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Title of paper: Lidocaine suppresses subthreshold oscillations by inhibiting persistent

Na⁺ current in injured dorsal root ganglion neurons

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Short title: Lidocaine suppresses I_{NaP} in injured DRG neurons

Summary

The aim of this study was to determine the effect and mechanism of low concentration of lidocaine on subthreshold membrane potential oscillations (SMPO) and burst discharges in chronically compressed dorsal root ganglion (DRG) neurons. (delete) DRG neurons were isolated by (delete) enzymatic disassociation method. SMPO, burst discharges and single spike were elicited by whole cell patch-clamp technique in current clamp mode. Persistent Na^+ current (I_{NaP}), transient Na^+ current (I_{NaT}) were elicited in voltage clamp mode. The results showed that SMPO was suppressed and burst discharges were eliminated by tetrodotoxin (TTX, 0.2 $\mu\text{mol/L}$) in current clamp mode, I_{NaP} was blocked by 0.2 $\mu\text{mol/L}$ TTX in voltage clamp mode. SMPO, burst discharges and I_{NaP} were also suppressed by low concentration of lidocaine (10 $\mu\text{mol/L}$) respectively. However, single spike and I_{NaT} could only be blocked by high concentration of lidocaine (5 mmol/L). From these results, it is suggested that I_{NaP} mediates the generation of SMPO in injured DRG neurons. Low concentration of lidocaine (10 $\mu\text{mol/L}$) suppresses SMPO by selectively inhibiting I_{NaP} , but not I_{NaT} , in chronically compressed DRG neurons.

Keywords lidocaine; neuropathic pain; dorsal root ganglion; ectopic discharges; subthreshold membrane potential oscillations; persistent Na^+ current

INTRODUCTION

Neuropathic pain is a chronic pain syndrome caused by drug-, disease-, or injury-induced damage or destruction of sensory neurons within the dorsal root ganglia of the peripheral nervous system. This type of pain represents a mixture of pathophysiologic mechanisms, a complex assortment of spontaneous and elicited pain states, and somewhat an unpredictable response to analgesics. Previous investigations reported the reduction of deafferentation pain with i.v. lidocaine, suggesting a possible therapeutic value of i.v. lidocaine for managing intractable neuropathic pain syndromes (Boas *et al.* 1982, Kastrup *et al.* 1987, Bath *et al.* 1990). The peripheral mechanisms of neuropathic pain relief from i.v. lidocaine are thought that low concentration of lidocaine suppresses ectopic spontaneous discharges of injured nerve without blocking normal nerve conduction (Devor *et al.* 1992).

Ectopic spontaneous discharges were known to be generated in injured sensory nerve axons and their cell bodies in dorsal root ganglia (DRG). These spontaneous discharges enter the spinal cord and sensitize dorsal horn neurons (Devor and Seltzer. 1999, Obata *et al.* 2003). In the chronically compressed DRG (CCD) model of neuropathic pain, an important characteristic of the spontaneous activity from CCD neurons is that the patterns of bursting discharges were displayed by 42% of the spontaneous active units within two weeks after the chronic compression, and mechanical and thermal hyperalgesia were also obvious in the same period (Hu *et al.* 1998). The amounts of spontaneous discharges are generally well correlated with the degree of pain behavior in neuropathic pain rats (Han *et al.* 2000). Therefore, the

ectopic spontaneous discharges play a critical role for both initiation and maintenance of the neuropathic pain state (Michaelis *et al.* 2000, Liu *et al.* 2000). A key mechanism underlying ectopic discharges was thought to be subthreshold membrane potential oscillations (SMPO) (Amir *et al.* 1999, Hu *et al.* 1997). SMPO is enhanced after nerve injury or injury of the DRG (Liu *et al.* 2000). In addition, burst discharges in primary sensory neurons are triggered by SMPO and maintained by depolarizing afterpotentials (Amir *et al.* 2002). Thus, SMPO is regarded as the pacemaker of the generation of neuropathic pain and the target of drugs for the treatment of neuropathic pain (Xing *et al.* 2003, Xing *et al.* 2001). However, the mechanism underlying the SMPO is still uncertain.

Persistent Na^+ current (I_{NaP}) observed in a variety of neuronal types is slowly inactivating, and is associated with control of membrane excitability in the voltage region just subthreshold to spike production (Crill. 1996, Hutcheon and Yarom. 2000). In some types of neurons, such as those within the dorsal column nuclei (Reboreda *et al.* 2003), and trigeminal mesencephalic V nucleus (Wu *et al.* 2001), the generation of SMPO is dependent upon I_{NaP} . Accordingly, it can be presumed that the SMPO observed in injured DRG cells **was** also generated on I_{NaP} . However, experimental evidence is still lacking. This study sought to determine the effect and mechanism of low concentration of lidocaine on SMPO, burst discharges in chronically compressed DRG neurons by using whole-cell patch-clamp techniques.

MATERIALS AND METHODS

Surgery Experiments were conducted on young Sprague-Dawley rats (n=45, 74 \pm 3 g) of both sexes under an institutionally approved protocol. The chronically compressed DRG group (CCD group) was prepared (n = 23) according to the method described previously (Hu and Xing. 1998). Another group of rats (n = 22) served as unoperated controls.

Preparation of DRG neurons L₄ or L₅ DRG neurons from ipsilateral side of either control or CCD group were dissociated using enzyme digestion according to Zhang et al with small modifications (Zhang *et al.* 2001). Briefly, rats were deeply anesthetized, L₄ or L₅ DRG was isolated and incubated in 0.1% collagenase containing phosphate-buffered solution for 20 min followed by 20 min in 0.1% collagenase/dispase and 5-10 min in 0.25% trypsin at 37 °C. After washing out of enzymes, DRG were triturated with fire polished pipettes in Dulbecco's Modified Eagle Medium (DMEM), and cells were plated in a polyethylenimine treated plate mounted on an inverted microscope (DM 1L, leica, Germany). After the cells attached the bottom, the bath was perfused with oxygenated bathing solution at a flow rate of 2mL/min.

Chemicals and solutions collagenase, dispase, trypsin, DMEM, HEPES, Mg-ATP, 1, 4-aminopyridine (4-AP), tetraethylammol-chloride (TEA-Cl), CdCl₂, CsF, tetrodotoxin (TTX), lidocaine were purchased from Sigma. Other reagents were products of Xi'an Chemical reagent plant.

The bathing solution contained (in mmol/L): NaCl 150, MgCl₂ 1.0, KCl 5.0, CaCl₂ 5.0, Glucose 10, HEPES 10; pH was adjusted to 7.4 with NaOH. The pipette solution contained (in mmol/L): KCl 140, MgCl₂ 2.0, HEPES 10, Mg-ATP 2.0; pH was adjusted to 7.4 with KOH. The bathing solution for the measurement of Na⁺ currents contained (in mmol/L): NaCl 140, MgCl₂ 1.0, KCl 3.0, CaCl₂ 4-AP 3.0, TEA-Cl 10, CdCl₂ 0.1, HEPES 10; pH was adjusted to 7.4 with NaOH. The pipette solution for the measurement of Na⁺ currents contained (in mmol/L): CsF 140, EGTA 1.0, NaCl 10, HEPES 10; pH was adjusted to 7.4 with Tris.

Whole cell patch-clamp techniques Micropipettes (3-5 MΩ) were pulled with a vertical puller (PP-83, Narishige, Japan). Recordings were obtained from DRG neurons with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, USA). Cell diameter was estimated before patch clamping using an eyepiece micrometer at 400× magnification. Gigaseal formation and whole cell configuration were achieved in the bathing solution. Offset potential was nulled directly before formation of the seal. Liquid junction potential (<4 mV) was not corrected. Whole-cell capacitance and series resistance were corrected (usually 60-70%). Neurons were examined in order, as patched, accepting only those exhibiting resting potential (*V_r*) more negative than -40 mV and an overshooting single spike.

In current-clamp experiment, SMPO and burst discharges were elicited from *V_r* levels by delivering depolarizing ramp pulses of 1.5-s and less than 2 nA amplitude. Single spike was elicited from *V_r* levels by delivering depolarizing step pulses of 100 ms duration. In voltage-clamp experiment, I_{NaP} was elicited by a 3.5-s depolarizing

voltage ramp from holding potential of -80 mV to -30 mV (Rizzo *et al.* 1994). Transient Na⁺ currents (I_{NaT}) were elicited by applying hyperpolarizing prepulses (100 ms) to -120 mV for removal of inactivation followed by depolarizing voltages steps from the holding potential of -70 mV to -20 mV, by steps of 10 mV.

Data Acquisition and Analysis Signals were acquired at 10 KHz and filtered at 5 KHz with a digidata 1322A (Axon Instruments) and pCLAMP 9 software (Axon Instruments). All data were expressed as mean \pm SD, and analyzed for statistical significance ($P < 0.05$) by Student's t-test (group comparisons) and Chi-square test.

RESULTS

Different effects of lidocaine on SMPO, burst discharges and single spike

In the current-clamp experiment, recordings were obtained from 127 DRG neurons (30-40 μm) from CCD group (n=13) and 93 DRG neurons from control group (n=10). Input resistance was $254 \pm 51 \text{ M}\Omega$, capacity was $67 \pm 9 \text{ pF}$ and V_r was $-54.7 \pm 3.1 \text{ mV}$. The ratio of neurons exhibit SMPO and burst discharges was greater in CCD group (27/127, 21.3%) than that in control group (7/93, 7.5%) ($P < 0.05$). Amplitude and frequency of oscillations had no significant difference between the two groups.

SMPO was elicited from V_r levels by delivering depolarizing ramp current (less than 2nA). The amplitude and frequency of SMPO were voltage-sensitive, and burst discharges were triggered by SMPO when the amplitude of SMPO reaches threshold (Xing *et al.* 2001, Xing *et al.* 2003) (Fig. 1A, 1B). SMPO and burst discharges were lidocaine-sensitive, SMPO was suppressed and burst discharges were blocked by low concentration of lidocaine (10 $\mu\text{mol/L}$, n=13, CCD group) (Fig. 1A, 1B). Single spike was elicited by delivering depolarizing step current on the same neurons that exhibit SMPO and burst discharges (less than 0.4 nA). When SMPO and burst discharges were eliminated by low concentration of lidocaine (10 $\mu\text{mol/L}$), single spike could still be evoked. Single spike could only be blocked by high concentration of lidocaine (5 mmol/L, n=13, CCD group) (Fig. 1C).

Characteristics of I_{NaP} and its role in SMPO In the cells tested (n=9, CCD group), SMPO and burst discharges were reversibly abolished by TTX (0.2 $\mu\text{mol/L}$, Fig. 2A), suggesting the contribution of I_{NaP} as previously described for similar

oscillations in other cell types (Llinás *et al.* 1991, Pape and Driesang. 1998).

To further examine the relationship of I_{NaP} and SMPO, the voltage dependence of I_{NaP} was measured on the same neurons that exhibit SMPO and burst discharges in voltage-clamp mode (n=9, CCD group) (Hu *et al.* 2002). The membrane potential was held at -70 mV and voltage steps were delivered from hyperpolarized potentials to depolarized potentials. The steady-state current was measured at the end of the 800-ms-long steps before and after applying TTX (0.2 μ mol/L). The result showed a TTX-sensitive inward current, obtained by subtraction, was activated at positive potentials to -65 mV (Fig. 2B, 2C).

We also used slow, ascending voltage ramps (from -80 to -30 mV; 15 mV s⁻¹) to detect I_{NaP} in voltage-clamp mode (Rizzo *et al.* 1994). 4-AP (3.0 mmol/L), TEA-Cl (10 mmol/L), CdCl₂ (0.1 mmol/L) were added to block K⁺ and Ca²⁺ currents. 140 mmol/L Cs⁺ was substituted for the same concentration of K⁺ in the pipette, to further block K⁺ currents from inside. Voltage-clamp experiments confirmed the presence of I_{NaP} in most DRG neurons tested (38/46 in CCD group and 31/39 in control group, $P > 0.05$). The result showed a low threshold (-62.9 ± 3.5 mV) inward current peaking at -48.3 ± 3.1 mV, which was strongly blocked by 0.2 μ mol/L TTX (n=12, CCD group), these characteristics matching those of I_{NaP} (Fig. 2D) (Crill. 1996). I_{NaP} peak amplitude varied widely from cell to cell (range 105 - 289 pA; median 201 pA) in CCD group and control group, much less than the amplitude of I_{NaT} (6.5-10.7 nA). When the currents were normalized for differences in cell size, as indicated by changes in cell capacitance, we found that the current density of I_{NaP} was greater in

CCD group (4.6 ± 0.6 pA/pF, $n=38$) than that in control group (2.5 ± 0.4 pA/pF, $n=31$)($P < 0.05$). There **was** no significant difference of activation potential and maximum activation potential of I_{NaP} between CCD group and control group.

Different effects of lidocaine on I_{NaP} and I_{NaT} In order to discriminate the different effects of lidocaine on I_{NaP} and I_{NaT} , different concentrations of lidocaine were applied and their effects were observed ($n=10$, CCD group). The result **showed** the current traces of I_{NaP} were inhibited by low concentration of lidocaine ($10 \mu\text{mol/L}$) obviously (Fig. 3A). Low concentration of lidocaine ($10 \mu\text{mol/L}$) **had** little effect on the amplitude of I_{NaT} , only high concentration of lidocaine (5 mmol/L) could block I_{NaT} apparently (Fig. 3B).

DISCUSSION

The effectiveness of systemic lidocaine in relieving chronic pain has been recognized for over 20 years (Mao and Chen. 2000). The peripheral mechanisms of neuropathic pain relief from lidocaine therapy are thought to have its suppressive effects on spontaneous ectopic discharges of the injured nerve without blocking normal nerve conduction (Devor *et al.* 1992). Spontaneous ectopic discharges play a critical role for both initiation and maintenance of the neuropathic pain. Blocking spontaneous discharges attenuate pain behaviors both in neuropathic animal models and clinical cases (Gracely *et al.* 1992, Yoon *et al.* 1996). SMPO, which **can** be observed spontaneously or elicited by ramp depolarization, **is** proved to be a fundamental factor in the generation of abnormal spontaneous discharges, and **is** regarded as the pacemaker of chronic pain (Hu *et al.* 1997, Xing *et al.* 2003). Our results indicated that SMPO and burst discharges, were eliminated by low concentration of lidocaine (10 $\mu\text{mol/L}$). Single spike, which represents the normal nerve conduction, was not blocked by the same concentration of lidocaine. Data from clinical or experimental evidences indicate that the effective plasma concentration of lidocaine for chronic pain therapy is 1-2 $\mu\text{g/ml}$, **which equals to** 3.5-7 $\mu\text{mol/L}$ (Mao and Chen. 2000). The effective plasma concentration of lidocaine for pain therapy is similar with the concentration of lidocaine on SMPO. Therefore, we **presumed** that the suppressive effect of low concentration of lidocaine (10 $\mu\text{mol/L}$) on the SMPO may be involved in the peripheral effect of neuropathic pain relief.

It is known that I_{NaT} mediates the generation of single spike, but what is the ion

mechanism underlying the SMPO? The oscillation sinusoids of DRG neurons are due to an interaction between voltage-dependent, TTX-sensitive Na^+ conductance and passive, voltage-independent K^+ leak (Amir *et al.* 2002). Recent researches reveal that TTX-sensitive I_{NaP} mediates SMPO in entorhinal cortex and dorsal column nuclei neurons (Agrawal *et al.* 2001, Reboreda *et al.* 2003). In the present study, the SMPO were suppressed and burst discharges were eliminated by 0.2 $\mu\text{mol/L}$ TTX, suggesting the contribution of a persistent Na^+ current (I_{NaP}) in the generation of SMPO as previously described in other cell types (Llinás *et al.* 1991, Klink and Alonso. 1993).

I_{NaP} has been found in various type of neurons. The inactivation of I_{NaP} is very slow and the activation potentials of I_{NaP} is 10–15 mV below the threshold of transient Na^+ current (Klink and Alonso. 1993). It is believed that I_{NaP} may contribute to oscillations of DRG neurons and affect neurons excitability (Amir *et al.* 2002). Steady state current–voltage (I–V) plots of the data from the injured DRG neurons (exhibit SMPO and burst discharges) revealed a TTX-sensitive inward current which activation potentials was around -65 mV. We also used slow, ascending voltage ramps to detect the inward Na^+ current. The result showed that (delete) the characteristics of the inward current matching those of I_{NaP} (Crill. 1996) and indicated that I_{NaP} contributes to the generation of SMPO in injured DRG neurons. Furthermore, it is presumed that I_{NaP} was generated on the up-expression of sodium channel subunit Nav1.3 in injured DRG neurons (Lai *et al.* 2003).

As low concentration of lidocaine (10 $\mu\text{mmol/L}$) suppresses SMPO, and I_{NaP} mediates the generation of SMPO, the effect of lidocaine on I_{NaP} was examined. The

result showed that low concentration of lidocaine (10 $\mu\text{mol/L}$) inhibits the amplitude of I_{NaP} apparently, but the same concentration of lidocaine has little effect on the amplitude of I_{NaT} . Only high concentration of lidocaine (5 mmol/L) inhibits I_{NaT} apparently.

A new finding in the present study was that mechanisms underlying SMPO, burst discharges and single spike were different. The persistent Na^+ current was found to be mediated in the generation of SMPO in injured DRG neurons. Burst discharges, triggered by SMPO, have close relationship with neuropathic pain. Thus, I_{NaP} can be associated with neuropathic pain by SMPO. However, single spike, generated on transient Na^+ current, represents the normal nerve conduction. Our result suggested that low concentration of lidocaine (10 $\mu\text{mol/L}$) suppresses SMPO and blocks burst discharges by selectively inhibiting I_{NaP} , but not I_{NaT} , in injured DRG neurons. This effect of low concentration of lidocaine on I_{NaP} may be involved in the peripheral mechanism of neuropathic pain relief from systemic lidocaine. Furthermore, blockade of I_{NaP} might be a good target for blocking spontaneous discharge in neuropathic pain.

Acknowledgements

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Legends for figures

Figure 1 Different effects of lidocaine on subthreshold membrane potential oscillations (SMPO), burst discharges and single spike in injured DRG neurons. (A) The first line shows an example of SMPO elicited by depolarizing ramp current (1 nA). The amplitude of SMPO enhanced with the voltage depolarization, and burst discharges were triggered by SMPO. The next line shows burst discharges were blocked and SMPO were suppressed by low concentration of lidocaine (10 $\mu\text{mol/L}$). The last line shows SMPO and burst discharges restored after washout. (B) Illustration of each recording around the arrow on the left on an enlarged scale. Spike height was truncated. (C) Single spike elicited by depolarizing step current (0.2 nA) in the same DRG neuron. Little effect was observed on spike amplitude by low concentration of lidocaine (10 $\mu\text{mol/L}$). Single spike was blocked by high concentration of lidocaine (5 mmol/L).

Figure 2 Properties of the persistent Na^+ current (I_{NaP}) in injured DRG neurons. (A) The first line shows an example of SMPO elicited by depolarizing ramp current (0.5 nA). The amplitude of SMPO enhances with the voltage depolarization, and burst discharges were triggered by SMPO. The next line shows the burst discharges were blocked and the SMPO were suppressed by tetrodotoxin (TTX, 0.2 $\mu\text{mol/L}$). The last line shows the SMPO and burst discharges restored after washout. (B) The membrane current of the same DRG neuron responding to voltage steps at different membrane potentials (-105 mV to -55 mV), before and after applying TTX (0.2 $\mu\text{mol/L}$). (C)

Steady state current–voltage (I–V) plots of the data from the same DRG neuron. The current was measured at the end of the 800-ms voltage step, before and after applying TTX, noted that applying TTX reduced the current at more depolarized potentials than -65 mV. (D) I_{NaP} was elicited under slowly increasing ramp voltage stimulation from -80 to -30 mV, illustrating the current was activated at -65 mV and increased with depolarization. I_{NaP} was blocked by TTX (0.2 μ mol/L).

Figure 3 Different effects of lidocaine on I_{NaP} and transient Na^+ current (I_{NaT}). (A) One example of inhibitory effect of lidocaine on I_{NaP} . I_{NaP} was elicited under slowly increasing ramp voltage stimulation from -80 to -30 mV, and was inhibited by low concentration of lidocaine (10 μ mol/L) apparently. (B) I_{NaT} was elicited under depolarizing step current (0.2 nA) in the same neuron. Little effect was observed on the amplitude of I_{NaT} by low concentration of lidocaine (10 μ mol/L). I_{NaT} was blocked by high concentration of lidocaine (5 mmol/L).

Fig.1

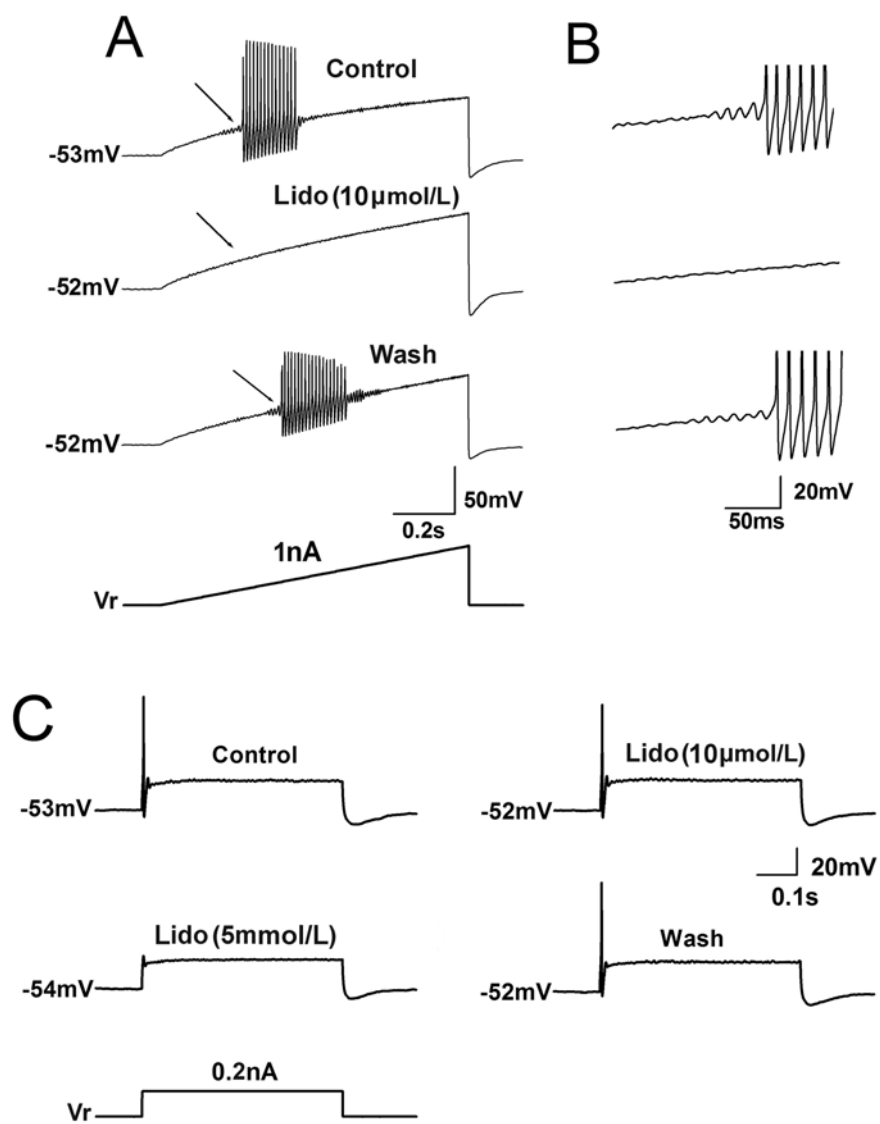


Fig.2

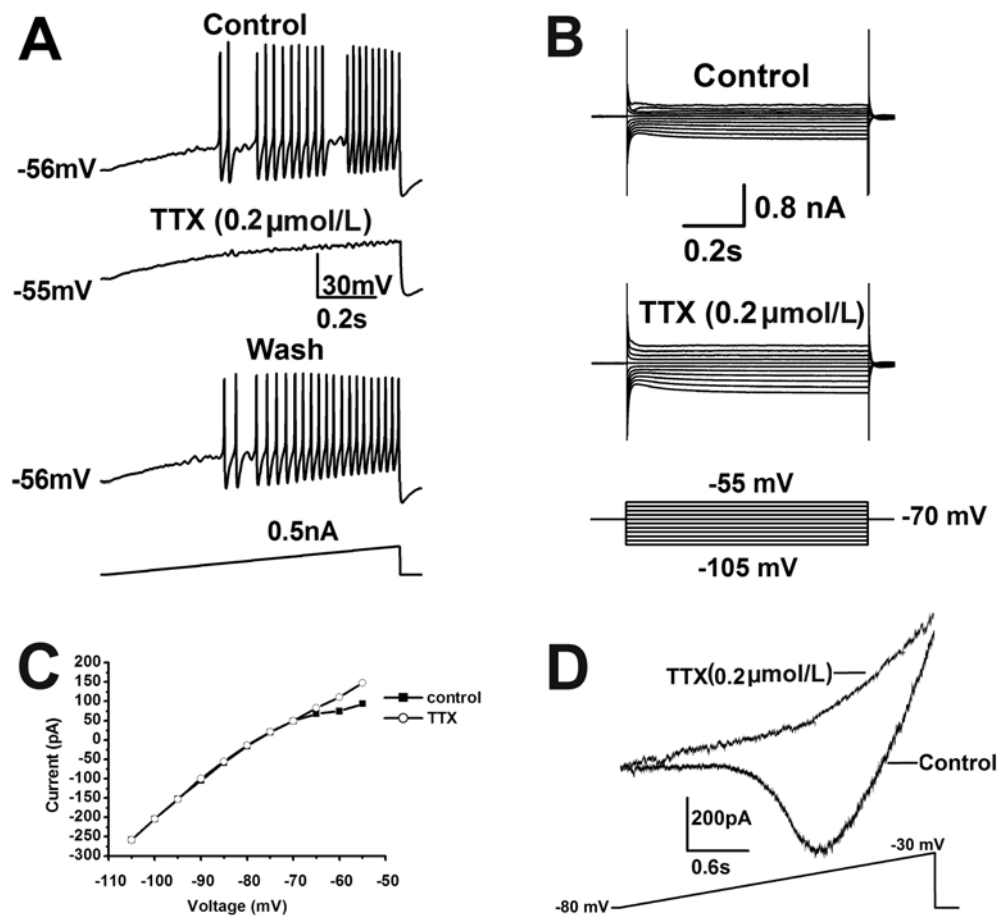


Fig.3

