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Role of Glycine in Nociceptive and Non-Nociceptive Bladder Reflexes and Pudendal Afferent Inhibition of These Reflexes in Cats

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Abstract

Aim—This study examined the role of glycinergic transmission in nociceptive and non-nociceptive bladder reflexes and in inhibition of these reflexes by pudendal nerve stimulation (PNS).

Methods—Cystometrograms (CMGs) were performed in α -chloralose anesthetized cats by intravesical infusion of saline or 0.25% acetic acid (AA) to trigger, respectively, non-nociceptive or nociceptive bladder reflexes. PNS at 2 or 4 times threshold (T) intensity for inducing anal twitch was used to inhibit the bladder reflexes. Strychnine (a glycine receptor antagonist) was administered in cumulative doses (0.001–0.3 mg/kg, i.v.) at 60–120 min intervals.

Results—Strychnine at 0.001–0.3 mg/kg significantly ($P < 0.05$) increased bladder capacity and reduced contraction amplitude during saline CMGs but did not change these parameters during AA CMGs except at the 0.3 mg/kg dose which increased bladder capacity. Strychnine did not alter PNS inhibition during saline CMGs except at the highest dose at 2T intensity, but significantly ($P < 0.05$) suppressed PNS inhibition during AA CMGs after 0.001–0.003 mg/kg doses at 2T and 4T intensities. During AA CMGs strychnine (0.3 mg/kg) also unmasked a post-PNS excitatory effect that significantly reduced bladder capacity after termination of PNS.

Conclusions—Glycinergic inhibitory neurotransmission in the central nervous system plays an unexpected role to tonically enhance the magnitude and reduce the bladder volume threshold for triggering the non-nociceptive bladder reflex. This is attributable to inhibition by glycine of another inhibitory mechanism. Glycine also has a minor role in PNS inhibition of the nociceptive bladder reflex.

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Keywords

bladder; cat; glycine; neuromodulation; pudendal

INTRODUCTION

Pudendal neuromodulation has been applied successfully to treat overactive bladder (OAB) symptoms.¹ However, the mechanisms of pudendal neuromodulation are not fully understood. Previous studies in cats^{2,3} have shown that electrical stimulation of pudendal afferent nerves reflexively activates sympathetic efferent pathways in the hypogastric nerves which in turn activate β -adrenergic receptors in the detrusor to inhibit bladder contractions. Our recent study in cats³ further showed that pudendal inhibition of spinal reflex bladder contractions remained after removing the sympathetic inhibition, indicating a central mechanism of action in addition to inhibition at the peripheral site. Understanding the central mechanisms underlying pudendal inhibition could be important for developing new therapies for OAB.

Micturition reflexes are mediated by two different afferent pathways.⁴ Under normal physiological conditions, bladder distention activates non-nociceptive A δ afferent fibers that trigger a supraspinal micturition reflex, while the nociceptive C-fiber afferents are silent.⁵ Under pathological conditions such as bladder infection/irritation, the nociceptive C-fiber afferents are activated which can trigger a spinal reflex and bladder overactivity.⁴

Recent experiments in cats⁶ revealed that spinal GABA_A receptors play an important role in pudendal inhibition of the nociceptive bladder reflex. Therefore, it is of interest to determine if glycine receptors also play a role in the inhibition because GABA and glycine are often co-localized in the same spinal interneurons and can interact synergistically.⁷ However, it is known that neuromodulation of nociceptive and non-nociceptive bladder reflexes can occur by different neurotransmitter mechanisms.⁶ Therefore, in this study using anesthetized cats, the bladder was distended with either saline to activate the non-nociceptive A δ -fiber afferent mediated supraspinal micturition reflex, or irritated with dilute (0.25%) acetic acid (AA) to activate the nociceptive C-fiber afferent mediated spinal micturition reflex. Pudendal nerve stimulation (PNS) was applied to inhibit these reflexes and mimic pudendal neuro-modulation. Strychnine (a glycine receptor antagonist) was administered intravenously (i.v.) to examine the possible role of glycine receptors in the two types of micturition reflexes and in PNS inhibition of these reflexes.

MATERIALS AND METHODS

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

Surgical Procedure

Sixteen cats (8 males and 8 females, 2.3–4.2 kg, Liberty Research, Waverly, NY) were anesthetized with isoflurane (2–3% in oxygen) during surgery and then with α -chloralose (65 mg/kg i.v. and supplemented as needed) during data collection. A tube was inserted via a

tracheotomy to maintain an open airway; and a catheter was inserted into the right carotid artery to monitor blood pressure. Catheters were also inserted into the left and right cephalic veins to allow injection of drugs and/or fluids during the experiment. A pulse oximeter (9847V, NONIN Medical, Inc., Plymouth, MN) was placed on the tongue to monitor heart rate and oxygen saturation. The ureters were exposed via a midline abdominal incision, cut and drained externally. A double lumen catheter was inserted through the urethra into the bladder and secured by a ligature around the urethra. One lumen was used to infuse (1–3 ml/min) either saline or 0.25% AA while the other lumen simultaneously monitored intravesical pressure. Via a small incision at the right sciatic notch the pudendal nerve was isolated and a tripolar cuff electrode (NC223pt, MicroProbe, Inc., Gaithersburg, MD) was placed around the nerve. Then, the cuff electrode was connected to a stimulator (S88, Grass Medical Instruments, Quincy, MA) via a constant voltage stimulus isolator (SIU5, Grass Medical Instruments, Quincy, MA). All incisions were closed by sutures.

Experimental Protocol

The PNS intensity threshold (T), which was defined as the minimal intensity for inducing observable anal sphincter twitching, was determined before administration of pancuronium (initial 0.05 mg/kg followed by 0.05 mg/kg/hr, i.v.) which was used to prevent striated muscle contractions. Based on our previous study,⁶ uniphasic rectangular pulses (5 Hz frequency, 0.2 ms pulse width) were used to stimulate the pudendal nerve at 2T or 4T intensity to inhibit bladder activity during the experiments. At the beginning of the experiments, cystometrograms (CMGs) were performed by slowly infusing saline into the bladder to determine the bladder capacity defined as the bladder volume threshold required to induce a micturition contraction of large amplitude (>30 cmH₂O) and long duration (>20 s). Multiple CMGs were performed to ensure reproducibility of the saline control capacity. Then, the animals were divided into two groups.

In the first group (n = 11), repeated CMGs were performed with infusion of 0.25% AA to irritate the bladder, activate nociceptive bladder C-fiber afferents, and induce bladder overactivity. When the bladder capacity stabilized, four CMGs were performed with AA infusion before administration of strychnine: (1) control CMG without PNS, (2) CMG during 2T PNS, (3) CMG during 4T PNS, and (4) control CMG without PNS to determine any post-stimulation effect. The bladder was emptied at the end of each CMG, and a 3- to 5-min rest period was inserted between CMGs. Then, cumulative doses (0.001, 0.003, 0.01, 0.03, 0.1, and 0.3 mg/kg, i.v.) of strychnine (Sigma–Aldrich, St. Louis, MO) were given at about 60–90 min intervals. Ten minutes after each dose, the four AA CMGs were again performed under the four conditions (control, 2T PNS, 4T PNS, and post-PNS control).

In the second group (n = 5), repeated saline CMGs were performed to activate the non-nociceptive A δ -afferent-initiated micturition reflexes. Before administration of strychnine, four saline CMGs were performed: (1) control CMG without PNS, (2) CMG during 2T PNS, (3) CMG during 4T PNS, and (4) control CMG without PNS to determine any post-stimulation effect. Then, cumulative doses (0.001, 0.003, 0.01, 0.03, 0.1, and 0.3 mg/kg, i.v.) of strychnine were given at 60–120 min intervals. Ten minutes after each dose, the saline

CMGs were again performed under the four conditions (control, 2T PNS, 4T PNS, and post-PNS control).

Data Analysis

Bladder capacity measured during each CMG was normalized to initial saline control capacity in the same cat. The maximal amplitude of bladder contractions was also measured. The measurements under the same experimental conditions were averaged across different animals and reported with standard error of mean. Statistical significance ($P < 0.05$) was determined by one-way ANOVA followed by Dunnett multiple comparisons or by two-way ANOVA followed by Bonferroni multiple comparisons.

RESULTS

Effect of Strychnine on Nociceptive Bladder Overactivity and the Magnitude of PNS Inhibition

As shown in Figure 1, AA-induced bladder overactivity significantly ($P < 0.05$) reduced bladder capacity to $24.9 \pm 3.8\%$ (3.4 ± 0.9 ml) of saline control capacity (11.6 ± 1.6 ml). PNS suppressed bladder overactivity and significantly ($P < 0.05$) increased capacity to $80.9 \pm 8.0\%$ at 2T and $90.1 \pm 7.8\%$ at 4T. After PNS, the bladder capacity returned to the volume prior to PNS, indicating that there was no post-stimulation effect (Fig. 1).

Strychnine at low doses of 0.001–0.003 mg/kg did not significantly change the small bladder capacity caused by AA irritation but significantly ($P < 0.05$) reduced the increase in bladder capacity induced by 2T or 4T PNS (Fig. 2A–E). Larger doses of strychnine (0.01–0.1 mg/kg) did not significantly affect the inhibition elicited by 2T or 4T PNS and produced a small increase in bladder capacity that was not statistically significant. The largest dose of strychnine (0.3 mg/kg) significantly ($P < 0.05$) increased bladder capacity but did not block the increase in bladder capacity elicited by either 2T or 4T PNS (Fig. 2A–E). However during the CMGs immediately following PNS after the largest dose of strychnine, bladder capacity was significantly ($P < 0.05$) reduced compared to the capacity before PNS (see the last row of 4 CMGs in Fig. 2A–D and the summary data in Fig. 2E). The time course of this decrease in capacity was not studied. Strychnine (0.001–0.3 mg/kg) did not significantly change the maximal amplitude of micturition contractions (Fig. 2F).

Effect of Strychnine on Non-Nociceptive Bladder Activity and the Magnitude of PNS Inhibition

During saline CMGs, PNS at 2T, and 4T significantly ($P < 0.05$) increased bladder capacity to $168.2 \pm 8.2\%$ and $181.4 \pm 10.6\%$, respectively (Fig. 3). During CMGs performed immediately after PNS, bladder capacity returned to the level prior to PNS, indicating that there was no post-stimulation effect (Fig. 3).

Strychnine dose-dependently inhibited the micturition reflex elicited during saline CMGs (Fig. 4). The control bladder capacity measured either before or after PNS was significantly ($P < 0.05$) increased by strychnine at doses of 0.01–0.3 mg/kg (Fig. 4E). The maximal amplitude of micturition contraction was significantly ($P < 0.05$) reduced by strychnine at

doses greater than 0.003 mg/kg during CMGs performed before, during or after PNS (Fig. 4F).

After strychnine doses of 0.001–0.1 mg/kg PNS at both 2T and 4T significantly ($P < 0.05$) increased bladder capacity (Fig. 4A–E) and significantly ($P < 0.05$) decreased the maximal amplitude of the micturition contractions (Fig. 4F). However, after the largest dose (0.3 mg/kg) of strychnine, PNS at 2T but not at 4T failed to significantly increase bladder capacity. After the largest dose of strychnine, bladder capacity also significantly ($P < 0.05$) decreased following PNS (see the last row of 4 CMGs in Fig. 4A–D and summary in Fig. 4E) as also noted during AA CMGs (Fig. 2E).

DISCUSSION

This study in cats revealed different effects of strychnine on nociceptive (Fig. 2) or non-nociceptive (Fig. 4) bladder reflexes and on pudendal inhibition of these reflexes (Fig. 2E and Fig. 4E). Strychnine at doses of 0.001–0.1 mg/kg did not alter the nociceptive bladder reflex (Fig. 2E–F) but significantly reduced PNS inhibition of this reflex at 0.001 and 0.003 mg/kg doses (Fig. 2A–E). However, strychnine at these doses significantly inhibited the non-nociceptive bladder reflex, increasing bladder capacity (Fig. 4E), and reducing contraction amplitude (Fig. 4F), but did not alter PNS inhibition of this reflex (Fig. 4A–E). The largest dose (0.3 mg/kg) of strychnine, significantly increased bladder capacity of both reflexes (Figs. 2E and 4E) and reduced the 2T PNS inhibition of the non-nociceptive bladder reflex (Fig. 4E), but did not change the 2T or 4T PNS inhibition of the nociceptive bladder reflex (Fig. 2E). These results indicate that the effects of strychnine are dependent on both dose and the type of bladder reflex (nociceptive or non-nociceptive). Time control experiments in our previous study⁸ in which both types of bladder reflexes were examined and vehicle (saline) was injected using a similar drug testing protocol and experimental duration showed that the bladder capacity was not changed during repeated vehicle control CMGs.

It is well known that subconvulsant doses of strychnine (<0.09 mg/kg) suppress inhibitory postsynaptic potentials in motoneurons, and suppress inhibitory but enhance excitatory spinal somato-motor reflexes.⁹ Thus, an inhibitory effect instead of an excitatory effect of subconvulsant doses of strychnine (0.003–0.03 mg/kg) on the non-nociceptive, supraspinal bladder reflex without an effect on the nociceptive bladder reflex was unexpected. The resistance to these doses of strychnine of nociceptive bladder overactivity which is unmasked by AA and mediated in part by activation of a spinal reflex pathway raises the possibility that the strychnine inhibition is due to suppression of excitatory transmission in the supraspinal relay centers (i.e., the periaqueductal gray or the pontine micturition center) or in the spinal ascending sensory limb of the non-nociceptive reflex. The data also indicate that the parasympathetic preganglionic efferent neurons which are common to both reflex pathways must be insensitive to the inhibitory effects of strychnine even though these neurons express glycine receptors.¹⁰

Because strychnine in low doses (0.001–0.01 mg/kg) acts primarily by blocking the receptors for glycine, an inhibitory neurotransmitter, it is reasonable to speculate that the inhibitory effect of strychnine on the non-nociceptive bladder reflex are mediated by

elimination of tonic glycinergic inhibition of a non-glycinergic inhibitory mechanism leading to an enhancement of the latter inhibitory mechanism and in turn suppression of the excitatory micturition reflex pathway. This proposal is consistent with a previous report¹¹ that intracisternal administration of glycine in rats induces the opposite effect, i.e., bladder contractions during saline distention of the bladder, indicating that glycine eliminates an inhibitory control of micturition and thereby facilitates bladder activity.

Although a high dose of strychnine (0.3 mg/kg) did increase bladder capacity during AA CMGs, this effect only occurred at three times the convulsant dose of the drug, thirty times the threshold dose (0.01 mg/kg) to increase bladder capacity and one hundred times the threshold dose (0.003 mg/kg) to suppress contraction amplitude during saline CMGs (Fig. 4E–F). Thus, it is likely that the two types of inhibition occur by different mechanisms and that the increase in bladder capacity during AA CMGs is due to a non-specific effect possibly related to ongoing seizure activity induced by strychnine. A reduction in bladder contraction amplitude by strychnine during saline distention of the bladder was also observed after intrathecal administration in rats¹² or after intravenous administration in cats.¹³ A previous study in cats¹⁴ also showed that firing of bladder parasympathetic preganglionic neurons induced reflexively by saline distention of the bladder could be suppressed by direct electrophoretic application of large amounts of strychnine in excess of the quantity needed to block the inhibitory effects of electrophoretically applied glycine. Thus, large doses of strychnine have the potential to induce effects in the central nervous system that are independent of glycinergic receptor blockade.

Strychnine in a narrow range of low doses (0.001–0.003 mg/kg) modestly (about 10% change) and significantly reduced the PNS-induced increase in bladder capacity during AA CMGs but did not affect the PNS-induced increase in bladder capacity during saline CMGs. However larger doses of strychnine (0.01–1.3 mg/kg) were surprisingly ineffective during AA CMGs. During saline CMGs the largest dose (0.3 mg/kg) did reduce the 2T PNS-induced increase in bladder capacity. However this effect only occurred with a convulsant dose and under conditions where the bladder capacity was already significantly increased by the drug. These responses to larger doses of strychnine are difficult to explain and may be related to effects of the drug unrelated to block of glycine receptors. On the other hand based on the effects of subconvulsant doses of strychnine it is clear that the role of glycine in PNS inhibition of bladder reflexes is modest and that this role is more important in PNS inhibition of the nociceptive reflex than in the inhibition of the non-nociceptive bladder reflex (Fig. 2E and Fig. 4E). This difference contrasts with the difference in the direct effect of strychnine on two types of bladder reflexes where the non-nociceptive reflex is more sensitive. A recent study in cats¹⁵ also showed that low doses of strychnine (0.025–0.25 mg/kg, i.v.) did not reduce PNS inhibition of saline distention-induced bladder contractions in agreement with our current result. However, intrathecal administration of strychnine in rats¹⁶ suppresses rectal distention-induced inhibition of reflex bladder contractions during saline distention of the bladder.

Our previous study in cats⁶ revealed that PNS inhibition of the nociceptive bladder reflex is mediated primarily by GABA and that GABA is not involved in PNS inhibition of the non-nociceptive bladder reflex. The present experiments indicate that glycine has a similar

selective albeit a more modest role in PNS inhibition of the nociceptive bladder reflex. Because some inhibitory interneurons in the spinal cord are known to synthesize and release GABA and glycine as inhibitory co-transmitters,⁷ it is possible that these substances are also co-transmitters in the PNS spinal inhibitory mechanism. A previous study in the cat¹⁷ revealed that neither picrotoxin (a GABA_A receptor antagonist) 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 at a spinal site to generate tonic inhibition of the micturition reflex pathway. These data are consistent with the results of patch clamp studies in neonatal rat spinal cord slices which showed that focal electrical stimulation in the region of the sacral preganglionic neurons elicited inhibitory postsynaptic currents mediated by glycine and GABA.¹⁸ Further studies of GABA and glycine interaction in the spinal cord by intrathecally administering drugs are certainly warranted.

It is known that strychnine can also allosterically inhibit both nicotinic and muscarinic receptors.^{19,20} However, strychnine binding to glycine receptor is about 10–100 times stronger than its binding to nicotinic or muscarinic receptors.^{19,20,21} The major effects in this study (Fig. 2 and Fig. 4) were observed at very low doses (0.001–0.01 mg/kg) that are about 10–100 times lower than the convulsant dose of strychnine. Therefore, it is less likely that the effects observed at these low doses are due to the action of strychnine on nicotinic or muscarinic receptors.

In summary, this study in anesthetized cats revealed a complex role of glycine receptors in nociceptive and non-nociceptive bladder reflexes and PNS inhibition of these reflexes. Currently sacral, pudendal, and tibial neuromodulation have been used clinically to treat OAB symptoms,²² but the mechanisms underlying these therapies are still not fully understood. This basic science study in cats provides evidence supporting the involvement of glycine receptors in pudendal neuromodulation therapy and suggests that glycinergic synapses modulate other synaptic inhibitory mechanisms that control the micturition reflex pathway. Revealing neurotransmitter mechanisms involved in neuromodulation of bladder activity will ultimately help to understand the therapeutic effect of neuromodulation in OAB treatment.

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Fig. 1.

Acetic acid (AA) irritation decreases bladder capacity and pudendal nerve stimulation (PNS) increases bladder capacity. **A:** CMGs during saline or 0.25% AA infusion with/without PNS. T—stimulation intensity threshold to induce anal sphincter twitch. Black bars under pressure trace indicate the duration of PNS (5 Hz, 0.2 ms, T = 1.2 V). Infusion rate = 2 ml/min. **B:** Summarized results from 11 cats. The bladder capacity is normalized to the measurement during saline infusion. *indicates significantly different (one-way ANOVA). Infusion rate = 1–2 ml/min.

Fig. 2.

The effect of strychnine (i.v.) on the inhibition induced by pudendal nerve stimulation (PNS) during CMGs with bladder infusion of 0.25% acetic acid (AA). **A–D:** The CMGs at increasing cumulative doses of strychnine were performed in sequence from left to right and from top to bottom in each figure. Black bars under the pressure trace indicate the duration of PNS (5 Hz, 0.2 ms, T = 1.5 V). Infusion rate = 2 ml/min. **E:** Summarized results of bladder capacity. *indicates significantly different from the bladder capacity measured before strychnine treatment (i.e., at 0 mg/kg) for each condition (one-way ANOVA). #indicates significantly different from control capacity (AA before PNS) measured before PNS (two-way ANOVA). Note: The control capacity measured after PNS (AA after PNS) is only significantly different from the control capacity measured before PNS (AA before PNS) at the largest dose of 0.3 mg/kg. **F:** Neither strychnine (i.v.) nor PNS has any effective on the maximal amplitude of micturition contractions. PNS: 5 Hz, 0.2 ms, T = 0.25–1.6 V. N = 11 cats.

Fig. 3.

Inhibition of the micturition reflex by pudendal nerve stimulation (PNS) during saline infusion. **A:** CMGs during saline infusion with/without PNS. T—stimulation intensity threshold to induce anal sphincter twitch. Black bars under pressure trace indicate the duration of PNS (5 Hz, 0.2 ms, T = 0.35 V). Infusion rate = 3 ml/min. **B:** Summarized results from 5 cats. The bladder capacity is normalized to the measurement during first CMG without PNS. *indicates significantly different (one-way ANOVA). PNS: 5 Hz, 0.2 ms, T = 0.25–1.6 V. Infusion rate = 1–3 ml/min.

Fig. 4.

The effect of strychnine (i.v.) on the inhibition induced by pudendal nerve stimulation (PNS) during CMGs with bladder infusion of saline. **A–D**: The CMGs at increasing cumulative doses of strychnine were performed in sequence from left to right and from top to bottom in each figure. Black bars under the pressure trace indicate the duration of PNS (5 Hz, 0.2 ms, T = 1.5 V). Infusion rate = 2 ml/min. **E**: Summarized result of bladder capacity. * indicates significantly different from the bladder capacity before strychnine treatment (i.e., at 0 mg/kg) for different conditions (one-way ANOVA). © indicates significantly different from the control capacity (Saline before PNS) measured before PNS (two-way ANOVA). Note: The control capacity measured after PNS (Saline after PNS) is only significantly different from the control capacity measured before PNS (Saline before PNS) at 0.3 mg/kg. **F**: Summarized results of contraction amplitude. & indicates the contraction amplitude for CMGs before strychnine treatment (i.e., at 0 mg/kg) and before PNS (Saline before PNS) is significantly different from the amplitudes after increasing doses of strychnine. * indicates significant reduction in contraction amplitude in other data groups (one-way ANOVA). © indicates that during 2T or 4T PNS the contraction amplitude is significantly different from the control amplitude (Saline before PNS) before PNS (two-way ANOVA). @ indicates that only during 2T PNS the contraction amplitude is smaller than the “Saline before PNS” data. Note: The control amplitude after PNS (Saline after PNS) is not significantly different from the control amplitude measured before PNS (Saline before PNS). PNS: 5 Hz, 0.2 ms, T = 0.35–1.6 V. N = 5 cats.