Contribution of opioid and metabotropic glutamate receptor mechanisms to inhibition of bladder overactivity by tibial nerve stimulation

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Running Head: Metabotropic glutamate and opioid in tibial inhibition

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Abstract

The contribution of metabotropic glutamate receptors (mGluR) and opioid receptors to inhibition of bladder overactivity by tibial nerve stimulation (TNS) was investigated in cats under α-chloralose anesthesia using LY341495 (a group II mGluR antagonist) and naloxone (an opioid receptor antagonist). Slow infusion cystometry was used to measure the volume threshold (i.e., bladder capacity) for inducing a large bladder contraction. After measuring the bladder capacity during saline infusion, 0.25% acetic acid (AA) was infused to irritate the bladder, activate the nociceptive C-fiber bladder afferents, and induce bladder overactivity. AA significantly (P < 0.0001) reduced bladder capacity to 26.6±4.7% of saline control capacity. TNS (5 Hz, 0.2 ms) at 2 and 4 times the threshold (T) intensity for inducing an observable toe movement significantly increased bladder capacity to 62.2±8.3% at 2T (P < 0.01) and 80.8±9.2% at 4T (P = 0.0001) of saline control capacity. LY341495 (0.1-5 mg/kg, i.v.) did not change bladder overactivity, but completely suppressed the inhibition induced by TNS at a low stimulus intensity (2T) and partially suppressed the inhibition at high intensity (4T). Following administration of LY341495, naloxone (0.01 mg/kg, i.v.) completely eliminated the high intensity TNS-induced inhibition. However without LY341495 treatment a 10 times higher dose (0.1 mg/kg) of naloxone was required to completely block TNS inhibition. These results indicate that interactions between group II mGluR and opioid receptor mechanisms contribute to TNS inhibition of AA-induced bladder overactivity. Understanding neurotransmitter mechanisms underlying TNS inhibition of bladder overactivity is important for development of new treatments for bladder disorders.

Keywords: Neurotransmitter, Neuromodulation, Bladder, Cat
Introduction

Glutamatergic neurotransmission involves activation of both ionotropic glutamate receptors (iGluR) and metabotropic glutamate receptors (mGluR) that consists of eight subtypes classified into group I (mGluR 1 and 5), group II (mGluR 2 and 3), and group III (mGluR 4, 6, 7, and 8). An interaction between iGluR and opioid receptor mechanisms is thought to be important in pain pathways, because block of ionotropic N-methyl-D-aspartate (NMDA) receptors enhances the antinociceptive effect of morphine (an opioid receptor agonist) (14, 28, 36, 37). Recently the interaction between mGluRs and opioid receptors has also been identified in studies of opioid-induced antinociception (15, 16, 29, 45). Opioid-induced antinociceptive effects were enhanced by antagonists (15, 16, 45) as well as agonists (29) of group II mGluR in different types of somatic nociception. Currently, it is unknown if this interaction between group II mGluR and opioid receptors occurs in visceral nociceptive mechanisms.

Recent results from this laboratory (38) revealed that tibial nerve stimulation (TNS) inhibits bladder overactivity elicited by intravesical infusion of acetic acid (AA) that stimulates nociceptive bladder afferent nerves. This TNS-induced antinociceptive effect is completely eliminated by naloxone (an opioid receptor antagonist), indicating a role of opioid receptor activation in TNS-induced antinociception (39). Tramadol which produces an active metabolite with opioid receptor agonist activity enhances the TNS-induced inhibition of AA-induced bladder overactivity (47).

The current study examined the contribution of group II mGluRs and the possible interaction between these receptors and opioid receptor mechanisms in TNS-induced inhibition of AA-induced bladder overactivity in α-chloralose anesthetized cats. Intravesical infusion of dilute AA (0.25%) was used to irritate the bladder and activate nociceptive C-fiber bladder
afferents. LY341495 (a group II mGluR antagonist) and naloxone were administered to evaluate
the role of glutamatergic and opioid receptors. The effects of these agents on TNS inhibition may
provide insights into the mechanisms underlying the clinical efficacy of tibial neuromodulation
for the treatment of overactive bladder (OAB) symptoms (30, 31).

**Materials and Methods**

The Animal Care and Use Committee at the University of Pittsburgh approved all
protocols involving the use of animals in this study.

*Experimental Setup*

Experiments were conducted in a total of 18 cats (10 male, 8 female, 2.7-3.9 kg, 6-12
month old domestic shorthairs, Liberty Research Inc., Waverly, NY, USA) anesthetized initially
with isoflurane (2-5% in oxygen) and maintained with α-chloralose (65 mg/kg i.v. with
supplementation as necessary). Heart rate and blood oxygen level were monitored by a pulse
oximeter (9847 V, NONIN Medical, Inc., Plymouth, MN, USA) with the sensor attached to the
tongue. Systemic blood pressure was monitored via a catheter in the right carotid artery. Drug
and fluid were administered via the right cephalic vein, and airway access was secured with a
tracheotomy tube.

The ureters were isolated via an abdominal incision, cut, and drained externally using
penrose drainage tubing. The bladder was cannulated through the urethra with a double lumen
catheter. One lumen was used to infuse saline or 0.25% AA at a rate of 0.5-2 ml/min, and the
other lumen was attached to a pressure transducer to record the bladder pressure. A ligature was
tied around the proximal urethra approximately 2 cm from the bladder to prevent leakage. The
tibial nerve was exposed on the medial side of right hindlimb above the ankle. A tripolar cuff electrode (NC223pt, MicroProbe, Inc., Gaithersburg, MD, USA) was applied around the nerve and connected to a stimulator (S88, Grass Medical Instruments, Quincy, MA, USA).

**Stimulation Protocol and Drug Administration**

Uniphasic rectangular pulses (5 Hz frequency, 0.2 ms pulse width) were delivered to the tibial nerve via the cuff electrode. The intensity threshold (T) for inducing toe movement was determined by gradually increasing the stimulation intensity. Because our previous study indicated that a 2T stimulus intensity was required to inhibit reflex bladder contractions (40), intensities of 2T or 4T were used in this study to suppress nociceptive bladder overactivity induced by 0.25% AA irritation.

A cystometrogram (CMG) was performed with saline infusion to measure bladder capacity that was defined as the bladder volume threshold to induce a large amplitude (>30 cmH₂O) and long duration (>20 sec) bladder contraction. Then, multiple (3-5) saline CMGs were repeated to evaluate the reproducibility in a 30-90 minute period. Once the bladder capacity was determined during saline infusion, 0.25% AA was infused into the bladder during repeated CMGs in order to activate nociceptive C-fiber bladder afferents and induce an overactive bladder reflex. After 15-40 minutes of AA infusion for bladder capacity to be stabilized, four CMGs were performed during AA infusion: (1) control without TNS, (2) during 2T TNS, (3) during 4T TNS, and (4) control without TNS (Fig. 1A). Then, pharmacological studies were performed.

Cumulative doses (0.1, 0.3, 1, 3, and 5 mg/kg, i.v.) of LY341495 {(2S)-2-Amino-2-[(1S,2S)-2-carboxycycloppr-1-yl]-3-(xanth-9-yl) propanoic acid, Abcam Inc., Cambridge, MA, USA} were given in 10 cats. LY341495 is an antagonist for both mGluR2 and mGluR3 receptors
that are classified as group II mGluR. Starting 15 minutes after administering each dose of LY341495, four CMGs were performed during AA infusion: (1) control without TNS, (2) during 2T TNS, (3) during 4T TNS, (4) control without TNS. In 9 cats following the testing of LY341495, cumulative doses (0.001, 0.01, and 0.1 mg/kg, i.v.) of naloxone (Sigma-Aldrich, St. Louis, MO, USA) were injected to block opioid receptors prior to repeated testing of TNS. Naloxone is a non-selective antagonist for \( \mu \), \( \kappa \), and \( \delta \) opioid receptors. Starting 5 minutes after administering each dose of naloxone, four CMGs were performed during AA infusion: (1) control without TNS, (2) during 2T TNS, (3) during 4T TNS, and (4) control without TNS. A 5-minute rest period was inserted between the repeated CMGs to allow the bladder to recover from previous contractions.

In another experimental group (N=8 cats), the animals were not treated with LY341495 but the same protocol for repeated CMG tests (control, 2T, 4T, and control) was also used during AA infusion. Naloxone alone was administered in cumulative doses (0.001, 0.01, and 0.1 mg/kg, i.v.) to evaluate the effect of varying levels of opioid receptor blockade on TNS-induced inhibition in the absence of LY341495.

Data Analysis

For the repeated CMG recordings, bladder capacities were measured and normalized to the measurement of the first saline control CMG or the AA control CMG before naloxone test in the same animal so that the results from different animals could be compared. Repeated measurements in the same animal under the same experimental conditions were averaged. The results from different animals are reported as mean ± standard error (SE). Statistical significance
(P<0.05) was detected by one-way ANOVA followed by Dunnett multiple comparison, or two-way ANOVA followed by Bonferroni multiple comparison.

**Results**

*TNS inhibition of bladder overactivity induced by AA irritation*

Intravesical infusion of 0.25% AA irritated the bladder, activated nociceptive C-fiber bladder afferents, and significantly (P < 0.0001) reduced bladder capacity to 26.6±4.7% of the saline control capacity (8.5 ± 1.3 ml, N=10 cats) (Fig. 1). TNS at 2T and 4T intensity suppressed AA-induced bladder overactivity and significantly increased bladder capacity to 62.2±8.3% (P < 0.01) and 80.8±9.2% (P = 0.0001) of the saline control capacity, respectively (Fig. 1B). After the 2T and 4T TNS, bladder capacity returned to 26.7±5.7% of the saline control capacity (Fig. 1B).

*Dose-dependent effect of LY341495 on TNS inhibition of bladder overactivity*

The effect of LY341495 on TNS inhibition of bladder overactivity was dependent on the drug dosage and the TNS intensity (Fig. 2 and Fig. 3). Administering cumulative doses of LY341495 (0.1, 0.3, 1, 3 and 5 mg/kg, i.v.) did not significantly (P > 0.05) change the control bladder capacity in the absence of stimulation (Fig. 2A and Fig. 3). However, LY341495 completely blocked the inhibition induced by low intensity (2T) TNS at doses of 1-5 mg/kg (Fig. 2A-B and Fig.3), and significantly (P < 0.001) reduced the inhibition induced by high intensity (4T) TNS at doses of 3-5 mg/kg (Fig. 2C and Fig. 3). When compared to the AA control capacity, the high intensity (4T) TNS could still significantly increase the bladder capacity at doses of 3-5 mg/kg. In these experiments following 2T and 4T TNS, bladder capacity returned to the control level prior to stimulation, i.e. a post-stimulation effect did not occur. Post-stimulation inhibition
following TNS has been observed during saline CMGs (40) but not during AA irritation of the bladder (38).

The number of small uninhibited detrusor contractions that occurred before the micturition contraction was not changed significantly (P>0.05) by LY341495 treatment or stimulation. On average from the 10 cat experiments, the number of uninhibited detrusor contractions was 1.8±0.5 under AA control conditions before LY341495 treatment and was 2.3±0.5 after the last dose (5 mg/kg) of LY341495 treatment. There were 1.7±0.8 uninhibited detrusor contractions during 4T TNS before LY341495 treatment and 1.4±0.5 contractions after the last dose of LY341495 treatment.

Dose-dependent effect of naloxone on TNS inhibition of bladder overactivity

After administering the last dose (5 mg/kg) of LY341495, the effect of cumulative doses of naloxone (0.001-0.1 mg/kg, i.v.) on the residual TNS inhibition elicited by 4T stimulation was examined in 9 cats. Naloxone did not change the AA control bladder capacity in the absence of TNS, but a dose of 0.01 mg/kg completely blocked the residual TNS inhibition (Fig.4 A and Fig.5 A).

In another group of experiments (N=8 cats) without LY341495 pre-treatment, cumulative doses of naloxone (0.001-0.1 mg/kg, i.v.) also did not change the AA control bladder capacity prior to TNS (Fig.4 B and Fig.5 B) but dose dependently reduced the magnitude of the TNS inhibition. However, compared to the results in animals pretreated with LY341495 (5 mg/kg) a 10 times higher naloxone dose (0.1 mg/kg) was needed to completely block the 4T TNS inhibition (Fig.4 and Fig.5). In these animals the 0.01 mg/kg naloxone dose eliminated the
inhibition induced by 2T TNS (Fig.5 B). Inhibition at this intensity was also eliminated by the 3 mg/kg and 5 mg/kg doses of LY341495 (Fig.3 and Fig.5 A).

**Discussion**

This study revealed that LY341495 (a group II mGluR antagonist) administered intravenously in a range of doses did not change the bladder overactivity induced by AA irritation, but partially suppressed in a dose dependent manner TNS inhibition of bladder overactivity (Fig. 2 and Fig. 3). After the maximal dose of LY341495 (5 mg/kg), naloxone (an opioid receptor antagonist) completely eliminated the remaining TNS inhibition (Fig. 4 and Fig. 5) at a dose (0.01 mg/kg) 10 times smaller than the dose (0.1 mg/kg) required to completely block the inhibition in animals that were not treated with LY341495. These results indicate that neurotransmitter mechanisms involving group II mGluRs and opioid receptors are essential for TNS inhibition of reflex bladder overactivity and that there may be a significant synergistic interaction between these two mechanisms.

Various studies have implicated group II mGluRs in peripheral and central nociceptive mechanisms. Group II mGluRs have been identified with immunohistochemical methods in the spinal cord and brain, as well as in small to medium size dorsal root ganglia (DRG) neurons, and in cutaneous sensory nerves that co-express TRPV1 receptors (5, 7, 27). Group II mGluR antagonists enhance nociceptive behavior and primary afferent firing induced by intraplantar injection of capsaicin; and a group II mGluR agonist suppressed this enhancement (9). Group II mGluR agonists are also thought to act in the spinal cord to produce analgesia (13, 34) by suppressing the release of glycine, GABA, or glutamate (35, 48), while knockdown of group II mGluR enhances nociceptive responses (46). These results indicate that somatic nociceptive
pathways are controlled by tonically active group II mGluR inhibitory mechanisms. However, our studies did not detect similar tonically active inhibitory mechanisms in the control of nociceptive bladder reflexes. Bladder overactivity was not altered after administration of LY341495 that induces hyperalgesia and enhances nociceptive behavior in somatic pain models (9, 34). However, the drug did suppress TNS-induced inhibition of bladder overactivity. This suggests that the group II mGluR inhibitory mechanism in bladder reflex pathways was not effective except during TNS.

Pharmacological studies of somatic nociceptive mechanisms have provided evidence for a linkage between group II mGluRs and opioid receptors. Group II mGluR antagonists (15, 16, 45) enhance the inhibitory effect of opioid receptor agonists on somatic nociceptive responses induced by thermal stimuli or formalin injection. On the other hand, a group II mGluR agonist also enhances opioid-induced antinociceptive effect on neuropathic pain (29). These results indicate that mGluR-opioid interaction could be complex depending on different types of nociception. Our study showing that LY341495 enhances the potency of naloxone in blocking TNS inhibition (Figs.4-5) raises the possibility that group II mGluR also facilitate endogenous opioid inhibitory mechanisms in the TNS inhibitory pathway and that removal of the facilitation by LY341495 reduces the opioid inhibition and makes it more susceptible to blockade by naloxone.

The inhibition of reflex bladder activity by TNS is thought to occur by modulation of the micturition reflex pathway at a site in the central nervous system. As shown in Fig.6, both spinal and supraspinal pathways can mediate reflex bladder activity. Therefore, TNS inhibition could occur in the brain or spinal cord. Because naloxone and LY341495 were both administered systemically, it is impossible to determine their site of action. However, it is likely that these
drugs interact at least in part at synapses in the lumbosacral spinal cord based on the following observations: (1) group II mGluRs and opioid receptors are expressed in the spinal dorsal horn (6, 8, 21, 22, 32, 41); (2) intrathecal administration of group II mGluR agonists suppress nociceptive behavior (13, 23, 24, 34), (3) AA-induced bladder overactivity is mediated by a reflex pathway organized in the spinal cord (17), (4) our previous study (39) suggested that TNS inhibition of AA-induced bladder overactivity could be mediated by suppression of transmission at a spinal interneuronal synapse prior to the sacral parasympathetic preganglionic neurons, (5) spinal opioid receptors have a prominent inhibitory effect on micturition (12, 20, 42, 43).

Fig.6 shows a hypothetical spinal mechanism for TNS inhibition, which is intended to facilitate the discussion about possible sites of interaction between naloxone and LY341495. Alternative sites of action in the brain are also indicated but detailed pathways are not shown. In the diagram TNS inhibition mediated by inhibitory interneuron-1 targets excitatory interneuron-3 on the spinal micturition pathway interposed between the bladder C-fiber afferent and a bladder preganglionic neuron (neuron-4). Electrophysiological evidence for this type of interneuronal inhibition by activation of somatic afferent nerves has been obtained in the cat spinal cord (11). Because TNS inhibition is blocked by naloxone, we propose that inhibitory interneuron-1 is enkephalinergic and releases an opioid peptide. Opioid peptides may be released as co-transmitters with glutamate acid because they are contained in subpopulations of glutamatergic neurons in the spinal dorsal horn (26, 44). Thus these two transmitters may interact to elicit a synergistic postsynaptic inhibitory effect at the synapse between interneuron-1 and interneuron-3. Group II mGluR and opioid receptors share a common intracellular signaling mechanism that inhibits adenylate cyclase activity and reduces cAMP levels (4, 10). This may account for the synergism and for the enhancement of opioid induced analgesia by exogenously administered
group II mGluR agonists. In addition, activation of group II mGluRs can inhibit voltage-gated Ca\(^{2+}\) channels, activate potassium channels to decrease neuronal excitability, and suppress synaptic transmission (4, 10, 18, 49). Elimination of the synergism following block of group II mGluR with LY341495 may explain the reduction in TNS inhibition after LY341495 and the increased potency of naloxone to block TNS inhibition.

Group II mGluR mechanisms could also indirectly affect TNS opioid inhibitory mechanisms via changes in NMDA ionotropic GluR. Activation of group II mGluR inhibits NMDA receptor mechanisms (1, 25, 33) and inhibition of NMDA receptors enhances opioid-induce antinociception (14, 28, 36, 37). Therefore, it is possible in our experiments that LY341495 reduced a tonic inhibition of NMDA receptors mediated by group II mGluR, thereby weakening an opioid antinociceptive effect and partially suppressing the TNS inhibition. This could be tested in future experiments by evaluating the sensitivity of TNS inhibition to other drugs that modulate NMDA receptors. Additionally, non-specific effects of the tested drugs could also play a role in the drug interaction such as changes in drug metabolism which could alter the potency of the drug.

An alternate hypothesis for the effect of LY341495 on TNS inhibition is that the drug acts at more proximal sites on the TNS reflex pathway to down regulate the firing of inhibitory interneuron-1. This mechanism would require a collateral inhibitory pathway that modulates TNS inhibition (interneuron-5, Fig. 6). It is known that group II mGluRs are located presynaptically on glutamatergic (10, 33) and GABAergic/glycinergic nerve terminals (35, 48) where they suppress transmitter release. Block of group II mGluR with LY341495 would increase the release of the transmitters, enhance transmission in the collateral inhibitory pathway, and therefore decrease the firing of inhibitory interneuron-1.
Perspectives and Significance

Our recent studies showing that tramadol which produces an active metabolite with opioid receptor agonist activity can significantly enhance TNS inhibition of AA-induced bladder overactivity (47) have provided additional support for the involvement of opioid receptor mechanisms in TNS inhibition of bladder nociception. Our current results raise the possibility that group II mGluR agonists might interact with tramadol to further enhance TNS inhibition of bladder nociception. However, whether these results obtained in anesthetized animals can be translated into clinical applications in human subjects still needs to be validated. Understanding neurotransmitter mechanisms underlying TNS inhibition of nociceptive bladder overactivity is important for development of new therapies to treat OAB or painful bladder syndrome/interstitial cystitis (2, 3, 19) and reduce adverse drug effects by combining drug therapy with neuromodulation.

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Disclosures

None of the authors have any conflicts of interest associated with this study.

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Figure Captions

Figure 1. Inhibition of bladder overactivity by TNS. A. Repeated CMGs during saline or 0.25 % acetic acid (AA) infusion with/without TNS. T - threshold intensity of stimulation (5 Hz, 0.2 ms, T = 1.0 V). Short arrows indicate the start and stop of the infusion. Infusion rate = 1 ml/min. B. Summarized results of TNS inhibitory effect on bladder capacity during AA infusion (N = 10 cats). Infusion rate = 0.5-2 ml/min. * indicates significantly different from AA control (one-way ANOVA).

Figure 2. Dose dependent effect of LY341495 on tibial inhibition of bladder overactivity induced by 0.25 % acetic acid (AA). The CMGs at each dose of LY341495 were performed in sequence from left to right in figures A-C. A. The AA CMGs without stimulation. B. The AA CMGs during 2T stimulation. C. The AA CMGs during 4T stimulation. The black bars under the pressure trace indicate stimulation duration. Stimulation: 5 Hz, 0.2 ms, intensity threshold T = 0.7 V. Short arrows indicate the start and stop of bladder infusion. Infusion rate = 1 ml/min.

Figure 3. Dose-dependent effect of LY341495 on TNS inhibition of bladder overactivity induced by 0.25 % acetic acid (AA) (N = 10 cats). * indicates significantly different from AA control at each dosage (two-way ANOVA). # indicates significantly different from the bladder capacity
measured during TNS before LY341495 treatment (i.e. at 0 mg/kg of LY341495) (one-way ANOVA). Bladder capacity was normalized to the capacity during saline CMG prior to infusion of AA. Stimulation: 5 Hz, 0.2 ms, T = 0.35-1.2 V.

Figure 4. Effect of naloxone on TNS inhibition of bladder overactivity induced by 0.25 % acetic acid (AA). A. CMGs during 0.25 % AA infusion after 5 mg/kg LY341495 pretreatment. B. CMGs during 0.25 % AA infusion without LY341495 pretreatment. Black bars under the bladder pressure traces indicate the stimulation duration. T - threshold intensity of stimulation (5 Hz, 0.2 ms, T = 0.7 V in A, but T = 1 V in B). Infusion rate = 1 ml/min.

Figure 5. LY341495 pretreatment significantly reduces the naloxone dosage required to eliminate TNS inhibition of bladder overactivity induced by 0.25% acetic acid (AA) infusion. A. Naloxone effect in LY341495 (5 mg/kg) pretreated animals (N = 9 cats). B. Naloxone effect without LY341495 pretreatment (N = 8 cats). Bladder capacities were normalized to the capacity measured during the AA control CMG before naloxone treatment (i.e. at 0 mg/kg naloxone). Stimulation: 5 Hz, 0.2 ms, T = 0.35-2.4 V. * indicates significantly different from control capacity at each dosage (two-way ANOVA).

Figure 6. Putative opioid receptor and group II mGluR mechanisms underlying the inhibitory effect of TNS on overactive bladder reflexes induced by nociceptive stimulation of the bladder with acetic acid (AA). Reflex pathways on the right side of the figure show spinal and supraspinal circuits mediating excitatory input to the bladder activated by C-fiber and Aδ bladder afferents, respectively. Left side of the figure shows an interneuronal pathway mediating TNS-induced inhibition of the bladder reflexes. Interneuron-1 that is activated by tibial afferents releases opioid peptides and glutamate that inhibit interneuron-3 on the spinal micturition reflex pathway by activating opioid receptors (*) and group II mGluR (#), respectively. Activation of
interneuron-5 by the tibial afferent modulates TNS inhibition by eliciting GABAergic/glycinergic inhibition of interneuron-1. Presynaptic group II mGluR at sites (#) on the interneuronal-5 pathway suppress transmitter release. Block of these receptors with LY341495 enhances the inhibitory modulation of interneuron-1 and reduces TNS inhibition of the micturition reflex. Opioid receptor (*) and group II mGluR (#) mechanisms in micturition centers (PAG/PMC) in the brain stem may also contribute to TNS inhibition. Note: The spinal mechanism for TNS enkephalinergic inhibition of the supraspinal micturition reflex pathway is not included in this figure.
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