Acute anal stretch inhibits NMDA-dependent pelvic-urethra reflex potentiation via spinal GABAergic inhibition in anesthetized rats

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Running title: Anal stretch inhibits SRP

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ABSTRACT

The impact of acute anal stretch on the pelvic-urethra reflex potentiation was examined in urethane-anesthetized rats by recording the external urethra sphincter electromyogram (EUSE) activity evoked by the pelvic afferent stimulation. Test stimulation (TS, 1 stimulation/30 sec) evoked a baseline reflex activity with a single action potential that was abolished by gallamine (5 mg/kg, i.v.). On the other hand, the repetitive stimulation (RS, 1 stimulation/1 sec) induced spinal reflex potentiation (SRP) that was attenuated by intrathecal CNQX (a glutamatergic AMPA receptor antagonist, 100 μM, 10 μl) and APV (a glutamatergic NMDA antagonist, 100 μM, 10 μl). Acute anal stretch using a mosquito clamp with a distance of 4 mm exhibited no effect, while with distances of 8 mm attenuated and of 12 mm abolished the repetitive stimulation-induced SRP. Intrathecal NMDA (100 μM, 10 μl) reversed the abolition on SRP caused by anal stretch. On the other hand, pretreated bicuculline (GABA A receptor antagonist, 100 μM, 10 μl) but not hydroxysaclofen (GABA B receptor antagonist) counteracted the abolition on the repetitive stimulation-induced SRP caused by the anal stretch. All the results suggested that anal stretch may be used as an adjunct to assist voiding dysfunction in patients with overactive urethra sphincter, and that GABAergic neurotransmission is important in the neural mechanisms underlying external urethra sphincter activity inhibited by anal stretch.

Key words: spinal cord, voiding dysfunction, glutamate, bicuculline, hydroxysaclofen
INTRODUCTION

The mechanism involved in micturition, which is the result of coordination between bladder detrusor and outlet, is intricate. Both elements, the detrusor and outlet, maintain a sound cycle of storage and evacuation that is controlled by a group of reflex and voluntary actions (Shefchyk, 2002). During the storage phase of a micturition cycle, detrusor relaxes and urethra contracts to produce continence; while detrusor contracts in associated with sphincter relax to void urine during the evacuation phase. Inhibition of external urethra sphincter (EUS) activity during evacuation is essential for sufficient bladder emptying (Fedirchuk et al., 1994; Sackman and Sims 1990). The absence of suppression on external urethra sphincter activity during voiding is one feature of pathological condition referred to as detrusor-sphincter dyssynergia (Sethi et al., 1989). Such a dyssynergic sphincter contraction results in high intravesical pressure and residual urine. Therefore, to achieve near normal voiding function in patients with detrusor-sphincter dyssynergia, outlet resistance should be reduced.

Arising from the puborectalis muscle, EUS innervated by the perineal branch of the pudendal nerve and external anal sphincter (EAS) innervated by the rectal branch of the pudendal nerve, are both striated skeletal muscles that contract and relax voluntarily. A study investigating the coordination between the EUS and EAS muscles demonstrated these muscles shared in reflex actions as in dilatation and closing anal reflexes (Shafik, 1991). It is interesting that inserting examining fingers into anus for anal stretch caused marked inhibition of the electromyogram (EMG) activity in both EUS and EAS (Rodriquez and Awad, 1979). In able-bodied persons, EMG recording from the EUS and EAS during micturition and cystometry also showed simultaneous electric activity in these muscles (Scott et al. 1964; Abramson
et al. 1966; Vereecken and Verduyn 1970). Results coming from clinical studies suggested anal stretch could be a useful technique to facilitate voiding in overactive urethra sphincter patients (Donovan et al., 1977; Low and Donovan 1981; O’shaughnessy et al., 1981). However, the effects of anal stretch to EUS in normal individuals have yet been established in the literatures.

Cross-talk via the convergent neural pathways within the lumbosacral spinal cord is important for the normal regulation of sexual, bowel and bladder functions (Janig and Koltzenbug, 1990; Pezzone et al, 2005). Alterations in these convergent neural pathways cause a pathological mechanism by which injury or inflammation in one organ may lead to modifications in the function of other organs (Aziz and Thompson, 1998). In the pelvis, chemical and mechanical irritation in urethra may enhance the activity of not only striated urethra sphincter muscle itself, but also external anal sphincter, implying a neural mediated cross-talk existed between external anal and urethra sphincter (Thor and Muhlhauser, 1999).

The pelvic-urethra reflex activity is presumed to be involved in the developing of urethral resistance (de Groat and Yoshimura 2001). Recent studies on pelvic-urethra reflex, using intact spinal cord preparations, have demonstrated a glutamatergic N-methyl-D-aspartate (NMDA)-dependent spinal reflex potentiation where the activity of this reflex was dynamically potentiated by repetitive (Lin 2003) and tetanic (Lin 2004) afferent inputs. Since pathological potentiation in this reflex activity was suggested to underlie the hyperactive urethra (Lin et al., 2006; Liao et al., 2007; Chen GD et al., 2007, 2008) the activity-dependent spinal reflex potentiation seems to be a novel animal model for studying urethra continent function (Chen KJ et al. 2006, 2007; Lin et al. 2006; Pan et al., 2008, Peng et al., 2008 a,b,c,d)
The identity of neurotransmitters responsible for EUS suppression during micturition, acting at receptors either on motoneurons, interneurons or central sensory afferent terminals, is not well known (Shefchyk et al. 1998). GABAergic terminals have been shown on EUS motoneuron cell bodies and dendrites of motoneurons within Onuf’s nucleus (Ramirez-Leon and Ulfhake 1993). In both human studies (Nanninga et al. 1989; Steers et al. 1992) and animal experiments (Maggi et al. 1987; Magora t al. 1989), administration of the γ-Aminobutyric acid (GABA) agonist baclofen has been shown to decrease not only limb reflex output but also bladder and EUS activity. It has also been reported that intrathecal injection of GABAergic antagonists promotes the micturition reflex (Igawa et al. 1993). All these studies suggested GABAergic neurons are involved in the inhibitory pathway of EUS.

To shed light on the issue, whether anus distension may relief or attenuate detrusor-urethra sphincter dyssynergia by suppressing the urethra activity, we investigated the impact of anal distension on the induction of spinal reflex potentiation in the pelvic-urethra reflex activity. In addition, the possible neurotransmitters involved were also studied to clarify the mechanisms underlies this phenomenon.

**EXPERIMENTAL PROCEDURES**

*Animal Preparations*

Adult female Wistar rats weighing 200 to 250 g were anesthetized using an intraperitoneal injection of urethane (i.p., 1.2 g/kg). The animal care and the experimental protocol were in accordance with the guidelines of the National Science Council of Taiwan and the guidelines of the National Institute of Health Care for the
Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996.

All efforts were made to minimize both animal suffering and the number of animals used throughout this study. This study was reviewed and approved by the Institutional Review Board of Chung-Shan Medical University in Taichung, Taiwan. The trachea of the animal was intubated to keep the airway clear. A PE-50 catheter (Portex, Hythe, Kent, U.K.) was placed in the left femoral vein for administration of anesthetics when needed. A midline abdominal incision was made to expose the pelvic viscera. A wide-bore bladder cannula (PE-50) was inserted into the lumen of the urinary bladder from the apex of the bladder dome and was secured with cotton thread. The right pelvic nerve was dissected carefully from the surrounding tissues and was then transected as distally as possible for stimulations, while the left pelvic nerve was left intact. In experiments exploring the effect of anal stretch on the pelvic-urethra reflex activity, the end of the cannula left open to the air and drained freely (figure 1A) to avoid urine accumulation in the bladder during experiment may alter the reflex activity. In cystometry experiments, after the trigone was ligated, the intravesical catheter was connected to a volume reservoir and a pressure transducer to test the effect of anal stretch on the urethra activity under bladder distension with both pelvic nerves intact (figure 5A). The rats were monitored for the corneal reflex and a response to noxious stimulation to the paw throughout the experiment. If either was present, a supplementary dose of anesthetics (urethane 0.4 g/kg) was given through the venous catheter. At the end of the experiment, the animals were sacrificed by an intravenous injection of potassium chloride saturation solution under deep anesthesia.

**Intrathecal catheter**

The occipital crest of the skull was exposed and the atlanto-occipital membrane was incised at the midline with the tip of an 18-gauge needle. A PE-10 catheter was
inserted through the slit and passed caudally to the dorsal arachnoid space at the T13 vertebrae level (which is the level at L5-S2 spinal cord). The volume of fluid within the cannula was kept constant at 10 μl in all experiments. A single, 10 μl volume of drug solution was administered followed by a 10 μl flush of vehicle solutio. At the end of each experiment, the location of the injection site was marked by an injection of Alcian blue (10 μl, 2 %) and a laminectomy was performed to verify the location of the cannula tip. The volume of drug injected into the spinal cord in this experiment has been reported to spread from 0.5-1.5 mm from the site of injection as described previously (Chen 2006). The data obtained from animals, whose cannula tip deviated by more than 0.5 mm from the upper and lower limits of the dorsal aspect of the arachnoid space along L5 to S2 were excluded from the statistical analysis.

Electromyogram recordings

Epoxy-coated copper wire (50 μm; M.T. Giken Co., Tokyo, Japan) electromyogram electrodes were placed into the peri-urethra area intra-abdominally using a 30-gauge needle with a hooked electromyogram electrode positioned at the tip. The needle was inserted into the sphincter approximately 1 to 2 mm lateral to the urethra and then was withdrawn, leaving the electromyogram wires embedded in the muscle. The electromyogram activity was amplified 20,000-fold and filtered (high frequency cut-off at 3,000 Hz and low at 30 Hz, respectively) by a preamplifier (Grass 7P1, Cleveland, OH, U.S.A.), then was continuously displayed on an oscilloscope (Tectronics TDS 3014, Wilsonville, OR, U.S.A.) and the recording system (Biopac, MP30, Santa Barbara, CA, U.S.A.). The dissected nerve and the stimulating/recording electrodes were bathed in a pool of warm paraffin oil (37°C) to prevent drying.

Application of drugs
Drugs dissolved in artificial cerebrospinal fluid that pH value was adjusted to 7.4 were used for intrathecal injections. Drugs used in the experiment were gallamine triethiodide (GL, 5mg/kg), 6-cyano-7-nitroquinoxaline-2,4-dione [CNQX, a glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionat (AMPA) receptor antagonist; 100 μM, 10 μl, Sigma], 2-amino-5-phosphonovalerate [APV, a glutamatergic N-methyl-D-aspartate (NMDA) receptor antagonist; 100 μM, 10 μl, Sigma], 1-glutamate (100 μM, 10 μl, Sigma), NMDA (100 μM, 10 μl, Sigma), bicuculline [γ-amino-butyric acid (GABA)A receptor antagonist; 100 μM, 10 μl, Sigma] and hydroxysacrofen (a GABA B receptor antagonist; 100 μM, 10 μl, Sigma). A solution of identical volume to the tested agents was dispensed intrathecally to serve as a vehicle. At the end of the experiment, the location of the injection site was marked by an injection of Alcian blue (2 %, 10 μl). The volume of drug injection into the spinal cord in such experiment was ever reported to spread 0.5 to 1.5 mm from the site of injection (9). Therefore, a cannula positioned more than 0.5 mm away from the intended site of injection was not included in the statistical analysis.

Experimental arrangement

Recording the numbers of action potentials evoked by the electric shocks assessed the pelvic-urethra reflex activity. The schematic arrangement of external urethra sphincter electromyogram (EUSE) recording as well as the pelvic afferent nerve fiber stimulation is shown in figure 1A. In the beginning of all experiments, we manipulated the stimulation intensity, and an electric intensity that caused a single spike action potential in the reflex activity was used to standardize the baseline reflex activity. This intensity was then used for stimulation throughout each experiment. The protocol for assessing the effects of electrical stimulation and different kinds of reagent/maneuver on the reflex activity was as follows:
Protocol 1. Pelvic afferent nerve test stimulation (TS): Single shock at a fixed suprathreshold strength was repeated at 30 sec interval (1 stimulation/30 sec), and given through a pair of stimulation electrodes for 30 min. This frequency of stimulation was chosen for sampling data because it did not result in response facilitation.

Protocol 2. Pelvic afferent nerve repetitive stimulation (RS): After a baseline period (usually 30 minutes), the repetitive stimulation (1 stimulation/1 sec, lasting for 30 minutes) with intensity identical to the test stimulation was applied to induce reflex potentiation.

Protocol 3. Glutamatergic agonists/antagonists: After the equilibrium (usually 30 min), glutamatergic agonists, glutamate or NMDA was injected intra-thecally 1 min before test stimulation onset to test their effects. In experiment test the effects of glutamatergic antagonists, CNQX or APV was intra-thecally injected 1 min before repetitive stimulation onset.

Protocol 4. Anal stretch: After a reflex potentiation has been established by the repetitive stimulation, anal stretch was carried out at 20 min following stimulation onset and maintained. Since balloon distension may cause vertical displacement that interferes the recording electrodes located at urethra, we stretch the anus horizontally using a mosquito clamp, of which tip was inserted into anus for about 2 cm. The distance used for anal stretch were 4, 8 or 12 mm.

Protocol 5. GABAergic antagonists: After the equilibrium (usually 30 min), GABA$_A$ or GABA$_B$ antagonists, bicuculline or hydroxysacrofen was injected intra-thecally 1 min before repetitive stimulation onset. Then the repetitive stimulation in associated with anal stretch was tested as protocol 4.

*Statistics*
All the data in the text and figures are mean value±standard error of mean (SEM).

Statistical analysis of the data was performed by means of ANOVA, in all cases, a $P$-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Baseline reflex activity and reflex potentiation

As shown in figure 1C, single pulse of pelvic afferent nerve test simulation (TS, 1 stimulation/30 sec) evoked a stable baseline reflex activity with single action potential in the external urethra sphincter electromyogram (EUSE) activity. In three animals, gallamine (5 mg/kg) was administrated intravenously after we connected these animals to a ventilator. Gallamine injection abolished the reflex activity evoked by the test stimulation (TS+GL). On the other hand, in animal received no gallamine injection, repetitive stimulation (RS, 1 stimulation/1 sec) induced reflex potentiation in the electromyogram (EUSE) activity in contrast to that test stimulation evoked a baseline reflex activity. The summarized data in figure 1B shows that the mean spike number evoked by the test stimulation varied little over the stimulation period ($p>0.05$, $N=35$), while that done by the repetitive stimulation increased shortly following onset of stimulation then reached a rather constant level and maintained until the cessation of stimulation. Moreover, the mean spike numbers evoked by the repetitive stimulation (RS) were significantly higher than that done by the test stimulation (TS, **$p<0.01$, $N=7$).

Glutamatergic agonists and antagonists

As shown in Figure 1C, test stimulation on the pelvic afferent nerve evoked a baseline reflex activity with single action potential. Intrathecal pretreatments of glutamate (TS+GLU, 100 μM, 10 μL) and NMDA (TS+NMDA, 100 μM, 10 μL)
both induced a longer-lasting reflex potentiation. On the other hand, repetitive stimulation (RS) produced a long-lasting potentiation in the reflex activity. Intrathecal pretreatments of CNQX (RS+CNQX, 100 uM, 10 uL) and APV (RS+APV, 100 uM, 10 uL) both blocked the repetitive stimulation-induced reflex potentiation. The summarized data in Figure 1D shows that pretreatments of glutamate (TS+GLU) and NMDA (TS+NMDA) significantly increased the mean spike numbers evoked by the test stimulation averaged within 1 min counted at 30 min following stimulation onset (TS, ** p<0.01 to TS, N=7). Moreover, pretreatment of CNQX (RS+CNQX) and APV (RS+APV) significantly decreased the mean spike numbers evoked by the repetitive stimulation (RS, ## p<0.01 to RS, N=7).

Effects of anal stretch

As showed in figure 2A, after a reflex potentiation has been established by the repetitive stimulation (RS), anal distension at 20 min following stimulation onset with a distance of 4 mm did not affected (RS+D4), while that with a distance of 8 mm attenuated the repetitive stimulation-induced reflex potentiation (RS+D8). Moreover, the established reflex potentiation was completely abolished by anal stretch with a distance of 12 mm (RS+D12). Mean spike number evoked by repetitive stimulation (RS) with anal stretch with distances of 4 (RS+D4), 8 (RS+D8) and 12 (RS+D12) mm was summarized in figure 2B. The stepwise increment of the distance of anal stretch attenuated and eventually abolished the repetitive stimulation-induced reflex potentiation when the distances of anal stretch were wider than 8 mm (## p<0.05, N=7)

The NMDA agonist

As shown in figure 2A, repetitive stimulation (RS) induced reflex potentiation in the electromyogram activity, which is abolished by anal stretch with a distance of 12 mm
(RS+D12). After the repetitive stimulation-induced reflex potentiation has been abolished, intrathecal NMDA injection reversed such an abolition caused by anal stretch (RS+D12+NMDA).

GABAergic antagonists

As shown in figures 3A and 4A, the repetitive stimulation-induced reflex potentiation was abolished by anal stretch with a distance of 12 mm (RS+D12), intrathecal pretreatment of hydroxysaclofen, a GABA_B receptor antagonist, exhibited no effect on the abolition caused by anal stretch (figure 4A, RS+D12+SCF). On the other hand, as shown in figure 3A, pretreatment of bicuculline, a GABA_A receptor antagonist, partly restored the reflex potentiation (RS+D12+BCL). The summarized data in figure 3B and 4B shows bicuculline (RS+D12+BCL, p<0.05, N=7) but not hydroxysaclofen (RS+D12+SCF, p>0.05, N=7) reversed the attenuation in the mean spike number caused by anal stretch with a distance of 12 mm (RS+D12).

Cystometric investigation

In three animals, after a ligation was made at the bladder trigone, the urinary bladder was connected to a volume reservoir and a pressure transducer (figure 5A) as described in elsewhere (Liao et al., 2007a). As shown in figure 5A, no spontaneous firing was recorded in the electromyogram activity when the pressure reservoir was positioned at the level identical to the urinary bladder (Control). We then elevated and hold the volume reservoir at the level 16 cm higher than the urinary bladder, and this maneuver caused firing in the electromyogram activity (BD). Anal stretch with a distance of 12 mm attenuated and eventually abolished the firing caused by bladder distension (BD+D12).
DISCUSSION

In this *in vivo* animal study, we make the first direct demonstration that acute anal stretch may abolish NMDA-dependent repetitive stimulation-induced pelvic-urethra reflex potentiation, which is presumed to be involved in the development of urethra hyperactivity. In addition, pharmacological tests showed that intrathecal injection of low dose bicuculline and NMDA both counteracted the abolition on the reflex potentiation caused by the anal stretch. These data suggest acute anal stretch may reflexively inhibit NMDA-dependent reflex potentiation via GABA<sub>A</sub>-ergic neurotransmssion at the spinal cord level.

GABA, which is readily accepted as a vital inhibitory neurotransmitter (Bowery and Smart, 2006), exhibits pre-synaptic or post-synaptic inhibition on the primary afferent fibers at the spinal cord level (Alford et al., 1991; Alford and Grilllner 1991; el Manira et al., 1996). GABA elicits inhibitory effects on the superficial dorsal horn neurons through activation of the chloride permeable GABA<sub>A</sub> receptors (Todd and Spike, 1993) or G-protein-coupled GABA<sub>B</sub> receptors (Bormann, 1988). GABA has also been identified as a critical inhibitory neurotransmitter for the spinal micturition circuitry and exerts its effect via activating either GABA<sub>A</sub> or GABA<sub>B</sub> receptors (Igawa et al., 1993; Maggi et al., 1987; Watanabe et al., 1997). GABAergic neurotransmission are known to have an inhibitory action on urethral function via effects on motor neurons to the urethra sphincter. When evacuation takes place, impulses descending from the pontine micturition center inhibit the motor neurons innervating the urethra sphincter via projections to the GABAergic premotor interneurons of Onuf’s nucleus (Blok et al., 1997).

Not only the spinal integrated reflex activity, GABAergic neurotransmission may also affect activity-dependent reflex potentiation. In studies investigating long-term
potentiation, a well-known form of activity-dependent reflex potentiation, GABAergic pre-synaptic and post-synaptic inhibitions were observed in rat hippocampal neurons (Gaiarsa et al., 1995). It is well established that activation of GABA receptor activity may attenuate or abolish the NMDA-dependent reflex potentiation via pre-synaptic or post-synaptic inhibition on the glutamatergic NMDA receptors (Buesa et al., 2006). All these results were quite correlated with the present study that pretreated GABA_A receptors antagonist, bicuculline, reversed the attenuation on the induction of NMDA-dependent spinal reflex potentiation indicating spinal GABA_Aergic neural inhibition plays a role in the abolition of the induction of reflex potentiation caused by anal stretch. However, despite we injected GABA antagonist intrathecally with a volume of 10 uL, and the possibility of such a low dose of test agents to exhibit effects on the higher brain center was minor. Since we did not transect the spinal cord in this study, possibility of descending modulation coming form higher neurological center to inhibit spinal pelvic-urethra reflex potentiation can not be ruled out.

However, in contrast to bicuculline, hydroxysaclofen, the GABA_B receptors antagonist used in this study, appeared to be of minimal effect on the reversal of the abolition on reflex potentiation caused by anal stretch. There are several possible causes may be taken into account. The first is, GABA_B antagonists have limited efficacy in modulating of synaptic transmission than GABA_A. It has been reported that application of weak GABA_B antagonists, such as hydroxysaclofen, at the soma did not reach distal synapse of neurons (Stuart and Redman, 1992). In addition, post-synaptic GABA_B receptors were less important in the regulation of motor neuron activity. Application of GABA_B agonist, baclofen, at a concentration sufficient to depress synaptic activity was not associated with changes in membrane potential,
conductance and excitability in the spinal motor neurons (Edwards et al., 1989; Jimenez et al., 1991; Lev-Tov et al., 1988). Another possibility is that GABA\(_B\) receptors on the afferent terminals are located extra-synaptically. Under such a condition, activation of extra-synaptic receptor is only like to occur during periods of massive GABA release or reduced re-uptake (Stuart and Redman, 1992). Moreover, GABA\(_B\) receptor may require longer exposure or a higher concentration of GABA for activation than GABA\(_A\) receptors (Pehrson and Anderson, 2002). Furthermore, hydroxysaclofen, the GABA\(_B\) receptor antagonist, is a weak antagonist with possible agonistic properties (Rekling et al., 2000). Therefore, clarify the detail GABAergic mechanism involved in anal stretch needs further investigations.

Arising from the puborectalis muscle, external urethra sphincter innervated by the perineal branch of the pudendal nerve and external anal sphincter innervated by the rectal branch of the pudendal nerve, are both striated muscles. In addition to voluntarily contract or relax while the other does not, both of these muscles reflexively contract or relax simultaneously (Shafik, 1991). Electrophysiological evidences demonstrated the basal activity of the external urethral sphincter was altered by electric stimulation of the external anal sphincter in the healthy volunteers (Shafik 1992). In addition, vigorous distension of anal sphincter leaded to inhibition of urethra and anal sphincter activity in most spinal cord injured patients (Rodriquez and Awad, 1979). Furthermore, recent study investigating urodynamic responses to anal stretch in patients with detrusor-sphincter dyssynergia also revealed that anal distension for 30 sec could significantly reduced the spasticity of external urethra sphincter without affecting the detrusor pressure (Huang et al., 2008). Not only the reflex activity of the urethra itself, in this study we demonstrated anal distension attenuated and eventually abolished the pelvic-urethra reflex potentiation, an novel
form of activity-dependent reflex potentiation, in a dose dependent manner. This result implying anal stretch may also modulate the activity-dependent physiological/pathological response of the urethra via cross-organ innervation at the lumbosacral spinal cord levels.

Incomplete bladder emptying in patients with detrusor-sphincter dyssynergia is often related to an increase in the external urethra sphincter activity during detrusor contraction. Such a dyssynergic sphincter contraction increases outlet resistance, which in turn contributes to an increased intravesical pressure and residual urine (Wu et al., 1986). In the present study, cystometric investigation demonstrated that anal distension may abolish the electromyogram activity of the urethra induced by bladder distension. These data offer neurophysiological evidence that anal distension can be an effective way to relax external urethra sphincter when the urinary bladder was filled, and may be used as an adjunct to assist voiding in patients with detrusor-sphincter dyssynergia. In addition, GABAergic neurotransmission seem to be a possible adjuvant therapeutic target for the treatment of voiding dysfunction caused by dyssynergic or overactive sphincter.

In summary, the results in this study demonstrated that anal stretch might abolish the repetitive stimulation-induced potentiation in the pelvic-urethra reflex, which is presumed to be essential for establishing urethra resistance. These data offer neurophysiological evidence that anal stretch can be an effective way to relax overactive external urethra sphincter. In addition, GABAergic inhibitory neurotransmission is important in the neural mechanisms underlying external urethra sphincter activity inhibited by anal stretch.
Acknowledgements

This research was supported by the National Science Council of Taiwan (NSC-96-2314-B-040-012-MY2) for Dr. G.D. Chen, (NSC-96-2320-B-040-012) for Dr. T.B. Lin and Chung-Shan Medical University Hospital (CSH-96-08) for Dr Y.L. Kao.
Figure Legends

Figure 1 Baseline pelvic-urethra reflex activity and reflex potentiation. (A) The experimental arrangements showing the external urethra sphincter electromyogram activity (EUSE) was recorded under the urinary bladder as it drains freely. (B) Summarized data show the mean spike number evoked by each pulse of the test stimulation varied little over the stimulation period, while that evoked by the repetitive stimulation increased shortly following stimulation onset then reached a rather constant level and maintained until the cessation of stimulation (** P<0.01 to TS, N=35). (C) Single pulses of pelvic afferent nerve test simulation (TS, 1 stimulation/30 sec, indicated by the marks at the bottom) evoked a baseline reflex activity with single action potentials, which was abolished by neuromuscular blockage using gallamine (TS+GL). In animal received no gallamine injection, test stimulation evoked a baseline reflex activity, whereas repetitive stimulation (RS, 1 stimulation/1 sec, indicated by the marks at the bottom) gradually induced reflex potentiation in the external urethra sphincter electromyogram activity. Intrathecal glutamate (TS+GLU) and NMDA (TS+NMDA) both exhibited excitation on the test stimulation-elicited reflex activities. Moreover, intrathecal CNQX and APV both exhibited inhibition on the repetitive stimulation-induced reflex potentiation. (D) Summarized data showing the mean spike number averaged at 30 min following stimulation onset evoked by test stimulation (TS) and test stimulation with intrathecal application of glutamate (TS+GLU) or NMDA (TS+NMDA) as well as repetitive stimulation (RS) and repetitive stimulation with intrathecal CNQX (RS+CNQX) or APV (RS+APV, ** p<0.01 to TS, ## P< 0.01 to RS, N=7).
Figure 2. Anal stretch inhibited the repetitive stimulation-induced reflex potentiation. (A) A single action potential in the external urethral sphincter electromyogram (EUSE) were evoked by the test stimulation (TS, 1 stimulation/30 sec, indicated by the arrow at the bottom) at the pelvic afferent nerve, while a longer-lasting reflex potentiation was induced by the repetitive stimulation (RS, 1 stimulation/1 sec, indicated by the arrows at the bottom). Onset and 30min indicate the reflex activity at the first 6 sec and 30 min following stimulation onset). Anal stretch with a distance of 4 mm (RS+D4) failed to affect, while with distances of 8 mm (RS+D8) attenuated and of 12 mm (RS+D12) abolished the repetitive stimulation-induced reflex potentiation. Intrathecal NMDA (RS+D12+NMDA) reversed the abolition on reflex potentiation caused by anal stretch with a distance of 12 mm (RS+D12+NMDA). (B) Summarized data shows the mean spike numbers evoked by the test stimulation (TS), repetitive stimulation (RS), repetitive stimulation with anal stretch with distances of 4, 8 and 12 mm (RS+D4, RS+D8 and RS+D12 respectively). Acute anal stretch with distances wider than 8 mm significantly inhibited the repetitive stimulation-induced reflex potentiation (## P< 0.01 to RS, N= 7).

Figure 3. Bicuculline, an GABA_A antagonist reversed the inhibition on the repetitive stimulation-induced reflex potentiation caused by anal stretch. (A) pelvic afferent nerve test stimulation (TS, 1 stimulation/30 sec, indicated by the arrow at the bottom) evoked a single action potential, while repetitive stimulation (RS, 1 stimulation/1 sec, indicated by the arrows at the bottom) induced a long-lasting reflex potentiation in the external urethra sphincter electromyogram (EUSE) activity that was abolished by anal stretch with a distance of 12 mm (RS+D12). Moreover, intrathecal bicuculline pretreatment reversed the inhibition exhibited by anal stretch (RS+D12+BCL). (B)
summarized data shows the mean spike number evoked by each pulse averaged with 1 min at 30 min following stimulation onset, elicited by the test stimulation (TS), repetitive stimulation (RS), repetitive stimulation in associated with anal stretch with or without bicuculline pretreatment (RS+D12 and RS+D12+BCL, respectively. ** P<0.01 to TS, ## P< 0.01 to RS, ++ P<0.01 to RS+D12, N=7).

Figure 4. Hydroxysacrofen, an GABA\textsubscript{B} antagonist failed to reverse the inhibition on the repetitive stimulation-induced reflex potentiation caused by anal stretch. (A) pelvic afferent nerve test stimulation (TS, 1 stimulation/30 sec, indicated by the arrow at the bottom) evoked a single action potential, while repetitive stimulation (RS, 1 stimulation/1 sec, indicated by the arrows at the bottom) induced a long-lasting reflex potentiation in the external urethra sphincter electromyogram (EUSE) activity that was abolished by anal stretch with a distance of 12 mm (RS+D12). Intrathecal hydroxysaclofen pretreatment failed to reverse the inhibition exhibited by anal stretch (RS+D12+SCF). (B) summarized data shows the mean spike number evoked by each pulse averaged within 1 min at 30 min following stimulation onset, caused by the test stimulation (TS), repetitive stimulation (RS), repetitive stimulation in associated with anal stretch with or without intrathecal hydroxysaclofen injection (RS+D12 and RS+D12+SCF, respectively. ** P<0.01 to TS, ## P< 0.01 to RS, ++ P<0.01 to RS+D12, N=7).

Figure 5. Anal stretch reverse the urethra electromyogram activity caused by bladder distension. (A) The schematic arrangements showing the external urethra sphincter electromyogram activity (EUSE) was recorded under the urinary bladder distension using a volume reservoir connected to the bladder via the bladder catheter. (B) when
the pressure reservoir was positioned at the level identical to the urinary bladder there was no spontaneous firing recorded in the electromyogram activity (Control). Elevating and then maintaining the volume reservoir at the level 16 cm higher than the urinary bladder caused firing in the electromyogram activity (BD, The arrow indicated initial of bladder distention) that was attenuated and eventually abolished the firing caused by bladder distension (BD+D12, The arrow indicated initial of anal stretch).
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