Influences of nanoparticle zinc oxide on acutely isolated rat hippocampal CA3 pyramidal neurons

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Abstract: The effects of zinc oxide nanoparticles (nano-ZnO) on the properties of voltage-dependent sodium, potassium currents and evoked action potentials were studied in acutely isolated rat hippocampal CA3 pyramidal neurons at postnatal ages of 10-14 days rats using the whole-cell patch clamp technique. The results indicated that: (1) In the present of final concentration of 10^{-4}g/ml nano-ZnO, the current-voltage curve of sodium current (I_{Na}) was decreased, and the peak amplitudes of I_{Na} were increased considerably from -50 to +20 mV($p<0.05$). Meanwhile, the inactivation and the recovery from inactivation of I_{Na} were also promoted by the nano-ZnO solution (10^{-4}g/L) ($p<0.01$). However, the steady-state activation curve of I_{Na} was not shifted by the nano-ZnO. (2) The amplitudes of transient outward potassium current (I_A) were increased by the nano-ZnO solution (10^{-4}g/ml), while the current-voltage curve of delayed rectifier potassium current (I_K) was significantly increased from +20 to +90 mV ($p<0.05$). However, it is apparent that the nano-ZnO solution didn't shift the steady-state activation curve of I_A and I_K, and neither had significant effects on the inactivation and the recovery from inactivation of I_A. (3) Peak amplitude and overshoot of the evoked single action potential were increased and half-width was diminished in the presence of the 10^{-4}g/ml nano-ZnO solution ($p<0.05$). Simultaneously, a prolonged depolarizing current injection enhanced ($p<0.05$) repetitive firing evoked firing rate. These results suggested that 10^{-4}g/ml nano-ZnO solution can lead to an enhancement in the current amplitudes of I_{Na} and I_K by increasing the opening number of sodium channels, delaying rectifier potassium channels, and enhancing the excitability of neurons, which lead to Na^+ influx and the accumulation of intracellular Na^+, as well as K^+ efflux plus the loss of cytoplasmic K^+. These may disturb the ionic homeostasis and the physiological functions of neurons.

Key words: zinc oxide nanoparticles (nano-ZnO); CA3 pyramidal neurons; sodium current (I_{Na}); transient outward potassium current (I_A); delayed rectifier potassium current (I_K); action potential.

1. Introduction

With the industrialization of nanotechnology, public exposure to nanoparticles will undoubtedly increase in the near future. Consequently, there has been rising concern over the biosafety of nanomaterials. Currently, there is increasing scientific evidence that these the physical and chemical properties of manufactured nanoparticles lead to an increase in bioavailability and toxicity (Nel et al., 2006). Toxicological studies have shown that nanoparticles could enter into the human body through several distinct routes including inhalation, ingestion, and dermal penetration. Subsequently they could elicit toxicological effects at different levels of biological systems. Recent experimental studies indicated that nanoparticles coated with thiamine surface ligands representing a transporter would cross the blood-brain barrier(Lockman et al., 2003), further, size and surface charges as well as coating all contribute to nanoparticle's ability to cross biological barriers (Lockman et al., 2004). And inhaled ultrafine particles via the olfactory neuronal pathway could enter into the central nervous system (CNS) of exposed animals (Oberdorster et al., 2004; Elder et al., 2006).

Nanostructures of ZnO have great potential applications in nanoelectronics, optoelectronics, field emission, light-emitting diodes, photocatalysis, nanogenerators, and nanopiezotronics due to their exceptional semiconducting, piezoelectric, and pyroelectric properties (Wang, 2008). And it can also be used in environmental remediation because of its high chemical catalytic and strong physical adsorption capability for elimination or degradation of pollutants in water or air (Jing et al., 2001). Additionally, nano-ZnO is also widely used commercially in dyes, paints, textiles, medical diagnosis, sunscreens and cosmetics (Yuranova et al., 2007; Serpone et al., 2007; Colvin, 2003). Current interest has been focused toward the application of nano-ZnO in biosensing because of its high isoelectric point (9.5), certain level of biocompatibility showing promising features on the use of ZnO as a biomimic membrane to immobilize and modify biomolecules (Kumar and Chen, 2008; Li et al., 2008). One of the distinctive properties of the nano ZnO is its heritage capability of antimicrobe (Zhang et al., 2007; Tam et al., 2008). Some researchers demonstrated that nanostructures of ZnO were toxic to varying degrees towards the bacteria (Zhang et al., 2007; Reddy et al., 2007; Nair et al., 2008; Heinlaan et al., 2008;), the aquatic biota or eco-relevant species (Adams et al., 2006; Franklin et al., 2007; Zhu et al., 2008;), the mammalian cells (Jeng and Swanson, 2006) and mammals (Sayes et al., 2007; Wang et al., 2008). Adverse effects were partly due to the result of the generation of reactive oxygen species (ROS)(Yamamoto et al., 2004; Ghule et al., 2006; Tam et al., 2008) or causing membrane damage through the direct nanoparticle-cell membrane wall interaction or generation of ROS (Brayner et al., 2006; Zhang et al., 2007; Reddy et al., 2007; Heinlaan et al., 2008) or release of zinc ions in the ZnO particle suspensions (Brunner et al., 2006; Franklin et al., 2007; Zhu et al., 2008; Heinlaan et al., 2008). Therefore, the toxicity mechanisms of nano-ZnO are still fully clear.

Ion channels are transmembrane proteins that mediate passive transport of ions, and the channels underlie a broad range of the most basic biological processes, from excitation and signaling to secretion and absorption. Studies of ion channels provide useful and informative clues for understanding the biophysics and pharmacology of these important and ubiquitous membrane proteins. Ion channels in cell membrane are targets for many toxins and drugs. Much damage on the CNS was caused by interrupting the function of ion channels (Taylor and Meldrum, 1995; Denac et al., 2000; Judge et al., 2007). Action potentials are a fundamental property of excitable cells in the mammalian CNS. Opening of Na^+ channels caused a rapid depolarization of membrane potential and constituted the upstroke phase of the action potential (Denac et al., 2000). Meanwhile, Na^+ influx through voltage-sensitive Na^+ channels has been implicated to play an important role in the mechanisms of neuronal cell damage under ischemia as well as in neurodegenerative disorders (Takahashi et al., 2000). Central neurons have multiple types of voltage-dependent potassium channels (Segal et al. 1984), and potassium currents are also especially important for the regulation of neuronal excitability, because they repolarize neurons in response to depolarizing events and help to stabilize the membrane potential below the firing threshold. The transient potassium current (I_A, fast activating, fast inactivating, and sensitive to 4-aminopyridine at 3 mM) is important to determine the spike threshold because it is activated near the resting membrane potential range and affects the latency of first spike. The "classcal" noninactivating delayed rectifier-type potassium current (I_K, slowly activating, noninactivating, and sensitive to external TEA at 3–25 mM), that is responsible for the fast repolarization of the membrane potential after action potentials (APs) in spiking neurons. It helps to determine the spike width and postspike hyperpolarization, and can help shape the maximal spike frequency of neurons (Mitterdorfer and Bean, 2002). However, little is known about the potential hazard of high concentration of manufactured nanoscale ZnO on the CNS. Moreover, a little knowledge about whether nanoparticle zinc oxide (nano-ZnO) have effect on ion channels and neuronal excitability, although zinc (Zn^{2+}) functions as a signaling molecule in the nervous system (Beyersmann and Haase, 2001) and regulates several voltage-gated ionic conductances, including K^+, Na^+ and Ca^{2+} conductances (Mathie et al., 2006). Therefore, it is necessary to examine the possible effects on ion channels, when neurons exposed to a commercially available nano-ZnO. In the present study, whole cell patch-clamp technique, a more direct experimental approach was used to determine the effects of nano-ZnO on voltage-gated ionic currents and changes in action potential waveforms in acutely isolated rat hippocampal CA3 neurons, and attempt to investigate the possible mechanisms of nano-ZnO on central nervous system.

2 Materials and methods

2.1 Cell preparation and solutions.

Wistar rats (postnatal day 10-14) were obtained from Experimental Animal Center,

Chinese Academy of Medical Sciences. The experiments were conducted in accordance with the guidelines of the Medical Experimental Animal Administrative Committee of the Nation, and all efforts were made to minimize the number of animal used and their suffering. Single rat hippocampal CA3 pyramidal neurons were acutely isolated by enzymatic digestion and mechanical dispersion according to the method (Zou et al., 2000) with the following modifications. Briefly, Wistar rats were anesthetized and decapitated, then brains were quickly removed and placed in ice-cold, oxygenated dissociation (95% O_2-5% CO_2) in artificial cerebrospinal solution fluids (aCSF) containing (in mM): NaCl 126, KCl 3.5, NaH_2 PO_4 1. 5, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 25, Glucose 10 (buffered to pH 7.4 with NaOH) within 1 min. Hippocampi were dissected and cut into approximately 400μm-thick transverse slices. The regions of the CA3 neurons were isolated and incubated for 50min at 32℃in aCSF, bubbled with 95% O_2-5% CO_2; and successively transferred into aCSF containing 0.5 mg/ml protease for 15 min at 32℃, bubbled with 95% O_2-5% CO_2. After digestion the tissue pieces were rinsed 3 times with aCSF to end the enzyme's function. The CA3 regions were dissected and triturated to release single cells through a series of fire-polished Pasteur pipette with tip diameter from 150 to 500μm. The cell suspension was transferred into a 35 mm culture dish filled with 2 ml extracellular solution, containing (in mM): NaCl 130, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, Glucose 10, Hepes 10, and pH 7.4. Twenty minutes later, the cells were attached to the bottom of the culture dish and were ready for experiments. Pyramidal neurons were easily identified morphologically with bright pyramidal-shaped soma and two or three short branched dendrites and a long axon. These neurons remained viable for electrophysiological studies up to 4-5 h.

The standard pipette solution for current-clamp experiments was (in mM): KCl 130, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 10, Hepes 10, Mg-ATP 2, buffered to pH 7.2 with KOH.

The standard pipette solution for recording sodium current containing (in mM): CsCl 140, $MgCl_2$ 2, HEPES 10, EGTA 10, TEA-Cl 10, Mg-ATP 2, buffered to pH 7.2 with CsOH.

The standard pipette solution for recording voltage-dependent potassium current containing (in mM): KCl 65, KF 80, KOH 5, Hepes 10, EGTA 10, Mg-ATP 2, buffered to pH 7.2 with KOH. Tetrodotoxin (TTX, 0.001mM) and cadmium chloride (CdCl, 0.2 mM) were added into the extracellular solution to block Na^+ and Ca^{2+} channels (Chi and Xu. 2000). Two types of voltage-dependent potassium channels known as I_A (fast activating, fast inactivating, and sensitive to 4-aminopyridine at 3 mM), and I_K (slowly activating, noninactivating, and sensitive to external tetraethylammonium chloride at 3-25 mM) (Mitterdorfer and Bean, 2002) were distinguished respectively in presence of tetraethylammonium chloride (TEA-Cl, 25 mM) and 4-aminopyridine (4-AP, 3 mM)(Segal et al., 1984).

2.2 Whole cell recordings

Whole cell currents were recorded with EPC10 patch clamp amplifier (HEKA, Germany). A micropipette puller (PIP5, HEKA, Germany) was used to pull the

electrodes. The patch electrodes had a tip resistance of 5~8 MΩ when filled with pipette solution. After the formation of a giga seal, the pipette resistance and capacitance were compensated electronically. After the rupture of the membrane and the establishment of a whole cell voltage-clamp configuration, compensation (80%) for series resistance was routinely used. Data were low-pass filtered at 2.9 kHz, digitized at 10 kHz (four pole Bessel filter) and stored in an Intel-based computer using Pulse 8.74 software (HEKA, Germany). All experiments were performed at room temperature (21-24℃).

2.3 Nanoparticle ZnO and solutions

Zinc oxide is nearly insoluble in water but soluble in acids and bases. It occurs as white powder known as zinc white which occurs in nature as the mineral zincite. Crystalline ZnO exhibits the piezoelectric effect and is thermochromic, changing from white to yellow when heated. Zinc oxide decomposes into zinc vapor and oxygen only at around 1975 °C, reflecting its considerable stability. Heating with carbon converts the oxide into zinc: $ZnO + C \rightarrow Zn + CO$. In this research, the nano-ZnO was analyzed and provided by IntriciQ Materials, Farnborough, Hants GU14 0LX, UK. The Transmission Electron Microscopy (TEM, Tecnai G2 20 S-TWIN, FEI, USA) images showed that ZnO nanoparticles were with the sizes of 20-80 nm, 2 or 3 types of crystal shapes(as shown in Fig.1), which might provide different functionalities such as photocatalytics and photovoltaic capabilities.

Stock solution(10^{-2}g/ml) of nano-ZnO was prepared in Milli-Q water and dispersed by ultrasonic vibration for 20 min. Nano-ZnO suspension (10^{-4}g/ml) in artificial cerebrospinal fluid (ACSF) were characterized by Dynamic Light Scattering(DLS) using a ZetaPALS+ BI-90Plus (Brookhaven Instruments Corp. USA) at a wavelength of 659 nm. The scattering angle was fixed at 90°. The particle size distribution had a wide range from 65 nm to1534 nm due to the aggregation, and the hydrodynamic diameter was 371 nm. Its suspension was stirred on vortex agitator before every use.

2.4 Drug application and data analysis

According to our experiment in nanoparticle CuO(Xu et al., 2008), and the preliminary experiment showed that 10^{-5}g/ml and 10^{-6}g/ml nano-ZnO had no significant effects on whole cell current in acutely isolated rat hippocampal CA3 pyramidal neurons($p>0.05$), however, 10^{-4}g/ml nano-ZnO could have obvious effects on whole cell current($p<0.05$). Therefore, final concentration of 10^{-4}g/ml nano-ZnO suspension was used to examine the effects on the properties of voltage-dependent sodium, potassium currents and evoked action potentials in acutely isolated rat hippocampal CA3 pyramidal neurons.

Final concentration of 10^{-4}g/ml nano-ZnO suspension was added into extracellular solution once currents were stable (about 3~5 min). The nano-ZnO deposited around the recording cell and played a direct role on cell. Drug actions were measured only after steady-state conditions reached, which were judged by the amplitudes and time courses of currents remaining constant.

Tetrodotoxin (TTX) was purchased from the Research Institute of the Aquatic Products of Hebei (China). 4-aminopyridine (4-AP), tetraethylammonium chloride (TEA-Cl), CsOH, CdCl, EGTA, Hepes, Mg-ATP and Protease were purchased from Sigma(USA), and other reagents were of AR grade.

All data were analyzed and fitted by using Igor Pro 5.04 (Wavemetrics, Lake Oswego, OR, USA) and Origin 7.5 (Microcal Software, USA) softwares. All values were represented as mean ± SEM and statistical comparisons were made using the Student's paired t-test and one-way analysis of variance (ANOVA). A probability value less than 0.05 was considered to be statistically significant.

3 Results

3.1 Effects of nano-ZnO on I_{Na}

TTX-sensitive sodium current carried the largest inward current. For recording sodium current, CdCl (0.2 mM) was added into the extracellular solution to suppress Ca^{2+} currents. In whole cell patch-clamp recording, cells were held at a holding potential of -100mV, and 20ms depolarizing potentials from -100 to +60mV at 10 mV steps activated inward currents which were completely and reversibly blocked by bath application of 0.001 mM TTX. Therefore, these inward currents were attributed to sodium currents I_{Na}.

Final concentration of 10^{-4}g/ml nano-ZnO was applied in the extracellular solution once sodium currents were stable (about 3~5 min). The peak amplitudes of I_{Na} were increased at more different membrane potentials as compared with the control, as shown in Fig.2A. The current-voltage curve of I_{Na} was decreased, and the peak amplitudes of I_{Na} were increased obviously from -50 to +20 mV ($n=11$, $P<0.05$ vs. control), as shown in Fig.2B.

3.1.1 Effects of nano-ZnO on activation kinetics of I_{Na}

The steady-state activation curves for I_{Na} under control conditions and after exposure to nano-ZnO were shown in Fig.2C. The curves were fitted well with the Boltzmann equation $G/G_{max} =1/\{1+\exp[(V_m–V_h)/k]\}$. First, the current amplitudes evoked by the step pulses were converted into conductance by using the equation $G = I/(V_m–V_r)$, where G is the conductance, I is the current, V_m is the membrane potential, and V_r is the reversal potential. In Boltzmann equation, V_h is the membrane potential at half-activation, and k is slope factor.

The values of V_h for activation of I_{Na} in control and nano-ZnO were -55.75± 2.59 mV and -59.60 ± 2.74mV ($n =11$, $P >0.05$), with a slope factor k of 5.57 ± 2.25mV and 6.09 ± 2.54mV ($n = 11$, $P >0.05$), respectively. There was no statistical difference between the values of V_h in control and nano-ZnO, and the slope factor remained unchanged.

3.1.2 Effects of nano-ZnO on inactivation kinetics of I_{Na}

The steady-state inactivation of I_{Na} was determined by a double-pulse protocol: applying 50 ms conditioning prepulses to potentials between -100 and -10mV in 5mV increments, followed by a 20ms depolarizing pulse to -10mV, and holding potential at -100mV(as shown in Fig.3A). Peak amplitudes for I_{Na} currents were normalized and plotted vs prepulse potentials. The inactivation curves were well fitted with the Boltzmann equation: $I/I_{max} = 1/\{1+\exp[(V_m - V_h)/k]\}$, where I_{max} is the maximal current, V_h is the membrane potential at half-inactivation, and k is slope factor(as shown in Fig.3B). The values of V_h for inactivation of I_{Na} before and after addition of nanoparticle ZnO are -52.54± 0.43 mV and -54.67 ± 0.39mV (n = 8, P<0.01), with a slope factor k of 9.32 ± 0.42mV and 8.72 ± 0.38mV (n = 8, P >0.05), respectively.

3.1.3 Effects of nano-ZnO on recovery from inactivation of I_{Na}

Once the I_{Na} is inactivated following membrane depolarization, a sufficient amount of hyperpolarizing time must elapse before the channel can recover and be fully activated again. In order to determine the kinetics of recovery from the inactivated channel state, cells were held at -90 mV, a 50ms conditioning depolarizing pulse of -40mV was applied to inactivate the sodium channels fully, and then a 50ms test pulse of -40mV was applied after a series of -90 mV intervals varying from 2 to 36 ms (in 2 ms increments, as shown in Fig.4A). The peak value of I_{Na} evoked by the conditioning pulse was designated as I_1, while the peak value of I_{Na} evoked by the test pulse was designated as I_2. The ratio of I_2 to I_1 represents the recovery of I_{Na} from inactivation. The plot of I_2/I_1 vs the duration of the -90 mV intervals was well fitted with a monoexponential function: $I/I_{max} = A + B\exp(-t/\tau)$, where t is the recovery interval of the conditioning prepulse and τ is the time constant for the recovery from inactivation of I_{Na}(as shown in Fig.4B). The time constants (τ), which described the recovery time course, before and after addition of nano-ZnO were 5.40 ± 0.19 ms and 3.95 ± 0.15 ms (n = 8, P < 0.01), respectively. The results indicated 10^{-4}g/ml nano-ZnO accelerated the recovery of I_{Na} from inactivation.

3.2 Effects of nano-ZnO on voltage-dependent potassium currents (I_A and I_K)

In whole cell patch-clamp recording, TTX(0.001 mM) and CdCl (0.2 mM) were added into the extracellular solution to block Na^+ and Ca^{2+} channels. In order to record transient outward potassium current (I_A), TEA-Cl (25 mM) was also used to inhibit delayed rectifier potassium current (I_K). And the holding potential was -70mV, using an 80ms constant depolarizing pulse from a command potential of -50 to +90 mV in increments of 10mV. I_K was obtained using a 160ms constant depolarizing pulse by a similar pulse protocol in present of 4-AP (3 mM).

After 5 min's stable current recordings, final concentration of 10^{-4}g/ml nano-ZnO was applied in the extracellular solution. The amplitudes of I_A and I_K were both increased at more different membrane potentials (as shown in Fig.5A and Fig.5B), the current-voltage curve of I_A was increased but there was no statistically significant (n=6, P>0.05 vs. control). While the current-voltage curve of I_K was significantly increased by nano-ZnO from +20 to + 90 mV (n=6, P <0.05 vs. control, as shown in Fig.5C and Fig.5D).

3.2.1 Effects of nano-ZnO on the activation kinetics of I_A and I_K

The steady-state activation curves for I_A and I_K under control conditions and after exposure to nano-ZnO are shown in Fig. 5E and Fig.5F. The curves were fitted well with the Boltzmann equation $G/G_{max} = 1/\{1+\exp[(V_m - V_h)/k]\}$(as described in Results 3.1.1). The values of V_h for activation of I_A in control and nano-ZnO groups were 23.97± 1.33 mV and 29.22 ± 1.73mV (n = 6, P > 0.05), with a slope factor k of 30.85 ± 1.85mV and 34.45 ± 2.42mV (n = 6, P > 0.05), respectively; and V_h for activation of I_k in control and nano-ZnO groups were 33.09 ± 1.06 mV and 32.07 ± 0.87 mV (n = 6, P> 0.05), with a slope factor k of 29.96 ± 1.25 mV and 27.34 ± 1.01 mV (n = 6, P > 0.05), respectively. Thus, there were no significant effects of nano-ZnO on the activation kinetics of I_A and I_K.

3.2.2 Effects of nano-ZnO on the inactivation kinetics of I_A

The steady state inactivation of I_A was determined by applying 80 ms conditioning prepulses to potentials between -120 and +10mV in 10mV increments, followed by a 100 ms depolarizing pulse to +50mV, and holding potential at -80mV(as shown in Fig.6A). Peak amplitudes for I_A currents were normalized and plotted vs prepulse potentials. The inactivation curves were well fitted with the Boltzmann equation: $I/I_{max} = 1/\{1+\exp[(V_m - V_h)/k]\}$, where I_{max} is the maximal current, V_h is the membrane potential at half-inactivation, and k is slope factor. The values of V_h for inactivation of I_A before and after addition of nano-ZnO were -69.09± 0.56 mV and -68.79 ± 0.65mV (n = 6, P > 0.05), with a slope factor k of 9.59 ± 0.49mV and 10.85 ± 0.57mV (n = 6, P > 0.05), respectively. Thus, there were no significant effects of nano-ZnO on the inactivation of I_A, as shown in Fig.6B.

3.2.3 Effects of nano-ZnO on the recovery from inactivation of I_A

In order to determine the kinetics of recovery from the inactivated channel state, cells were held at -70 mV, and an 80 ms conditioning depolarizing pulse of +50 mV was applied to inactivate the transient outward potassium channels fully, and then an 80 ms test pulse of +50 mV was applied after a series of -80 mV intervals varying from 10 to 260 ms (in 10 ms increments, as shown in Fig.7A). The peak value of I_A evoked by the conditioning pulse was designated as I_1, while the peak value of the I_A evoked by the test pulse was designated as I_2. The ratio of I_2 to I_1 represents 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 function $I/I_{max} = A + B\exp(-t/\tau)$, where t is the recovery interval of the conditioning prepulse and τ is the time constant for the recovery from inactivation of I_A. The time constants (τ), which described the recovery time course, before and after addition nano-ZnO were 34.67 ± 2.47 ms and 31.76 ± 2.26 ms (n = 6, P> 0.05), respectively. The nano-ZnO had no marked effect on the recovery of I_A from inactivation, as shown in Fig.7B.

3.3 Effects of nano-ZnO on action potential

Action potential properties and the pattern of repetitive firing were examined using whole cell current-clamp recordings. Evoked action potentials were generated from a

holding potential of -70 mV. Single action potential was elicited by brief depolarizing current pulses (5 ms, 100pA), and repetitive firing was evoked by a prolonged depolarizing current injection (500 ms, 50 pA). Peak amplitude, overshoot, spike half-width of single action potential and firing rate of repetitive firing were measured before and after drug applications (as shown in Table 1). Peak amplitude and overshoot of the evoked single action potential were increased and half-width was diminished in the present of the 10^{-4}g/ml nano-ZnO solution, and the firing rate of repetitive firing was also increased (n=6, $p<0.05$)(as shown in Fig.8 and Fig.9).

4. Discussion

Recent experimental studies indicated that surface modified nanoparticle can cross most strong biological barriers such as blood-brain barrier (Lockman et al., 2003, 2004) and inhaled ultrafine particles via the olfactory neuronal pathway enter into the CNS of exposed animals (Oberdorster et al., 2004; Elder et al., 2006). It has been demonstrated that nanosize titanium dioxide stimulates reactive oxygen species in brain microglia and damages neurons in vitro (Long et al., 2007). More studies about nano-ZnO showed that the antibacterial activity or adverse effects were partly due to the result of the generation of reactive oxygen species (ROS)(Yamamoto et al., 2004; Ghule et al., 2006; Tam et al., 2008) or causing membrane damage through the direct nanoparticle-cell membrane wall interaction or generation of ROS (Brayner et al., 2006; Zhang et al., 2007; Reddy et al., 2007; Heinlaan et al., 2008) or release of zinc ions in the ZnO particle suspensions (Brunner et al., 2006; Franklin et al., 2007; Zhu et al., 2008; Heinlaan et al., 2008). Studies in mammals suggested that nanoscale ZnO oral exposure could cause the increase in blood viscosity and the pathological lesions in the gastric, liver, renal, pancreas and spleen (Wang et al., 2008). However， the potential hazard of high concentration of manufactured nanoscale ZnO on the CNS is known little. Moreover, it has little knowledge about whether nano-ZnO has effect on ion channels and neuronal excitability.

This is the first study to evaluate the acute effects of nano-ZnO on the properties of voltage-dependent sodium, potassium currents and evoked action potentials by using the whole-cell patch clamp technique in acutely isolated rat hippocampal CA3 pyramidal neurons. The results showed that (1) In the present of final concentration of 10^{-4}g/ml nano-ZnO, the peak amplitudes of I_{Na} were increased considerably from -50 to +20 mV($p<0.05$). The kinetic analysis demonstrated that the inactivation and the recovery from inactivation of I_{Na} were also promoted by the nano-ZnO solution ($p<0.01$). However, the steady-state activation curve of I_{Na} was not altered. (2) The amplitudes of I_A were increased by the nano-ZnO solution, while amplitudes of I_K was significantly increased from +20 to + 90 mV ($p<0.05$). However, the kinetic analysis demonstrated that the steady-state activation curve of I_A and I_K remained unchanged, and nano-ZnO had no significant effect on the inactivation and the recovery from inactivation of I_A. (3) Peak amplitude and overshoot of the evoked single action potential were increased and half-width was diminished in the present of

nano-ZnO ($p<0.05$). Simultaneously, firing rate of repetitive firing evoked by a prolonged depolarizing current injection was increased ($p<0.05$).

The effect of nano-ZnO on voltage-gated sodium channels in acutely isolated rat hippocampal CA3 pyramidal neurons

Voltage-gated sodium channels are responsible for the largest inward current during the depolarization phase of action potential in excitable cells. Na^+ channels open and inactivate in response to a depolarization of the resting membrane potential. When these channels open, an influx of Na^+ occurs, depolarizing the membrane potential, and generating the upstroke phase of the action potential. Inactivation of Na^+ channels stops Na^+ influx, preventing a permanent depolarization of the resting membrane potential. Recovery from inactivation allows the cell membrane to regain its resting excitable properties. The channels of Na^+ play important roles both in vital physiological functions and several pathological processes of the CNS. Results from in vitro and in vivo studies support the idea that Na^+ channels are involved in Alzheimer's disease (AD) and injury in CNS neurons, and have been proposed as a potential target for drug treatment as well. It has also been widely accepted that an increase Na^+ influx through voltage-gated Na^+ channels upon depolarization exacerbates neuronal cell damage (Lynch et al., 1995; Stys, 1998). Growing evidence also suggests that the reduction in voltage-gated Na^+ channel activity improves cell survival and reduces hypoxia-induced injury (Lynch et al., 1995; Taylor and Meldrum, 1995; Martínez-Sánchez et al., 2004). Meanwhile activation of Na^+ channels by veratridine has been used as a depolarization-induced neuronal injury model eliciting Ca^{2+}-dependent neuronal damage (Takahashi et al., 1999, 2000; Takano et al., 2003; Akasofu et al., 2008). Epilepsy, chronic pain, neurodegenerative diseases, and spasticity are all characterized by an over-excited state of specific groups of central neurons that is accompanied by an abnormally increased activity of sodium channels. Therefore, an efficient strategy of controlling such diseases is to use blockers that preferentially act on these over-excited cells. Various blockers of the Na^+ channel, including phenytoin, or lamotrigine, leave normal physiological functions relatively intact, resulting in a favorable therapeutic window (Tarnawa et al., 2007). Our experimental results suggested that the peak amplitudes of I_{Na} were increased obviously from -50 to +20 mV ($n=11$, $P<0.05$ vs. control) in the present of 10^{-4}g/ml nano-ZnO, but the steady-state activation curve of I_{Na} was not altered. The kinetic analysis demonstrated that the inactivation and the recovery from inactivation of I_{Na} were also promoted by the nano-ZnO solution ($p<0.01$), which suggested that the nano-ZnO may increase the neuronal excitability. Therefore, the results support the above point that activation of Na^+ channels by nano-ZnO solution could lead to depolarization-induced neuronal injury. Additionally, the increase of peak amplitudes of I_{Na} by nano-ZnO solution could contribute to increase Na^+ influx and add to the intracellular Na^+ load . The consequent increases in intracellular Na^+ concentration may contribute to an increase intracellular Ca^{2+} due to activation of voltage-gated Ca^{2+} channels and a reversed operation of the Na^+-Ca^{2+} exchanger (Xiao et al., 2002). An increase in intracellular Ca^{2+} is generally believed to be a trigger for excitotoxicity

of necrotic cell death (Choi, 1988). Furthermore, the increase in intracellular Na^+ favors the passive influx of chloride (Cl^-) and water. This causes neuronal swelling and other signs of acute neuronal injury, such as the formation of blebs in the membrane (Jiang et al., 1991; Friedman and Haddad, 1994).

The effect of nano-ZnO on voltage-gated potassium channels in acutely isolated rat hippocampal CA3 pyramidal neurons

In the nervous system, voltage-gated K^+ currents determine a large number of neuronal properties, such as resting membrane potential, action potential(AP) waveform, and fire frequency, and are known to play important roles in all cell types underlying both normal and pathophysiological functions. I_A and I_K are the two main neuronal voltage-gated K^+ current; they contribute to AP repolarization and repetitive firing. In the present study, the current-voltage curve of I_K was significantly increased from +20 to + 90 mV ($n=6$, $P<0.05$ vs. control). However, the nano-ZnO solution didn't shift the steady-state activation curve of I_A and I_K, and neither had a significant effect on the inactivation and the recovery from inactivation of I_A. The results suggest that activation of K^+ currents by the nano-ZnO solution could be preferential for I_k, and cause the increase of K^+ efflux and the loss of cytoplasmic K^+. In addition, I_K is responsible for the fast repolarization of the membrane potential after action potentials in spiking neurons, and helps to determine the spike width and postspike hyperpolarization, and can help shape the maximal spike frequency of neurons (Mitterdorfer and Bean, 2002). Therefore, the activation of I_k may reduce the spike half-width and increase the spike frequency by rapid K^+ efflux, and further increase the excitability of neurons. Emerging evidence supports the idea that changes in ionic concentrations, primarily K^+, play a pivotal role in the activation and progression of apoptosis (Bortner et al., 1997; Bortner and Cidlowski, 1999; Hughes and Cidlowski, 1999; Montague et al., 1999; Vu et al., 2001). K^+ is the predominant cation in the cytosol. Maintenance of a high [K^+] in the cytoplasm (140-150 mM) is essential for governing cell excitability, setting resting E_m, regulating apoptotic enzyme activity, and controlling cell volume (Remillard and Yuan, 2004). Cytoplasmic K^+ concentration is mainly regulated by the activity of Na^+-K^+-ATPase and various K^+ channels in the plasma membrane. The loss of cytoplasmic K^+ due to increased K^+ efflux through K^+ channels, perhaps in particular via I_K, results in cell shrinkage, a major characteristic of apoptosis, and caspase activation, a triggering process in apoptosis (Yu et al., 1997,1999; Krick et al., 2001; Remillard and Yuan, 2004). Therefore, inhibiting K^+ efflux by reducing the K^+ electrochemical gradient with high extracellular K^+ concentrations or pharmacological blockade of K^+ channels by TEA significantly attenuated the apoptosis. (Bortner et al., 1997; Yu et al., 1999; Thompson et al., 2001). It has also been shown that cytoplasmic K^+ at normal concentration (~140 mM) inhibits some morphological, biochemical and molecular hallmarks of apoptosis such as cell shrinkage, DNA fragmentation and caspase-3-like protease activation (Bortner et al., 1997; Thompson et al., 2001). Based on the above investigations, we speculated that the nanoparticle ZnO could involve in the neuronal apoptosis caused by the loss of cytoplasmic K^+ due to increased K^+ efflux through

delayed rectifier potassium channels.

Additionally, there is still compelling evidence indicating an early increase in intracellular Na^+ followed by a decrease in both intracellular K^+ and Na^+ suggesting a regulatory role for these cations during both the initial signaling, and the execution phase of apoptosis (Fernandez-Segura et al., 1999; Bortner and Cidlowski, 2003; Arrebola et al., 2005). The studies point to a role for intracellular sodium increase not only in controlling cell size changes but also in controlling the initial signaling of the cell death cascade (Panayiotidis et al., 2006). Our experimental results showed that the enhancement of Na^+ influx via activating voltage-gated Na^+ channels and K^+ efflux through delayed rectifier potassium channels by nano-ZnO would lead to the increase and the loss in intracellular Na^+ and K^+ respectively, which are associated with neuronal apoptosis.

The influence of nano-ZnO on evoked action potential in acutely isolated rat hippocampal CA3 pyramidal neurons

The channel blockers were usually added into the intracellular or extracellular solution to suppress other channel currents in whole cell patch clamp recording when the effects of nano-ZnO on kinds of ion channels in hippocampal CA3 pyramidal neurons were detected. Action potentials are a fundamental property of excitable cells in the mammalian central nervous system. Many channel types present in a particular neuron work together to determine its firing properties. Excitability may also reflect changes in the properties of voltage-gated membrane channels. So action potential properties and the pattern of repetitive firing were examined using whole cell current-clamp recordings for further detecting the effects of nano-ZnO. The results suggested peak amplitude and overshoot of the evoked single action potential were increased and half-width was diminished in the present of nano-ZnO ($p<0.05$). Simultaneously, firing rate of repetitive firing evoked by a prolonged depolarizing current injection was increased ($p<0.05$). Therefore, the results confirmed that the increase of the neuronal excitability resulted from the enhancement of current amplitudes of I_{Na} and I_K by the nano-ZnO solution. Although there are multiple types of potassium channels involved in the repolarization of the action potential and together to determine its firing properties, the neuronal excitability, as a whole, was increased in the present of the nano-ZnO solution. Therefore, the results support the widely accepted idea that the enhancement of Na^+ influx and the accumulation of intracellular Na^+ via activating voltage-gated Na^+ channels would contribute to depolarizing neurons, causing Ca^{2+} influx, reversed Na^+-Ca^{2+}exchange, K^+ efflux, glutamate release, increasing the neuronal excitability, and eventually lead to the injury of CNS (Choi, 1988; Taylor and Meldrum, 1995; Xiao et al., 2002; Martínez-Sánchez et al., 2004).

In CNS, the increase of the neuronal excitability would consume more cell energy. It has been shown that approximately one half or more of total ATP generated in the brain at resting state is consumed by Na^+-K^+-ATPase to maintain proper transmembrane ionic gradients (Erecinska and Silver, 1989). Establishing and

maintaining high K^+ and low Na^+ in the cytoplasm are required for normal resting membrane potentials and various cellular activities. The ionic homeostasis maintained by the Na^+-K^+-ATPase is also critical for cell growth, differentiation, and cell survival (Yu, 2003). Our results also showed that peak amplitude and overshoot of the first action potential of repetitive firing were increased in the presence of nanoparticle ZnO, however, peak amplitude and overshoot of the subsequent action potentials of repetitive firing were decreased and membrane potential was raised in contrast to control. Based on the idea that cellular energy expenditure does increase linearly with the frequency of action potentials (Kadekaro et al., 1985), and repetitive firing will result in large increases of Na^+ influx and K^+ efflux. So it is likely that Na^+-K^+-ATPase could not export intracellular redundant Na^+ and/or import extracellular K^+ timely, which would result in the accumulation of intracellular Na^+ and loss of intracellular K^+, and consequently, reducing the Na^+ and K^+ electrochemical gradient, and thereby decrease peak amplitude and overshoot of the subsequent action potentials of repetitive firing. Therefore, we speculated that the decrease of peak amplitude and overshoot of the subsequent action potentials of repetitive firing may due to the dysfunction or deficiency of Na^+-K^+-ATPase, which was failure of maintenance of proper transmembrane ionic gradients. On the other hand, it has been shown that during the execution phase of apoptosis an early increase in intracellular Na^+, a decrease in intracellular Cl^- and K^+, and a decrease in K^+/ Na^+ ratio were related with inhibition of the Na^+-K^+-ATPase (Arrebola et al., 2005).

While zinc, the second most abundant trace metal (after iron) in mammalian tissues, is an essential element for growth, development, DNA synthesis, immunity, and other important cellular processes. The binding of zinc to L-histidine (His) both in plasma and cerebrospinal fluids seems to be involved in transferring zinc to target sites, which regulate its uptake across the brain barrier systems (Takeda et al., 2002). Even within the brain, 90% of total brain zinc is bound to zinc metalloproteins, with much of the remaining 10% found in presynaptic vesicles, either loosely bound or free (and therefore, histochemically reactive) (Takeda, 2001). Free Zn^{2+} modulates many membrane receptors, transporters and channels (Mathie et al., 2006). Taking into account the release of zinc ions in the ZnO particle suspensions (Franklin et al., 2007; Zhu et al., 2008), we performed the dissolution tests of different concentration of nano- ZnO suspensions in artificial cerebrospinal fluid (ACSF, pH 7.4). Zinc concentration in supernatants of the nanoparticle suspensions after centrifugation （13,000 rpm, for 30min） and filtration (100nm filter) was determined by an inductively coupled plasma optical emission spectrometry (ICP-OES, ICP-9000 (N+M), Thermo Jarrell-Ash Corp. USA). The concentrations of soluble zinc ions is 0.86~2.07 mg/L(13.28~30.8 μM) in the nano ZnO suspension(from 10^{-5}g/ml to 10^{-3}g/ml). The results of nano-ZnO (about 26μM; 1.69mg/L from 10-4g/ml nano-ZnO) were not consistent with the modulatory effect of Zn^{2+} to voltage-gated K^+ and Na^+ conductance(Harrison et al., 1993; Kuo and Chen, 1999). Therefore, we speculated soluble zinc ions may not mainly contribute to the effects.

In summary, our results suggested that nano-ZnO solution may involve in

depolarization-induced neuronal injury by activation of voltage-gated Na^+ channels, which lead to the enhancement of Na^+ influx, the accumulation of intracellular Na^+, Ca^{2+} influx, and glutamate release, increasing the neuronal excitability. Additionally, the nano-ZnO could participate the neuronal apoptosis caused by the loss of cytoplasmic K^+ due to increased K^+ efflux through delayed rectifier potassium channels. The two possible mechanisms seemed associated with the dysfunction or deficiency of Na^+-K^+-ATPase, which was failure of maintenance of proper transmembrane ionic gradients.

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Table 1 Effects of nano-ZnO on properties of single action potential and number of APs in repetitive firing

	peak amplitude(mV)	overshoot(mV)	spike half-width(ms)	number of APs
control	94.07 ± 2.15	24.07 ± 2.15	2.62 ± 0.31	13.83 ± 0.48
nano-ZnO	103.37 ± 2.19**	33.37 ± 2.19**	2.29 ±0.24*	15.50 ± 0.76*

Significance levels are given as P<0.05 (*) and P<0.01 (**) for differences from control. n=6.

Figure Legends

Fig.1. TEM image of nano-scaled ZnO.

Fig.2. Effect of 10^{-4}g/ml nano-ZnO on sodium currents (I_{Na}). (A) Currents were obtained by 20 ms depolarizing pulses from holding potential of -100 to +60 mV at 10 mV steps.(B) The current-voltage curves of I_{Na}. (C) The steady-state activation curves of I_{Na}, peak amplitudes of I_{Na} were converted into conductance by using the equation $G = I/(V_m–V_r)$, normalized conductance of sodium channels were plotted against the voltages of conditioning pulses, and fitted with a Boltzmann function. Each point represents mean ± SEM (n = 11, *P < 0.05 and **P<0.01 vs control).

Fig.3. Effect of 10^{-4}g/ml nano-ZnO on inactivation kinetics of I_{Na}. Currents were elicited with a 20ms depolarizing pulse to -10mV preceded by 50 ms prepulses to potentials between -100 and -10mV in 5mV increments, and holding potential at -100mV. (A)and (B) are steady-state inactivation of I_{Na} before and after application of nano-ZnO; (C) is the expanded traces taken from (A). (D) The steady-state inactivation curves of I_{Na}. Peak amplitudes for I_{Na} currents were normalized and plotted vs prepulse potentials, and the data are fitted with Boltzmann function. Each point represents mean ± SEM (n = 8). The values of V_h for inactivation of I_{Na} before and after addition of nanoparticle ZnO are -52.54± 0.43 mV and -54.67 ± 0.39mV (P<0.01).

Fig.4. Effect of 10^{-4}g/ml nano-ZnO on recovery from inactivation of I_{Na}. (A) The currents were obtained as followed protocol: holding potential at -90 mV, a 50ms conditioning depolarizing pulse of -40mV was applied to inactivate the sodium channels fully, and then a 50ms test pulse of -40mV was applied after a series of -90 mV intervals varying from 2 to 36 ms(in 2 ms increments). (B) The recovery from inactivation curves of I_{Na}. The peak value of I_{Na} evoked by the conditioning pulse was designated as I_1, while the peak value of I_{Na} evoked by the test pulse was designated as I_2. The ratio of I_2 to I_1 represents the recovery of I_{Na} from inactivation. The plot of I_2/I_1 vs the duration of the -90 mV intervals was fitted with a monoexponential function. Each point represents mean ± SEM (n = 8). The time constants (τ) for recovery from inactivation of I_{Na} before and after addition of nano-ZnO were 5.40 ± 0.19 ms and 3.95 ± 0.15 ms (P < 0.01).

Fig.5. Effect of 10^{-4}g/ml nano-ZnO on $I_A$and I_K. (A) and (B): I_A and I_K were obtained

by 80 ms and 160 ms depolarizing pulses respectively, from a command potential of -50 to +90 mV in increments of 10 mV, and the holding potential was -70mV. (C) and (D): The current-voltage curve of I_A and I_K. (E) and (F): The steady-state activation curves of I_A and I_K. Peak amplitudes of I_A and I_K were converted into conductance by using the equation $G = I/(V_m–V_r)$, normalized conductance of potassium channels were plotted against the voltages of conditioning pulses, and fitted with a Boltzmann function. Each point represents mean ± SEM (n = 6, *P < 0.05 vs control)

Fig.6. Effect of 10^{-4}g/ml nano-ZnO on inactivation kinetics of I_A. (A)Currents were elicited with a 80ms depolarizing pulse to +50mV preceded by 80 ms prepulses to potentials between -120 and +10mV in 10mV increments, and holding potential at -70mV.(B) The steady-state inactivation curves of I_A. Peak amplitudes for I_A currents were normalized and plotted vs prepulse potentials, and the data are fitted with Boltzmann function. Each point represents mean ± SEM (n = 6).

Fig.7. Effect of 10^{-4}g/ml nano-ZnO on recovery from inactivation of I_A. (A) The currents were obtained as followed protocol: holding potential at -70 mV, an 80 ms conditioning depolarizing pulse of +50 mV was applied to inactivate the transient outward potassium channels fully, and then an 80 ms test pulse of +50 mV was applied after a series of -80 mV intervals varying from 10 to 260 ms(in 10 ms increments). (B)The recovery from inactivation curves of I_A. 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 to I_1 represents the recovery of I_A from inactivation. The plot of I_2/I_1 vs the duration of the -80 mV intervals was fitted with a monoexponential function. Each point represents mean ± SEM (n = 6).

Fig.8. Effect of nano-ZnO on evoked single action potential. Single action potential was elicited by brief depolarizing current pulses (5 ms, 100pA) from a holding potential of -70 mV.

Fig.9. Effect of nano-ZnO on repetitive firing evoked by a prolonged depolarizing current injection (500 ms, 50 pA) from a holding potential of -70 mV.