron 2011; 26: 4124-32.
Yoshida T, Fukaya M, Uchigashima M, Miura E, Kamiya H, Kano M, et al. Localization of diacylglycerol lipase around postsynaptic spine suggests close proximity between production site of an endocannabinoid, 2-arachidinoyl- glycerol, and presynaptic cannabinoid CB1 receptor. J Neurosci 2006; 26: 4740–51.
Zhang S, Uludağ H. Nanoparticulate Systems for Growth Factor Delivery. Pharm Res 2009; 26: 1561-80.
Zhao DC, Alizadeh D, Zhang LY, Liu W, Farrukh O, Manuel E, et al. Carbon nanotubes enhance CpG uptake and potentiate antiglioma immunity. Clin Cancer Res 2011; 17: 771–82.