Contribution of GABA<sub>A</sub>, Glycine, and Opioid Receptors to Sacral Neuromodulation of Bladder Overactivity in Cats

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Abstract

In α-chloralose anesthetized cats, we examined the role GABA\textsubscript{A} receptors in sacral neuromodulation-induced inhibition of bladder overactivity elicited by intravesical infusion of 0.5% acetic acid (AA). AA irritation significantly (p<0.01) reduced bladder capacity to 59.5±4.8% of saline control. S1 or S2 dorsal root stimulation at threshold intensity for inducing reflex twitching of the anal sphincter or toe significantly (p<0.01) increased bladder capacity to 105.3±9.0% and 134.8±8.9% of saline control, respectively. Picrotoxin, a GABA\textsubscript{A} receptor antagonist administered i.v. blocked S1 inhibition at 0.3 mg/kg and blocked S2 inhibition at 1.0 mg/kg. Picrotoxin (0.4 mg, i.t.) did not alter the inhibition induced during S1 or S2 stimulation, but unmasked a significant (p<0.05) post-stimulation inhibition that persisted after termination of stimulation. Naloxone, an opioid receptor antagonist, (0.3 mg, i.t.) significantly (p<0.05) reduced pre-stimulation bladder capacity and removed the post-stimulation inhibition. Strychnine, a glycine receptor antagonist, (0.03-0.3 mg/kg, i.v) significantly (p<0.05) increased pre-stimulation bladder capacity but did not reduce sacral S1 or S2 inhibition. After strychnine (0.3 mg/kg, i.v.) picrotoxin (0.3 mg/kg, i.v.) further (p<0.05) increased pre-stimulation bladder capacity and completely blocked both S1 and S2 inhibition. These results indicate that supraspinal GABA\textsubscript{A} receptors play an important role in sacral neuromodulation of bladder overactivity, while glycine receptors only play a minor role to facilitate the GABA\textsubscript{A} inhibitory mechanism. The post-stimulation inhibition unmasked by blocking spinal GABA\textsubscript{A} receptors was mediated by an opioid mechanism.
Introduction

Overactive bladder (OAB) symptoms are characterized by urinary urgency, frequency, and nocturia with or without incontinence (Abrams et al., 2002). OAB affects more than 30 million adults in United States (Coyne et al., 2011). Currently, antimuscarinic drugs are the first-line pharmacotherapy for OAB, but have a limited efficacy with significant adverse effect (Andersson et al., 2003, 2004; Chapple et al., 2008). If pharmacotherapy fails, sacral neuromodulation is one of the alternative treatment options for OAB. Although this therapy has been approved by the US Food and Drug Administration to treat OAB for more than a decade (Schmidt et al., 1999; van Kerrebroeck et al., 2007), its mechanism of action is still uncertain (Elkelini et al., 2010). The initial event in sacral neuromodulation is the activation of primary afferent nerves that project into the spinal cord and trigger the release of neurotransmitters that in turn modulate the neural pathways controlling bladder function. Unfortunately, little is known about the identity of the neurotransmitters, the receptors that they activate, or their site of action. The present experiments were undertaken to address these issues.

Our previous studies in cat revealed that spinal GABA_A receptors play an important role in pudendal neuromodulation of bladder overactivity (Xiao et al., 2014), while opioid and glycine receptors have no or a minor role (Mally et al., 2013; Rogers et al., 2015). On the other hand we showed that opioid receptors have an essential role in tibial neuromodulation of bladder overactivity in the cat (Tai et al., 2012). Because afferent axons passing through the pudendal and tibial nerves enter the spinal cord through the sacral S1-S2 dorsal roots, it is possible that sacral neuromodulation activates these afferents in S1-S2 dorsal roots and might mimic some or all of the effects of pudendal/tibial neuromodulation. Therefore in this study we examined the effects of a GABA_A receptor antagonist (picrotoxin), a glycine receptor antagonist (strychnine), and an opioid receptor antagonist (naloxone) on the modulation of
Methods and Materials

The protocol and animal use in this study were approved by Animal Care and Use Committee at the University of Pittsburgh.

Surgical procedures

A total of 20 cats (9 males and 11 females, 2.7-5.0 kg; Liberty Research, Waverly, NY) were used in this study. The animals were anesthetized with isoflurane (2-5% in oxygen) during surgery and then switched to α-chloralose anesthesia (initial 65 mg/kg followed by slow i.v. infusion at 2 mg/kg per hour) during data collection. Pancuronium (initial 0.1 mg/kg followed by slow i.v. infusion at 0.1 mg/kg per hour) was also given during data collection to prevent striated muscle contractions and movement of the animal. Right and left cephalic veins were catheterized for i.v. administration of drugs and fluid. A tracheotomy was performed and a tube was inserted to keep the airway patent. A catheter was inserted into right carotid artery to monitor systemic arterial blood pressure. The mean systemic blood pressure was measured as the average of systolic and diastolic blood pressures. Heart rate and blood oxygen were monitored by a pulse oximeter (9847V; NONIN Medical, Plymouth, MN) attached to the tongue. Through an abdominal incision, the ureters were isolated, tied, and cut for external drainage. A double lumen catheter was inserted through the urethra into the bladder and secured by a ligature around the urethra. One lumen was connected to a pump to slowly (1-3 ml/min) infuse saline or 0.5% acetic acid (AA) in saline. The other lumen was attached to a pressure transducer to measure bladder pressure. After the surgery, the skin and muscle layers were closed by sutures.

The spinal cord and cauda equina were exposed between the L7 and Cx1 vertebrae.
via a dorsal laminectomy. The spinal dura was cut and the S1 and S2 dorsal roots on the right side were separated for electrical stimulation. A bipolar stainless steel hook electrode was used during the experiment to stimulate individual S1/S2 dorsal roots by delivering electrical pulses that were generated by an electrical stimulator (S88, Grass Medical Instruments, Quincy, MA). The animal was mounted in a modified Narishige “Eccles” spinal cord frame in which the hip was supported by metal pins, and the spinous process at the rostral end of the laminectomy was secured with a clamp. The skin, cut mid-sagittally from L4 to S3, was tied along each margin to form a pool that was filled with warmed (35-37°C) mineral oil. The temperature of the animal was maintained at 36-38°C using a heating pad during the experiments. In 5 cats, a small catheter (PE10) was inserted rostrally underneath the dura to position the catheter tip between S1 and S2 spinal cord for i.t. administration of picrotoxin or naloxone. The location of the intrathecal catheter was confirmed by a postmortem laminectomy between the L5-L6 spinal processes.

**Stimulation protocol and drug administration**

Our previous study in cats (Zhang et al., 2013) showed that reflex bladder activity could be inhibited by electrical stimulation (5 Hz frequency and 0.2 ms pulse width) of S1 or S2 dorsal roots at threshold (T) intensity for inducing reflex twitching of the anal sphincter or toe, while stimulation of S3 dorsal root or S1-S3 ventral roots was not effective. Therefore, stimulation (5 Hz, 0.2 ms) of S1 or S2 dorsal roots at motor threshold intensity was used in this study to inhibit reflex bladder activity. The motor threshold was determined before administering pancuronium.

At the beginning of each experiment, multiple cystometrograms (CMGs) were performed by slowly infusing the bladder with saline to determine the bladder capacity that was defined as the bladder volume threshold to induce a bladder contraction of large
amplitude (>30 cmH₂O) and long duration (>20 secs). Then, 0.5% AA was infused into the bladder to irritate the bladder and induce bladder overactivity. Once the control bladder capacity stabilized during repeated AA CMGs, the inhibitory effect of sacral dorsal root stimulation was determined by additional four AA CMGs: (1). Control CMG without stimulation; (2). CMG during S1 dorsal root stimulation; (3). CMG during S2 dorsal root stimulation; (4) Control CMG again to examine any post-stimulation effect. Then, the animals were divided into 3 experimental groups.

In the first group (N=9 cats), cumulative doses (0.01, 0.03, 0.1, 0.3 and 1.0 mg/kg, i.v.) of picrotoxin (Sigma-Aldrich, St. Louis, MO) were given. In the second group (N=6 cats), strychnine (Sigma–Aldrich, St. Louis, MO) was administered in cumulative doses (0.001, 0.003, 0.01, 0.03, 0.1, and 0.3 mg/kg, i.v.) followed by picrotoxin (0.3 mg, i.v.). In the third group (N=5 cats), a single dose (0.4 mg in 0.2 mL saline, i.t.) of picrotoxin was given, which was followed by a single dose (0.3 mg in 0.1 mL saline, i.t.) of naloxone. The dosage of each drug is chosen based on our previous studies (Hisamitsu and de Groat, 1984, Rogers et al., 2015, Xiao et al., 2014). After administering each dose of drug, the four CMGs (control, S1 stimulation, S2 stimulation, control) were repeated to determine the drug effects. A ten minute waiting period for each i.v. dose of picrotoxin or strychnine and a five minute period for i.t. picrotoxin or naloxone were used to allow time for the drugs to take effect. A waiting period of 2-3 minutes was also used between CMGs to allow the bladder reflex to recover. Our previous studies (Rogers et al., 2015, Xiao et al., 2014) showed that the effects of picrotoxin or strychnine lasted long enough to perform the four repeated CMGs that required about 30-40 minutes.

Data analysis

The bladder capacity was measured from each CMG and normalized to the capacity
measured during the first control CMG in different test groups. Repeated measurements in the same animal under the same conditions were averaged. The normalized data from different animals were presented as mean ± standard error. Statistical significance (p<0.05) was determined by a paired Student’s $t$-test or ANOVA followed by Bonferroni multiple comparisons.

**Results**

*Inhibition of bladder overactivity by S1 or S2 dorsal root stimulation*

AA irritation induced bladder overactivity and significantly (p<0.01) reduced bladder capacity to 59.5±4.8% of saline control capacity (N=20 cats, Fig.1). S1 or S2 dorsal root stimulation at threshold intensity for inducing reflex twitching of anal sphincter or toe inhibited bladder overactivity and significantly (p<0.01) increased bladder capacity to 105.3±9.0% and 134.8±8.9% of saline control, respectively. After the stimulation, AA control capacity returned to pre-stimulation level, indicating that there was no post-stimulation effect (Fig. 1B).

*Effect of i.v. picrotoxin on sacral inhibition of bladder overactivity*

Picrotoxin (i.v.) slightly increased the pre-stimulation bladder capacity at 0.3-1.0 mg/kg doses (first column CMGs in Fig.2A), but the increase was not statistically significant (p>0.05, N=9 cats, Fig.2B). The 0.3 mg/kg dose of picrotoxin blocked (p<0.05) the increase in bladder capacity elicited by S1 dorsal root stimulation but not the increase elicited by S2 dorsal root stimulation (Fig.2B). Picrotoxin at 1 mg/kg blocked (p<0.05) the increase in bladder capacity induced by either S1 or S2 dorsal root stimulation (Fig.2B). After the stimulation, the bladder capacity returned to pre-stimulation level at every dose of picrotoxin, i.e. no post-stimulation effect. Mean systemic blood pressure (control: 163.9±4.6 mmHg)
was not changed after any dose of picrotoxin (162.0±4.5 mmHg).

Combined effect of i.v. strychnine and picrotoxin on sacral inhibition of bladder overactivity

Strychnine at 0.03-0.3 mg/kg (i.v.) significantly (p<0.05) increased the pre-stimulation bladder capacity without affecting the increase in bladder capacity caused by S1 or S2 dorsal root stimulation (N=6 cats, Fig.3). Mean systemic blood pressure (control: 154.2±1.8 mmHg) was not changed after any dose of strychnine (150.6±3.8 mmHg). Following strychnine treatment, picrotoxin (0.3 mg/kg, i.v.) further significantly (p<0.05) increased the pre-stimulation bladder capacity and blocked the inhibition induced by S1 or S2 dorsal root stimulation (N=6 cats, Fig.4), while the same dose of picrotoxin without strychnine pre-treatment only blocked the inhibition induced by S1 but not S2 dorsal root stimulation (see Fig.2B). There was no post-stimulation effect at any dose of the drugs.

Effect of i.t. picrotoxin and naloxone on sacral inhibition of bladder overactivity

Picrotoxin (0.4 mg, i.t.) did not significantly change the pre-stimulation bladder capacity and had no effect on the capacity increase induced by either S1 or S2 dorsal root stimulation (N=5 cats, Fig.5). However, after sacral dorsal root stimulation the post-stimulation bladder capacity was significantly (p<0.05) increased (about 100%), i.e. the stimulation induced a significant post-stimulation inhibitory effect (Fig.5). Following picrotoxin treatment, naloxone (0.3 mg, i.t.) significantly (p<0.05) reduced the pre-stimulation bladder capacity and removed the post-stimulation inhibition induced by sacral dorsal root stimulation (Fig.6). However, even after i.t. administration of both picrotoxin and naloxone S1 or S2 dorsal root stimulation still significantly (p<0.05) increased bladder capacity during stimulation (Fig.6).
Discussion

In this study the effects of selective receptor antagonists administered alone or in combination revealed that GABA, glycine and opioids contribute in varying ways to sacral neuromodulation of bladder overactivity in anesthetized cats. GABA acting on GABAA receptors at supraspinal sites plays a major role in sacral neuromodulation (Figs.2 and 5); while glycine seems to have a minor role to facilitate the GABAergic inhibition (Figs.3 and 4). On the other hand, spinal opioid mechanisms have an unusual function. They do not contribute to the increase in bladder capacity elicited during stimulation of the S1 or S2 dorsal roots but do contribute to the post-stimulation increase in capacity that is unmasked by blocking GABAA receptors in the spinal cord with i.t. administration of picrotoxin (Figs.5 and 6). The latter observation suggests that in anesthetized cats sacral neuromodulation activates a spinal opioid inhibitory pathway which however is suppressed by a tonic GABAergic mechanism. These results suggest that sacral neuromodulation of bladder overactivity is mediated by a complex interplay between multiple transmitter mechanisms at spinal and supraspinal sites.

The involvement of GABAA receptors in sacral neuromodulation is very different from their involvement in pudendal neuromodulation. Our previous study in cats (Xiao et al., 2014) showed that spinal GABAA receptors play a critical role in pudendal inhibition of bladder overactivity because i.t. picrotoxin can completely remove the inhibition. In contrast, our current study indicates that spinal GABAA receptors are not involved in inhibition of bladder overactivity elicited by sacral neuromodulation (Fig.5). However, this inhibition is reduced by high doses (0.3-1 mg/kg, i.v) of picrotoxin (Fig.2) while pudendal inhibition is reduced by low doses (0.01-0.1 mg/kg) (Xiao et al., 2014). These results indicate that GABAA receptors in the spinal cord are important for pudendal neuromodulation of bladder overactivity while the receptors in the brain play a critical role in sacral neuromodulation of...
bladder overactivity. However, the exact sites of action in the brain still need to be determined in future studies.

The involvement of glycine receptor in sacral neuromodulation is also different from its involvement in pudendal neuromodulation. Our previous study in cats (Rogers et al., 2015) showed that strychnine at low doses (0.001-0.003 mg/kg, i.v.) reduces pudendal inhibition of bladder overactivity and at a high dose (0.3 mg/kg, i.v.) unmasks a post-stimulation excitatory effect on the overactive bladder reflex. However, in this study strychnine (i.v.) did not change the inhibition of bladder overactivity elicited by sacral neuromodulation and did not cause post-stimulation excitation (Fig.3). Instead, strychnine (0.3 mg/kg, i.v.) reduced the intravenous dosage of picrotoxin from 1 mg/kg to 0.3 mg/kg necessary to completely eliminate sacral inhibition of bladder overactivity (Fig.2 and Fig.4), indicating a synergistic interaction between glycine and GABA_A mechanisms. Please note that the 0.3 mg/kg of picrotoxin is a cumulative dose in Fig.2 but it is a single dose in Fig.4 which includes the data for the interaction between strychnine and picrotoxin.

Due to the pharmacokinetics of the drug, a cumulative dose may not be exactly same as the single dose. However, the different dosing methods should produce similar effects in this study because: 1. a logarithmic increase of the drug dosage was used which allowed an overlap of the effects of the two largest doses of the drug; 2. our previous study (Xiao et al., 2014) showed that the effect of picrotoxin can last more than 50 minutes for the period of performing the 4 repeated CMGs. A better designed dosing study is warranted to further determine the interaction between GABA_A and glycine mechanisms.

It is known that inhibitory interneurons in the spinal cord can synthesize and release GABA and glycine as inhibitory co-transmitters (Todd et al., 1996). A previous study in cat (Thomson and Franz, 1981) also revealed that neither picrotoxin nor strychnine administered alone increased firing in the parasympathetic efferent excitatory pathway to the bladder
induced by stimulation of bulbospinal excitatory axons; while co-administration of the two antagonists markedly increased the firing. The authors concluded that GABA and glycine act synergistically to generate tonic inhibition of the micturition reflex. In addition, a patch clamp study in neonatal rat spinal cord slices showed that focal electrical stimulation in the region of the sacral preganglionic neurons elicited inhibitory postsynaptic currents mediated by glycine and GABA (Araki, 1994). However, the interaction between glycine and GABA mechanisms revealed in this study probably occurs in the brain rather than in the spinal cord, because spinal GABA_A receptors do not contribute to sacral inhibition of bladder overactivity (Fig.5). Further studies to determine the supraspinal interaction between glycine and GABA_A mechanisms in sacral inhibition of bladder overactivity are certainly warranted.

There are some similarities between sacral neuromodulation and tibial neuromodulation. Similar to tibial neuromodulation (Ferroni et al., 2015), sacral neuromodulation only produced post-stimulation inhibition of reflex bladder activity during saline cystometry (Zhang et al., 2013) but not during AA cystometry that induces bladder overactivity (Fig.1). However, blocking spinal GABA_A receptors by picrotoxin (i.t.) unmasked a significant post-stimulation inhibition during AA infusion (Figs.5-6). Naloxone (i.t.) eliminated this post-stimulation inhibition, indicating that the inhibition was mediated by spinal opioid receptors. Similarly, tibial neuromodulation in cats produced a significant post-stimulation inhibition during AA infusion after activating opioid receptors by administration (i.v.) of tramadol (an opioid receptor agonist) (Zhang et al., 2012). These results indicate that opioid mechanisms play a critical role in the post-stimulation inhibition induced by both tibial and sacral neuromodulation during AA irritation of the bladder. It is worth noting that the vehicle (saline) control for i.t. picrotoxin (Fig.5) was not performed because it is assumed that the small amount (0.2 mL) of i.t. saline would have no effect. In addition, the interaction between GABA_A and opioid mechanisms on post-stimulation effect...
should be further investigated with systemic dosing of these drugs.

This study only tested a single drug (picrotoxin or strychnine) for each neurotransmitter mechanism. Further testing of different drugs is needed to confirm the results of this study and eliminate possible nonspecific effects related to testing a single drug. In addition, the small number of cats in each drug test (6 or 9 cats/group) cannot determine the variability related to sex, size, and age of the animals. More studies are certainly needed to further examine these issues.

In summary, comparing this study of sacral neuromodulation to previous studies of pudendal or tibial neuromodulation showed that the GABA_A and glycine mechanisms are involved differently in sacral and pudendal neuromodulation, while the post-stimulation inhibition induced by either sacral or tibial neuromodulation under different conditions is mediated by the same neurotransmitter (opioid) mechanism. These results suggest that the mechanisms underlying sacral neuromodulation could be different from pudendal/tibial neuromodulation, but at the same time they may also share some properties. Understanding neurotransmitter mechanisms involved in sacral neuromodulation could further improve this effective OAB treatment and identify molecular targets for developing new treatments for OAB.

Authorship Contributions

Participated in research design: Jiang, Fuller, Bandari, Bansal, Zhang, Shen, Wang, Roppolo, de Groat, Tai.

Conducted experiments: Jiang, Fuller, Bandari, Bansal, Zhang, Shen, Wang, Roppolo, de Groat, Tai.

Contributed new reagents or analytic tools: Jiang, Fuller, Bandari, Bansal, Zhang, Shen, Wang, Roppolo, de Groat, Tai.
Performed data analysis: Jiang, Fuller, Bandari, Bansal, Zhang, Shen, Wang, Roppolo, de Groat, Tai.

Wrote or contributed to the writing of the manuscript: Jiang, Fuller, Bandari, Bansal, Zhang, Shen, Wang, Roppolo, de Groat, Tai.

References


Footnotes

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Figure Legends

Fig.1. Inhibition of bladder overactivity by S1 or S2 dorsal root (DRT) stimulation at motor threshold (T) intensity. A. Repeated CMGs during saline or 0.5% acetic acid (AA) infusion with or without DRT stimulation. Black bars under the bladder pressure traces indicate stimulation duration. Stimulation: 5 Hz, 0.2 ms, T=0.3 V for S1, T=0.18 V for S2. Infusion rate = 3 ml/min. B. Normalized bladder capacity measured under different conditions (N = 20 cats). * indicates significantly (p<0.01) different from the AA control (one-way ANOVA).

Fig.2. Effect of picrotoxin (i.v.) on the inhibition of bladder overactivity by S1 or S2 dorsal root (DRT) stimulation at motor threshold (T) intensity. A. Repeated CMGs at different cumulative doses of picrotoxin were performed during acetic acid (AA) infusion with or without S1 or S2 DRT stimulation. Black bars under the pressure trace indicate stimulation duration. Stimulation: 5 Hz, 0.2 ms, T=0.3 V for S1, T=0.16 V for S2. Infusion rate = 1 ml/min. B: Normalized bladder capacity measured under different conditions (N=9 cats). * indicates significantly (p<0.05) different from the pre-stimulation group (two-way ANOVA). # indicates significantly (p<0.05) different from the untreated condition in the S2 DRT group (one-way ANOVA).

Fig.3. Effect of strychnine (i.v.) on the inhibition of bladder overactivity by S1 or S2 dorsal root (DRT) stimulation at motor threshold (T) intensity. A. Repeated CMGs at different cumulative doses of strychnine were performed during acetic acid (AA) infusion with or without S1 or S2 DRT stimulation. Black bars under the pressure trace indicate stimulation duration. Stimulation: 5 Hz, 0.2 ms, T=0.3 V for S1, T=0.18 V for S2. Infusion rate = 3 ml/min. B: Normalized bladder capacity measured under different conditions (N=6 cats). * indicates significantly (p<0.05) different from the pre-stimulation group (two-way ANOVA).
# indicates significantly (p<0.05) different from the untreated condition in the same group (one-way ANOVA).

Fig.4. Effect of picrotoxin (i.v.) on the inhibition of bladder overactivity by S1 or S2 dorsal root (DRT) stimulation at motor threshold intensity in strychnine (i.v.) pretreated cats. A: Repeated CMGs were performed during acetic acid (AA) infusion with or without S1 or S2 DRT stimulation in a cat pretreated with strychnine (0.3 mg/kg, i.v) followed by picrotoxin (0.3 mg/kg, i.v.). Note: the CMG traces for 0.3 mg/kg strychnine are from the same cat as shown in Fig.3A. Black bars under the pressure trace indicate stimulation duration. Stimulation: 5 Hz, 0.2 ms, T=0.3 V for S1, T=0.18 V for S2. Infusion rate = 3 ml/min. B: Normalized bladder capacity measured under different conditions (N=6 cats). * indicates a significant (p<0.05) difference between the pre-stimulation capacities before and after picrotoxin treatment (t-test). # indicates significantly (p<0.05) different from the pre-stimulation in strychnine pre-treated group (one-way ANOVA).

Fig.5. Effect of picrotoxin (i.t.) on the inhibition of bladder overactivity by S1 or S2 dorsal root (DRT) stimulation at motor threshold (T) intensity. A: Repeated CMGs were performed during acetic acid (AA) infusion with or without S1 or S2 DRT stimulation. Black bars under the pressure trace indicate stimulation duration. Stimulation: 5 Hz, 0.2 ms, T=0.4 V for S1, T=0.18 V for S2. Infusion rate = 2 ml/min. B: Normalized bladder capacity measured under different conditions. # indicates significantly (p<0.05) different from the pre-stimulation in the untreated or picrotoxin treated group (one-way ANOVA).

Fig.6. Effect of naloxone (i.t.) on the inhibition of bladder overactivity by S1 or S2 dorsal root (DRT) stimulation at motor threshold (T) intensity in picrotoxin (i.t.) pretreated cats. A: Repeated CMGs were performed during acetic acid (AA) infusion with or without S1 or S2
DRT stimulation in a cat pretreated with picrotoxin (i.t.) followed by naloxone (i.t.) treatment. Black bars under the pressure trace indicate stimulation duration. Stimulation: 5 Hz, 0.2 ms, T=0.3 V for S1, T=0.24 V for S2. Infusion rate = 2 ml/min. B: Normalized bladder capacity measured under different conditions. * indicates a significant (p<0.05) difference between the pre-stimulation capacities before and after naloxone treatment (t-test). # indicates significantly (p<0.05) different from the pre-stimulation in picrotoxin or naloxone treated group (one-way ANOVA).
**Figure 1**

(A) Start Infusion

Saline

AA

AA

S1 DRT

AA

S2 DRT

Stop infusion

(B) Nomorlized Capacity (%)

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>AA</th>
<th>S1 DRT</th>
<th>S2 DRT</th>
<th>AA</th>
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<tr>
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* indicates significant difference.
Figure 2

A

Pre-Stimulation

Start Infusion

Untreated

0.01 mg/kg picrotoxin

0.03 mg/kg picrotoxin

0.1 mg/kg picrotoxin

0.3 mg/kg picrotoxin

1.0 mg/kg picrotoxin

Start Infusion

Stop infusion

S1 DRT

Start Infusion

Stop infusion

S2 DRT

Start Infusion

Stop infusion

200 sec

100 cmH₂O

B

Normalized Capacity (%)

0 100 200 300 400

0 0.01 0.03 0.1 0.3 1.0

Picrotoxin (mg/kg)

S2 DRT

S1 DRT

Pre-Stimulation

Figure 2
Figure 3
Figure 4
Figure 5

(A) Pre-Stimulation  S1 DRT  S2 DRT  Post-Stimulation

Start Infusion
Untreated

0.4 mg picrotoxin

100 cmH₂O  200 sec

Stop Infusion

(B) Normalized Capacity (%)

Untreated  Picrotoxin

Pre-Stimulation  S1 DRT  S2 DRT  Post-Stimulation

# # # #
Figure 6

(A) Figure 6A shows the experimental protocol with pre-stimulation, S1 DRT, S2 DRT, and post-stimulation phases. The infusion starts with picrotoxin at 0.4 mg and naloxone at 0.3 mg. The infusion is stopped after 80 cmH₂O pressure for 200 sec.

(B) Figure 6B depicts the normalized capacity (%) comparison between picrotoxin and naloxone. The normalized capacity is measured pre-stimulation, S1 DRT, S2 DRT, and post-stimulation. Significant differences are indicated by * and #.