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Role of GABA receptors in nitric oxide inhibition of dorsolateral periaqueductal gray neurons

Jihong Xing, De-Pei Li, Jianhua Li*

Heart and Vascular Institute and Department of Medicine, The Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, Hershey, PA 17033, USA

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Abstract

Nitric oxide (NO) affects neuronal activity of the midbrain periaqueductal gray (PAG). The purpose of this report was to investigate the role of GABA receptors in NO modulation of neuronal activity through inhibitory and excitatory synaptic inputs within the dorsolateral PAG (dl-PAG). First, spontaneous miniature inhibitory postsynaptic currents (mIPSCs) and excitatory postsynaptic currents (mEPSCs) were recorded using whole cell voltage-clamp methods. Increased NO by either *S*-nitroso-*N*-acetyl-penicillamine (SNAP, 100 μM) or L-arginine (50 μM) significantly augmented the frequency of mIPSCs of the dl-PAG neurons without altering their amplitudes or decay time constants. The effects were eliminated after bath application of carboxy-PTIO (NO scavenger), and 1-(2-trifluorom-ethylphenyl) imidazole (NO synthase inhibitor). In contrast, SNAP and L-arginine did not alter mEPSCs in dl-PAG neurons. However the frequency of mEPSCs was significantly increased with prior application of the $GABA_B$ receptors antagonist, CGP55845. In addition, NO significantly decreased the discharge rate of spontaneous action potentials in the dl-PAG neurons and the effect was reduced in the presence of the $GABA_A$ receptor antagonist, bicuculline. Our data show that within the dl-PAG NO potentiates the synaptic release of GABA, while NO-induced GABA presynaptically inhibits glutamate release through $GABA_B$ receptors. Overall, NO suppresses neuronal activity of the dl-PAG via a potentiation of GABAergic synaptic inputs and via $GABA_A$ receptors.

Keywords: Synaptic transmission; GABA; Patch clamp; Autonomic regulation

1. Introduction

The midbrain periaqueductal gray (PAG) is an important neural site for a number of physiological functions including behavioral reactions, autonomic regulation and pain modulation (Bandler et al., 1991; Behbehani, 1995; Lovick, 1996). The PAG including the dorsolateral (dl), lateral and ventrolateral regions receives abundant somatic afferent inputs from the dorsal horn of the spinal cord (Craig, 1995; Keay et al., 1997; Wiberg and Blomqvist, 1984). Those regions of the PAG further send descending neuronal projections to the medulla (Hudson and Lumb, 1996; Odeh and Antal, 2001) in regulating pain and autonomic activity (Boscan and Paton, 2005; Tjen-A-Looi et al., 2006; van Bockstaele et al., 1991; Verberne and Guyenet, 1992). The dl-PAG is considered as a pressor area and activation of this region contributes to an increase in arterial blood pressure and antinociception (Bandler et al., 1991; Behbehani, 1995).

Nitric oxide (NO) that is produced within the PAG (Vincent and Kimura, 1992) is involved in cardiovascular regulation (D'Amico et al., 1994; Hamalainen and Lovick, 1997; Hironaga et al., 1998). For example, enhanced NO in the dl-PAG decreases renal sympathetic nervous activity and blood pressure. It has been shown using c-Fos expression that both somatic receptor and baroreceptor afferent inputs activate neuronal cells in various regions of the PAG (Li, 2002; Li and

* Corresponding author at: Heart and Vascular Institute and Division of Cardiology H047, The Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, 500 University Drive, Hershey, PA 17033, USA. Tel.: +1 717 531 5051; fax: +1 717 531 1792.
E-mail address: jzl10@psu.edu (J. Li).

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Mitchell, 2000). Also, ~18% of the neurons activated by somatic nerve fibers are in close proximity with neuronal processes containing nNOS in the dl-PAG (Li, 2002), suggesting that NO production in the PAG may affect neuronal activity and influence the cardiovascular responses.

In addition, electrophysiological evidence has shown that NO inhibits neuronal discharge of the dl-PAG (Hall and Behbehani, 1998; Lovick and Key, 1996). In the first of these studies, spontaneous inhibitory postsynaptic currents (IPSCs) and excitatory postsynaptic currents (EPSCs) were examined and the results suggested that NO modulates the release of GABA and glutamate in the dl-PAG (Hall and Behbehani, 1998). However, EPSCs were recorded without inhibition of GABA receptors in this experiment. Activation of presynaptic $GABA_B$ receptors inhibits synaptic glutamate release in the CNS (Iydomi et al., 2000; Lei and McBain, 2003; Takahashi et al., 1998). The role of $GABA_B$ receptors in NO effects on glutamate release in the dl-PAG have yet to be determined, but the possibility that NO indirectly modulates glutamate release by directly modulating GABA release and its subsequent activation of presynaptic $GABA_B$ receptors was not ruled out (Hall and Behbehani, 1998).

In the present study, therefore, we first examined the effect of NO on spontaneous miniature IPSCs (mIPSCs) in dl-PAG neurons. We also examined the effect of NO on miniature EPSCs (mEPSCs) in the presence and absence of a $GABA_B$ receptor antagonist. We hypothesized that NO would increase synaptic release of GABA and that elevated GABA would then attenuate presynaptic glutamate release via activation of $GABA_B$ receptors. Finally, we further examined the role of GABAergic synaptic inputs and $GABA_A$ receptors in the inhibitory action of NO on the firing activity of dl-PAG neurons.

2. Methods

2.1. Brain slice preparations

All procedures outlined in this study were approved by the Animal Care Committee of Penn State College of Medicine. Sprague–Dawley rats of either gender (4–6 weeks old) were anesthetized by inhalation of isoflurane oxygen mixture (5% isoflurane in 100% oxygen), and then were decapitated. Briefly, the brain was quickly removed and placed in ice-cold artificial cerebral spinal fluid (aCSF) perfusion solution. A tissue block containing the midbrain PAG was cut from the brain and glued onto the stage of the vibratome (Technical Product International, St. Louis, MO). Coronal slices (300 μm) containing the midbrain PAG were dissected from the tissue block in ice-cold aCSF solution. The slices were incubated in the aCSF at 34 °C for an equilibrium period of 60 min. The slices were then transferred to the recording chamber. During the procedures described above, aCSF was saturated with 95% O_2–5% CO_2. The aCSF perfusion solution contained (in mM) 124.0 NaCl, 3.0 KCl, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 1.4 NaH_2 PO_4, 10.0 glucose, and 26.0 $NaHCO_3$ (Li et al., 2002, 2004).

2.2. Electrophysiological recordings

2.2.1. Postsynaptic currents of dl-PAG neurons

A whole cell voltage-clamp technique was used to record postsynaptic currents in the dl-PAG neurons. Borosilicate glass capillaries (1.2 mm OD, 0.69 mm ID; Harvard, South Natick, MA) were pulled to make the recording pipettes using a puller (Sutter Instrument, Novato, CA). The resistance of the pipette was 4–6 MΩ when it was filled with the internal solution (contained in mM: 130.0 potassium gluconate, 1.0 $MgCl_2$, 10.0 HEPES, 10.0 EGTA, 1.0 $CaCl_2$, 4.0 ATP-Mg) (Li et al., 2002, 2004). When mIPSCs and mEPSCs were examined, the internal solution contained 3.0 mM of lidocaine *N*-ethyl bromide (QX-314) in order to block sodium currents and possible postsynaptic effect in these voltage-clamp experiments. The solution was adjusted to pH 7.25 with 1 M of KOH and osmolarity of 280–300 mOsm. The slice was placed in a recording chamber (Warner Instruments, Hamden, CT) and fixed with a grid of parallel nylon threads supported by a U-shaped stainless steel weight. The aCSF saturated with 95% O_2–5% CO_2 was perfused into the chamber at 3.0 ml/min. The temperature of the perfusion solution was maintained at 34 °C by an in-line solution heater with a temperature controller (model TC-324; Warner Instruments). Whole cell recordings from the dl-PAG neurons were performed visually using differential interference contrast (DIC) optics on an upright microscope (BX50WI, Olympus, Tokyo, Japan). The tissue image was captured and enhanced through a camera and displayed on a video monitor. A tight giga-ohm seal was subsequently obtained from dl-PAG neuron viewed using DIC optics. A 5–10 min equilibration period was allowed after whole cell access was established and the recording reached a steady state. The recording was abandoned if the monitored input resistance changed >15%.

2.2.2. Spontaneous action potentials of dl-PAG neurons

A whole cell current-clamp technique was used to record the spontaneous firing activity of the dl-PAG neurons. The recording procedures were described as above. Note that TTX and QX-314 weren't used in this experiment. Recordings of the firing activity of the dl-PAG neurons began 5–10 min after the whole cell access was established and the firing activity reached a steady state.

2.2.3. Drugs and their application

The mIPSCs were recorded in the presence of 1 μM of tetrodotoxin (TTX, Sigma Co.) and 20 μM of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Sigma Co.) at a holding potential of 0 mV. The mEPSCs were recorded in the presence of 1 μM of TTX and 20 μM of bicuculline (Sigma Co.) at a holding potential of −70 mV.

S-nitroso-*N*-acetyl-penicillamine (SNAP), L-arginine, 2 (4-carboxypheny)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), 1-(2-trifluoromethylphenyl) imidazole (TRIM), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and baclofen were obtained from Sigma Co. CGP55845 and QX-314 were obtained from Tocris Inc and Alomone Labs (Jerusalem, Israel), respectively. All drugs were dissolved in the aCSF perfusion solution immediately before they were used. According to experimental protocol, the drugs were delivered into the recording chamber at final concentrations using syringe pumps during the experiment (Li et al., 2002, 2004). After 3 min of control was obtained the responses of mIPSCs and mEPSCs to application of drugs were recorded.

2.3. Data acquisition and analysis

Signals were recorded with a MultiClamp 700B amplifier (Axon Instruments, Foster City, CA), digitized at 10 kHz with a DigiData 1322, filtered at 1–2 kHz, and saved in a PC-based computer using pClamp 9.0 software (Axon Instruments). A liquid junction potential of −15.0 mV (for the potassium gluconate pipette solution) was corrected during off-line analysis (Li et al., 2002, 2004). The mIPSCs, mEPSCs, and the firing activities of the PAG neurons were analyzed off-line with a peak detection program (Mini-Analysis, Synaptosoft, Leonia, NJ). Detection of mIPSCs and mEPSCs was accomplished by setting a threshold above the baseline level in the presence of the $GABA_A$ antagonist bicuculline and non-NMDA antagonist CNQX, respectively. The distribution of cumulative probability of the amplitude and inter-event interval of mIPSCs and mEPSCs was estimated using the Komogorov–Smirnov test (Li et al., 2002, 2004). Experimental data (amplitude, frequency and decay time of mIPSCs and mEPSCs, and the firing rate of dl-PAG neurons) were analyzed with one-way ANOVA. Tukey's post hoc analyses were utilized to determine the differences between groups, as appropriate. All values were expressed mean ± SE. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS for windows version 13.0.

3. Results

At the end of each experiment, the location of the recording pipette in the PAG slice was visualized and identified under a microscope using differential interference contrast (×40 magnification) (Xing and Li, 2007). It was confirmed that all the cells included for data analysis in this experiment located in the dl-PAG. Whole cell patch-clamp experiments were performed and experimental data were collected from 96 dl-PAG neurons.

3.1. Effect of NO on GABAergic mIPSCs

Spontaneous mIPSCs were recorded in the dl-PAG neurons ($n = 18$) in order to examine the effect of NO on synaptic GABA release onto the neurons (Fig. 1). Addition of the NO donor SNAP at 100 μM significantly increased the frequency of mIPSCs from 0.71 ± 0.03 to 1.19 ± 0.08 Hz ($P < 0.05$), but did not alter either the amplitude (41.2 ± 4.5 to 43.2 ± 4.3 pA, $P > 0.05$) or the decay time constants of mIPSCs (Fig. 1A,B). The mIPSCs recovered during washout of the perfusion solution and were completely abolished after bath application of 20 μM of bicuculline, a $GABA_A$ receptor antagonist (Fig. 1A). The responses of mIPSCs to SNAP developed at a latency of 15 ± 1 s and gradually increased over the next 0.5–5 min and declined within 5–10 min of washout. The onset of the SNAP effect was determined as the mIPSC frequency became significantly different from control. The effect of SNAP on mIPSCs was also analyzed by measuring the time constant of the decay phase of the mIPSCs. The decay phase of mIPSCs after application of SNAP was not different from the control (Fig. 1B). The decay time in control was 16.67 ± 0.44 ms and after SNAP 16.36 ± 0.58 ms ($P > 0.05$ vs. control). The cumulative probability analysis of mIPSCs also shows that the distribution pattern of the inter-event interval of mIPSCs shifted toward the left but the distribution pattern of the amplitude was not changed as SNAP was applied (Fig. 1C,D). Average data further show the effect of SNAP on the frequency and amplitude of mIPSC of the dl-PAG neurons (Fig. 1E,F).

A total of 1 μM of carboxy-PTIO, a specific NO scavenger, was applied in order to determine if the effect of SNAP on mIPSCs was mediated via an increase in NO ($n = 8$). Following the examination of the initial effect of SNAP (100 μM) on mIPSCs, carboxy-PTIO was perfused into the recording chamber. Subsequent application of SNAP failed to increase the frequency of mIPSCs (Fig. 1G–I). In addition, SNAP was repeatedly applied. The second application of SNAP still increased significantly the frequency of mIPSCs (Fig. 1G&H), indicating that no desensitization to SNAP had occurred. Finally, on the basis of previous reports (Li et al., 2003; Wang et al., 2006) 20 μM of ODQ, the soluble guanylate cyclase (sGC) inhibitor, was perfused into the recording chamber for 20 min (Fig. 1J&K, $n = 6$). This significantly attenuated SNAP effects. The frequency of mIPSCs in control was 0.69 ± 0.03 and after SNAP 0.71 ± 0.04 Hz ($P > 0.05$). This result suggests that the effect of SNAP was via the sGC pathway.

Similar to SNAP, 50 μM of L-arginine, an NO precursor, also significantly increased the frequency of spontaneous mIPSCs of the dl-PAG neurons from 0.68 ± 0.04 to 1.29 ± 0.11 Hz ($P < 0.05$, $n = 16$). A recovery of the mIPSCs was seen after the washout and the mIPSCs were then completely abolished in the presence of 20 μM bicuculline (Fig. 2A). However, L-arginine did not affect either the amplitude of the mIPSCs (41.5 ± 3.8 to 42.2 ± 3.4 pA, $P > 0.05$) or the decay time constants (15.86 ± 0.59 ms in control; and 16.51 ± 0.71 ms after L-arginine, $P > 0.05$ vs. control). Average data show the effect of L-arginine on the frequency and amplitude of mIPSCs of the dl-PAG neurons (Fig. 2B,C). When an average of 100 consecutive mIPSCs was superimposed during control and L-arginine application, the decay phase of mIPSCs was identical in control and during L-arginine perfusion (not illustrated). The cumulative probability analysis further suggests that L-arginine decreased the inter-event interval of mIPSCs but did not alter the amplitude distributions of the mIPSCs.

In the next group of experiments, the effect of L-arginine on the mIPSCs of the dl-PAG neurons during application of a specific nNOS inhibitor, TRIM, was examined ($n = 6$). L-arginine failed to increase the frequency of mIPSCs when applied in the presence of 50 μM TRIM (Fig. 2D–F) indicating that effect of L-arginine on mIPSCs was mediated via NO production. Furthermore, repeated administration of L-arginine still increased the frequency of mIPSCs (Fig. 2D,E). TRIM alone didn't significantly alter the frequency of mIPSCs (0.72 ± 0.06 to 0.69 ± 0.08 Hz, $P > 0.05$, $n = 6$).

3.2. Effect of NO on glutamatergic mEPSCs

Spontaneous mEPSCs were recorded from dl-PAG neurons in order to examine the effect of NO on synaptic glutamate release. SNAP (100 μM; $n = 10$) did no affect either the frequency (SNAP: 2.74 ± 0.32 to 2.71 ± 0.28 Hz, $P > 0.05$) or amplitudes (SNAP: 17.8 ± 1.1 to 18.4 ± 0.9 pA, $P > 0.05$) of mEPSCs in the dl-PAG neurons. The mEPSCs were completely eliminated by application of 20 μM of CNQX, an antagonist of non-NMDA glutamate receptors. The effect of SNAP on mEPSCs was also analyzed by measuring the time constant of the decay phase of mEPSCs. When average of 100 consecutive mEPSCs was superimposed during control and SNAP application, the decay time constant was similar during control (8.12 ± 0.44 ms) and during SNAP application (8.21 ± 0.50 ms, $P > 0.05$ vs. control). The cumulative probability analysis further shows that SNAP did not alter the distribution pattern of either the inter-event interval or the amplitude of the mEPSCs.

Similar to SNAP, 50 μM of L-arginine perfused into the chamber ($n = 8$) did not significantly alter either the frequency (2.61 ± 0.42 to 2.52 ± 0.36, $P > 0.05$) or amplitudes (16.2 ± 1.4 to 17.0 ± 0.8 pA, $P > 0.05$) of mEPSCs of the dl-PAG neurons. Also, L-arginine failed to alter the decay time constant of mEPSCs (8.46 ± 0.68 ms during control vs.

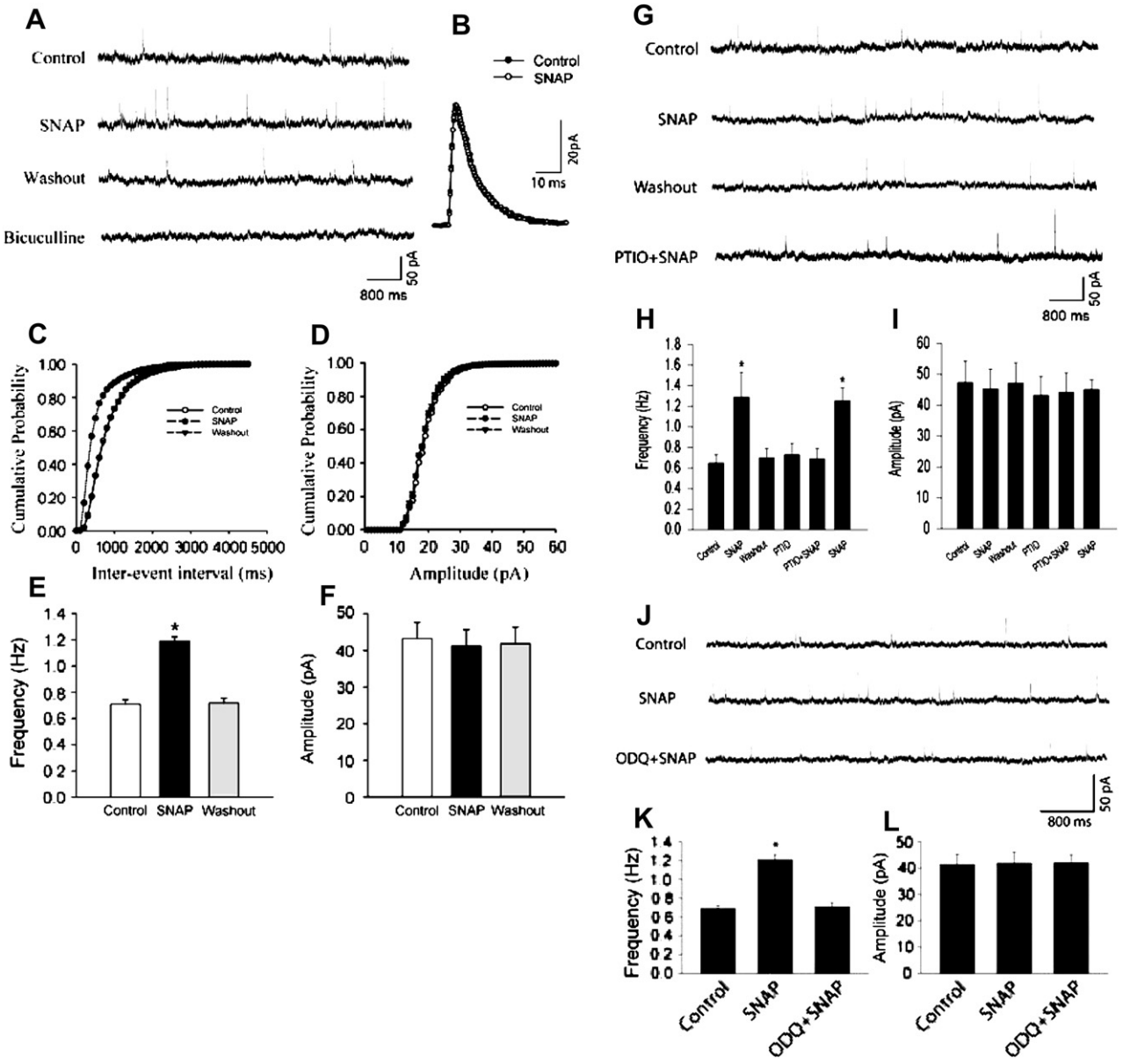

Fig. 1. Left panel: Effect of increased NO by *S*-nitroso-*N*-acetylpenicillamine (SNAP, an NO donor) on GABAergic miniature inhibitory postsynaptic currents (mIPSCs) of the dl-PAG neurons. **A:** Representative tracings from a dl-PAG neuron show that 100 μM of SNAP increased the frequency of spontaneous mIPSCs as compared with control, and that the mIPSCs recovered during washout. It is noted that the mIPSCs were completely abolished in the presence of 20 μM of bicuculline, a $GABA_A$ receptors antagonist. **B:** Average of 100 consecutive mIPSCs was superimposed during control and SNAP application. The decay phase of mIPSCs was identical in control and during SNAP. **C&D:** The cumulative probability analysis shows that SNAP decreased the inter-event interval of mIPSCs but did not alter the distribution pattern of the amplitude of the mIPSC. **E&F:** Average data show the effect of SNAP on the frequency and amplitude of mIPSC of the dl-PAG neurons ($n = 18$). $^*P < 0.05$, vs. control and washout. Right panel: Effect of SNAP on the mIPSCs of the dl-PAG neurons during application of a specific NO scavenger 2-(4-carboxypheny)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO). **G:** Representative tracings from a dl-PAG neuron show that 100 μM of SNAP increased the frequency of spontaneous mIPSCs and that this effect was abolished while 1 μM of carboxy-PTIO was perfused into the recording chamber. **H&I:** Average data show the effects of SNAP and SNAP plus carboxy-PTIO on the frequency and amplitude of mIPSC of the dl-PAG neurons ($n = 8$). $^*P < 0.05$, vs. control and SNAP plus carboxy-PTIO. **J–L:** Original tracings and average data ($n = 6$) show that 20 μM of ODQ attenuated SNAP effects on the frequency of the mIPSCs.

8.81 ± 0.80 ms during L-arginine, $P > 0.05$ vs. control). Neither the distribution pattern of the inter-event interval nor amplitude of the mEPSCs was shifted during L-arginine application.

In another group of experiments, in order to determine the effect of NO-released GABA on presynaptic glutamate release via $GABA_B$ receptors, we tested whether SNAP affected mEPSCs after their prior blockade with CGP55845. The frequency of mEPSCs was 2.61 ± 0.32 Hz in control and 2.59 ± 0.28 Hz after 100 μM of SNAP ($P > 0.05$) without blocking $GABA_B$ receptors. However, after bath application of 2 μM of CGP55845, a $GABA_B$ receptors antagonist, the

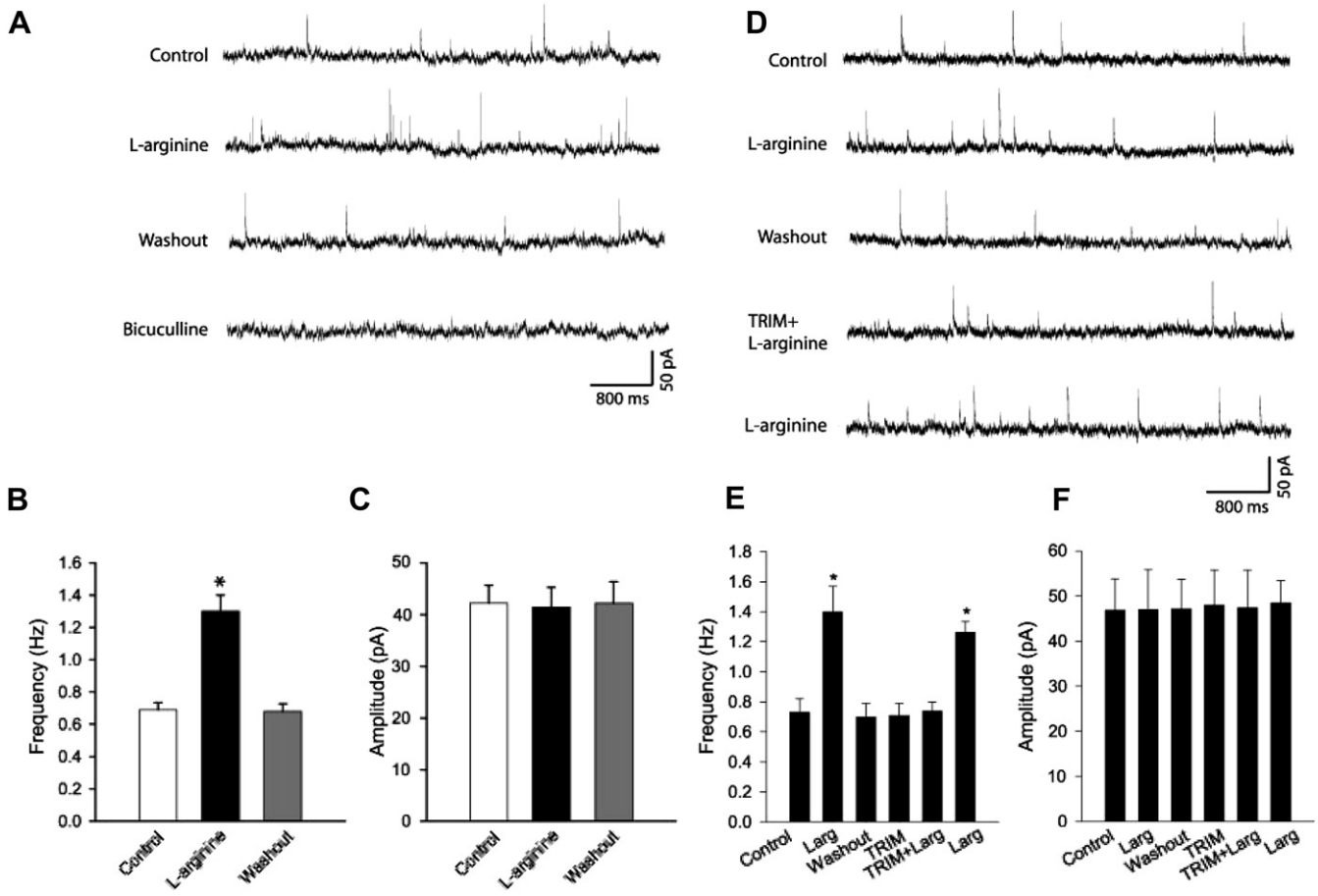

Fig. 2. Left panel: Effect of increased NO by L-arginine (an NO precursor) on GABAergic mIPSCs of the dl-PAG neurons. **A:** Representative tracings from a dl-PAG neuron show mIPSCs during control, 50 μM of L-arginine perfusion and washout. L-arginine increased the frequency of spontaneous mIPSCs. The mIPSCs were recovered during washout and were completely eliminated in the presence of 20 μM of bicuculline blocking $GABA_A$ receptors. **B&C:** Average data show the effect of L-arginine on the frequency and amplitude of mIPSC of the dl-PAG neurons ($n = 16$). $^*P < 0.05$, vs. control and washout. Right panel: Effect of L-arginine on the mIPSCs of the dl-PAG neurons during application of 1-(2-trifluoromethylphenyl) imidazole (TRIM, a specific nNOS inhibitor). **D:** Representative tracings from a dl-PAG neuron show that 50 μM of L-arginine increased the frequency of spontaneous mIPSCs and this effect was abolished while 50 μM of TRIM was perfused into the recording chamber. **E&F:** Average data show the effects of L-arginine during application of TRIM on the frequency and amplitude of mIPSC of the dl-PAG neurons ($n = 6$). $^*P < 0.05$, vs. control and L-arginine plus TRIM.

frequency of mEPSCs was significantly increased from 2.83 ± 0.30 Hz to 4.36 ± 0.32 Hz ($P < 0.05$, $n = 9$) without altering either the amplitudes or decay time constants (Fig. 3A–E). CGP55845 alone didn't have a distinct effect on the mEPSCs (frequency: 2.72 ± 0.32 Hz in control vs. 2.98 ± 0.31 Hz after CGP55845, $P > 0.05$, $n = 9$; and amplitude: 21.9 ± 1.75 pA in control vs. 20.8 ± 1.88 pA after CGP55845, $P > 0.05$, $n = 9$). The role of $GABA_B$ activation in the attenuation of mEPSCs was also confirmed (Fig. 4A–E). A total of 10 μM of baclofen significantly reduced the frequency of mEPSCs from 2.81 ± 0.15 Hz to 1.01 ± 0.18 Hz ($n = 10$, $P < 0.05$). The effect was eliminated with the prior application of 2 μM CGP55845.

3.3. Effect of NO on discharge of the dl-PAG neurons

The effect of SNAP on the discharge of the dl-PAG neurons was examined using whole cell current-clamp recordings (Fig. 5A–C). SNAP (100 μM) significantly decreased the discharge rate of the dl-PAG neurons from 3.97 ± 0.58 to 0.93 ± 0.27 Hz ($P < 0.05$, $n = 10$).

It seemed likely that NO inhibited the activity of the dl-PAG neurons via GABA because NO increased the inhibitory GABAergic inputs to the dl-PAG neurons but didn't alter the excitatory glutamatergic synaptic activity without blocking $GABA_B$ receptors. Thus, the effect of GABAergic synaptic inputs and $GABA_A$ receptors on NO inhibition of the dl-PAG neurons was determined ($n = 9$). The firing activities of dl-PAG neurons were examined in the presence of the $GABA_A$ receptor antagonist, bicuculline, following application of SNAP (Fig. 5D–F). The spontaneous discharge rates of the PAG neurons were increased following perfusion of 20 μM of bicuculline, suggesting that endogenously released GABA tonically inhibits dl-PAG neurons. However, subsequent application of 100 μM of SNAP failed to inhibit the spontaneous firing of neurons in the presence of bicuculline, suggesting that NO effects are mediated by activation of $GABA_A$ receptors. Since the increase in firing frequency after application

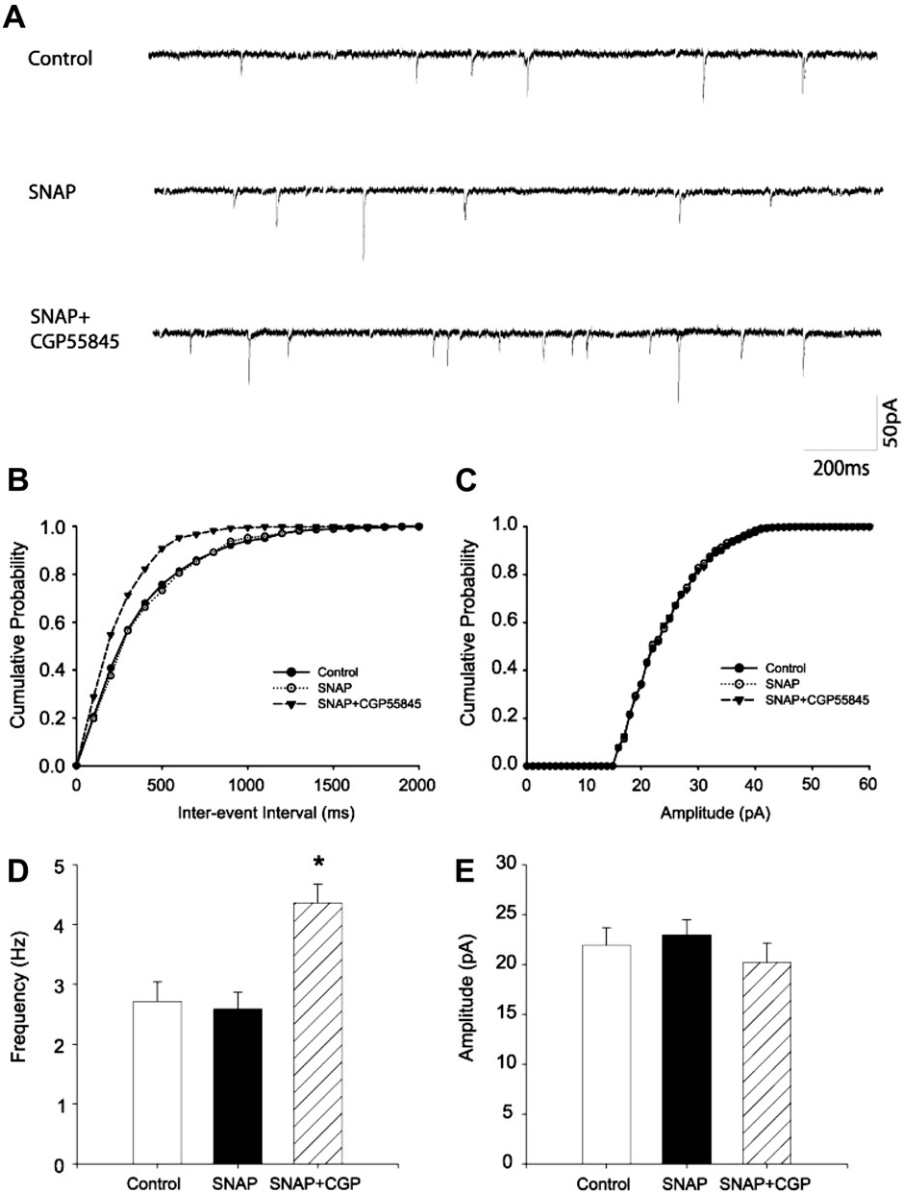

Fig. 3. Effect of SNAP on glutamatergic mEPSCs of the dl-PAG neurons with the prior application of CGP55845. **A:** Original recordings show mEPSCs during control, application of SNAP (100 μM) and application of CGP55845 (2 μM) plus SNAP. **B&C:** The cumulative probability plot shows the distribution of the inter-event interval and amplitude of the mEPSC. **D&E:** Average data show the frequency of mEPSC of the dl-PAG neurons was unaffected by SNAP but increased by SNAP after application of CGP55845 blocking $GABA_B$ ($n = 9$). Values are means ± SE. $^*P < 0.05$, vs. SNAP.

of bicuculline could be due to its effects on channels regulating spike accommodation, 50 μM of picrotoxin, another $GABA_A$ receptor antagonist was also used (Sanhueza and Bacigalupo, 2005; Hallworth and Bevan, 2005). Similar to bicuculline, picrotoxin increased the discharge rate of the dl-PAG neurons (3.85 ± 0.38 to 6.92 ± 0.42 Hz, $P < 0.05$, $n = 6$) and attenuated the effect of SNAP (6.53 ± 0.38 Hz after SNAP, $P > 0.05$, SNAP plus picrotoxin vs. picrotoxin alone, $n = 6$).

4. Discussion

In the present study, an in vitro PAG slice preparation allowed us to determine the modulatory actions of NO on inhibitory GABAergic and excitatory glutamatergic synaptic activity in the dl-PAG (Kabashima et al., 1997; Ozaki et al., 2000; Sulzer and Pothos, 2000). Our results demonstrated that bath application of an NO donor, SNAP as well as the NO precursor, L-arginine, increased the frequency of mIPSCs in dl-PAG neurons but did not significantly affect their amplitude (Figs. 1 and 2). These data suggest that NO increases synaptic GABA release in the PAG, and that the site of action of NO is most likely to locate at presynaptic GABAergic terminals (Sulzer and Pothos, 2000). Furthermore, the effects of SNAP and L-arginine on mIPSCs of the dl-PAG were completely eliminated by the specific NO scavenger carboxy-PTIO (Akaike et al., 1993) and the specific nNOS inhibitor TRIM (Handy et al., 1995), respectively (Figs. 1 and 2).

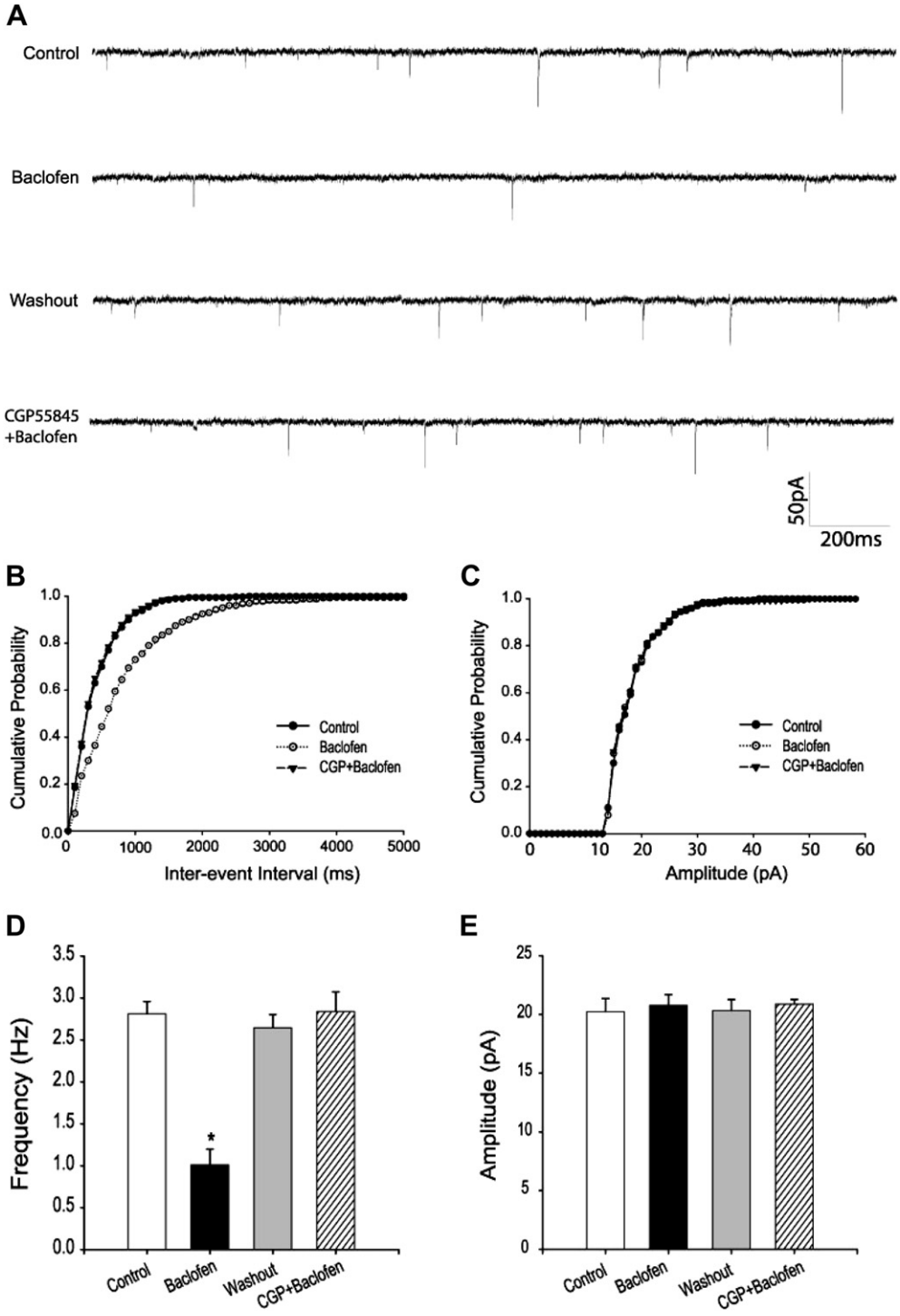

Fig. 4. Activation of $GABA_B$ inhibited the frequency of mEPSCs of the dl-PAG neurons. **A:** Original recordings show mEPSCs during control, application of 10 μM of baclofen and application of CGP55845 (2 μM) plus baclofen. **B&C:** The cumulative probability plot shows the distribution of the inter-event interval and amplitude of the mEPSC during control, application of baclofen and application of CGP55845 plus baclofen. **D&E:** Average data show the frequency of mEPSC of the dl-PAG neurons was inhibited by baclofen and the effect was antagonized by CGP55845 ($n = 10$). The amplitude of mEPSC of the dl-PAG neurons was unaffected by baclofen. Values are means ± SE. $^*P < 0.05$, vs. control and washout.

This finding further suggests that the effects of SNAP and L-arginine on increasing GABA were due to NO generation.

In agreement with the findings of Hall and Behbehani (1998) and Lovick and Key (1996), we presently observed that SNAP significantly inhibited the discharge activity of the dl-PAG neurons, and that the effect was abolished after blocking $GABA_A$ receptors with the prior application of bicuculline (Fig. 5). These findings indicate that the action of NO within the PAG is likely to be mediated by increasing synaptic release of GABA. Moreover, bicuculline significantly

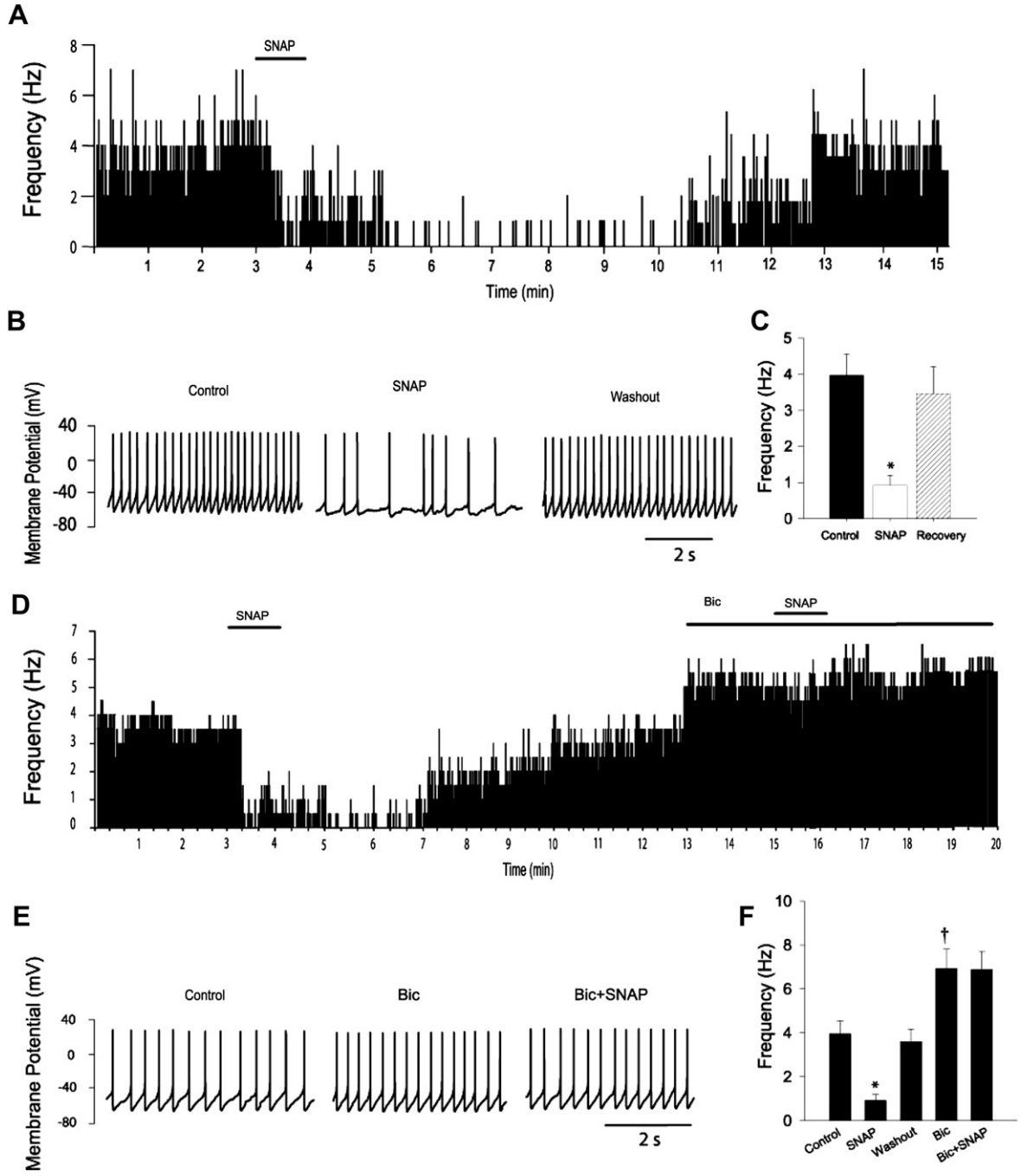

Fig. 5. First, SNAP had an inhibitory effect on the firing activity of the dl-PAG neurons. **A:** A histogram shows the time course of 100 μM of SNAP effect in a dl-PAG neuron. **B:** Original tracings from a dl-PAG neuron show the spontaneous discharge activity during control, SNAP perfusion and washout. **C:** Average data ($n = 10$). *$P < 0.05$, vs. control and recovery. Second, SNAP inhibition of the firing activity of the dl-PAG neurons was blunted after blocking $GABA_A$ receptors following perfusion of bicuculline (Bic). **D:** A histogram shows the time course of effects of SNAP (100 μM) plus bicuculline (20 μM) in a dl-PAG neuron. **E:** Original tracings from a dl-PAG neuron show the spontaneous discharge activity during control, bicuculline and SNAP perfusion with bicuculline. **F:** Average data ($n = 9$). *$P < 0.05$, vs. control. †$P < 0.05$, vs. control and washout. There is no difference for Bic vs. SNAP after Bic. Values are means ± SE.

increased the discharge rate of the dl-PAG neurons (Fig. 5), suggesting that GABA has a tonic influence within the dl-PAG.

It is noted that NO has also been shown to modulate GABA transmission in other brain regions including, but not restricted to, hypothalamus, hippocampus, cerebellum, spinal cord, etc. (Getting et al., 1996; Li et al., 2002; McLean and Sillar, 2004; Segovia et al., 1994; Wall, 2003).

The previous study also reported that NO had an effect on EPSPs (Hall and Behbehani, 1998). However, mIPSCs and mEPSCs were recorded in the presence of TTX in the present study. Thus, we monitored TTX-resistant mIPSC and mEPSC as an index of a presynaptic effect. Using this approach, TTX-resistant release events were found to be different from ''normal'' synaptic potentials evoked by invading action potentials. It has been reported that the key mechanism of NO on GABA

release is potentiation of Ca^{2+}-induced Ca^{2+} release from ryanodine/cADRP sensitive stores (Wang et al., 2006). This mechanism was suggested to play a fundamental role in action potential-induced release, but its involvement in TTX-resistant release is not clear. Hence, it is possible that while TTX-resistant mEPSCs were not very sensitive to NO without removing the depressant effect of $GABA_B$ receptors, the action potential-induced release, e.g. conventional EPSPs, could still be modulated (Hall and Behbehani, 1998). This could explain the lack of effect of NO on EPSCs by a concomitant increase in release of GABA, which affects the TTX-resistant glutamate release via GABA receptors.

In contrast to its actions on GABAergic mIPSCs, the effect of NO on the frequency of glutamatergic mEPSCs recorded from the dl-PAG neurons is dependent on activation of presynaptic $GABA_B$ receptors. Our results show that SNAP significantly increased the frequency of mEPSC when $GABA_B$ receptors were blocked (Fig. 3). Activation of presynaptic $GABA_B$ receptors inhibits synaptic glutamate release in the CNS (Iydomi et al., 2000; Lei and McBain, 2003; Takahashi et al., 1998). The role of $GABA_B$ receptors in attenuation of presynaptic glutamate release in the dl-PAG was also observed in this study (Fig. 4). Note that blocking $GABA_B$ receptors with CGP55845 alone had no effect on the mEPSCs. Findings suggest that there were no tonic $GABA_B$-mediated effects, in contrast to the tonic $GABA_A$-mediated inhibition.

In this experiment, either SNAP or L-arginine can increase NO production. However, it was not determined where NO was increased after application of those drugs since NOS can appear in neuronal cells and fibers of the dl-PAG. It is interesting that a recent study has also shown that there is a source of vascular NO that contributes the tone of NO influence in the hippocampus (Hopper and Garthwaite, 2006). Thus, NO might also be produced from a vascular source in the present experiment after SNAP or L-arginine was applied. The purpose of this report was to examine if NO could affect on GABA and glutamate releases from presynaptic sites. Despite of where NO was generated, our data support an idea that NO acts on presynaptic sites and increases GABA release, which inhibits activity of the dl-PAG neurons.

Nonetheless, the results of this study demonstrate that within the dl-PAG, NO enhances synaptic GABA release. Overall, NO suppresses neuronal activity of the dl-PAG via increase of GABAergic synaptic inputs and via $GABA_A$ receptors. This provides the first evidence suggesting that GABA receptors play a novel role in NO modulating the inhibitory GABAergic and excitatory glutamatergic inputs to the dl-PAG neurons (Fig. 6).

Inhibition of the dl-PAG neuronal firing by NO donors appears to be reduced after application of the sGC inhibitor, ODQ. Our data support the conclusion that the effect of NO generated within the dl-PAG is via the sGC pathway. NO in nanomolar concentrations can engage GABAergic inhibition via cGMP-mediated Ca^{2+} release from ryanodine/cADRP sensitive stores in brain cells (Wang et al., 2006). Considering 100 μM of SNAP perfused into the recording chamber, NO may also affect other pathways. A previous study using patch

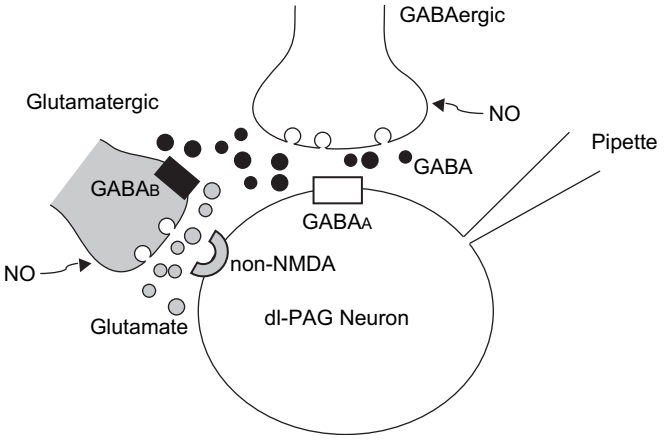

Fig. 6. Cartoon to illustrate the likely effect of NO on the release of GABA and glutamate in the dl-PAG. We proposed that NO increases synaptic release of GABA in the dl-PAG, and overspill of GABA acts at presynaptic $GABA_B$ receptors on glutamatergic terminals to attenuate release of glutamate.

methods has shown that 100 μM SNAP increases GABA release from presynaptic sites through sGC-cGMP-protein kinase G in the hypothalamic slice (Li et al., 2003).

GABAergic neurons, which constitute ~50% of the total population of neurons, play a crucial role in the intrinsic neuronal circuitry of the PAG (Mugnaini and Oertel, 1985; Reichling, 1991), and GABA synaptic inputs make up ~50% of the synaptic innervation of PAG neurons (Barbaresi, 2005). In the present study, blocking NO with the NOS inhibitor TRIM had no effect on GABA release within the dl-PAG. This indicates that NO does not tonically increase GABA release. It has been reported that an increase of NO production in the PAG attenuates the cardiovascular responses to activation of somatic sensory nerves and this effect is reduced by the blockade of $GABA_A$ receptors (Li, 2004). Therefore, it is very likely that enhanced NO within the PAG modulates autonomic responses by affecting GABAergic synaptic inputs when somatic nerves are activated. Comparing a previous study demonstrating the presence of NOS in GABAergic neurons in the dl-PAG (Lovick and Paul, 1999) to the present study, we see that it has important implications for interpretation of the data shown in the present report that increasing NO can alter GABAergic synaptic inputs in the dl-PAG. In the PAG, the majority of GABAergic neurons are tonically active interneurons (Barbaresi, 2005). Thus, the release of GABA from those neurons and the role played by NO in modulation of the presynaptic GABAergic terminals may have important contributions in integrating a variety of physiological functions of the PAG (Bandler et al., 1991; Behbehani, 1995; Brack and Lovick, 2007; Lovick, 1996, 2000). This also supports the idea that the GABA and GABA/NOS-containing populations of neurons play an essential role in amplifying neuronal inhibition in the PAG (Lovick, 2000). The results of the current study provide strong evidence that NO potentiates the presynaptic GABA release within the PAG and suppresses the neuronal activity via the increase of GABAergic synaptic inputs.

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Dr. Jihong Xing is a visiting scholar from Jilin University First Hospital, Changchun, Jilin Province 130021, PR China. Dr. De-Pei Li currently works at Department of Anesthesiology and Critical Care, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030.

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