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Title Page

Modulation of synaptic transmission to second-order peripheral chemoreceptor neurons in caudal NTS by α_1-adrenoreceptors

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Running Title Page

Running title: α_1-adrenoreceptors and peripheral chemoreflex

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ABSTRACT

NE is an important neurotransmitter in central autonomic regulation. Peripheral chemoreceptor stimulation activates central noradrenergic structures. These structures innervate and therefore could modulate neurons in caudal NTS (cNTS), which receives the first central projections from peripheral chemoreceptors. However, the role of α_1-adrenoreceptors in synaptic transmission of peripheral chemoreceptor inputs in cNTS is unknown. We investigated the responses to activation of α_1-adrenoreceptors on glutamatergic and GABAergic inputs in NTS slices using whole-cell recording. Second-order neurons were identified by DiA labeling of carotid bodies. Electrical stimulation of ipsilateral TS was used to evoke EPSCs (eEPSCs), while eIPSCs were evoked by electrically stimulating NTS near the recorded neuron. Application of α_1-adrenoreceptor agonist phenylephrine (PE) 20 μM significantly decreased amplitudes of eEPSCs (78±1% of control, n=16, $p<0.01$), and increased amplitudes of eIPSCs (120±13% of control, n=7, $p<0.01$). Both effects were blocked by the α_1-adrenoreceptor antagonist prazosin (10 μM). PE did not change holding current, input resistance, and I-V relationship in cNTS neurons. PE significantly changed paired-pulse ratios of eE/IPSCs, increased the frequency of miniature IPSCs (mIPSCs, 329±10% of control, n=6, $p<0.05$), but decreased that of mEPSCs (69±6% of control, n=5, $p<0.01$). PE-induced inhibition of eEPSCs was independent of NMDA or $GABA_B$ receptors. These results suggest that activation of α_1-adrenoreceptors reduces excitatory and enhances inhibitory inputs to second-order peripheral chemoreceptor neurons in cNTS via a pre-synaptic mechanism.

These actions result in the inhibition of synaptic transmission and could play a role in the autonomic responses to hypoxia.

INTRODUCTION

Norepinephrine (NE) is an important neurotransmitter in central autonomic regulation and sympathetic nerve discharge (Baker et al., 2001; Guyenet, 1991). Recently it has been found that NE is essential in central chemoreception (Li and Nattie, 2006). However, its role in peripheral chemoreflexes remains controversial (Joseph et al., 1998; McCrimmon et al., 1983; Schreihofer and Guyenet, 2000). Stimulation of peripheral chemoreceptors with systemic hypoxia or carotid sinus nerve stimulation increases *c-fos* expression in noradrenergic neural structures in the brain, including the A6 region (locus coeruleus) and the A5 cell groups in pons, and A1 and A2 noradrenergic cell groups in medulla (Buller et al., 1999; Erickson and Millhorn, 1994; Smith et al., 1995; Teppema et al., 1997). Electrophysiological studies also revealed that neurons within these noradrenergic neural structures responded to peripheral chemoreceptor stimulation (Guyenet et al., 1993; Li et al., 1992). The involvement of NE in peripheral chemoreflexes was further supported by *in vivo* studies showing that excitation or inhibition of these noradrenergic neural structures could modulate cardiorespiratory responses to peripheral chemoreceptor stimulation (Hayward, 2001; Koshiya and Guyenet, 1994a; Koshiya and Guyenet, 1994b; Perez et al., 1998). These data strongly suggest that NE has a potent influence on peripheral chemoreflexes within the central nervous system (CNS).

The caudal nucleus tractus solitarius (cNTS), where peripheral chemoreceptor afferents and other visceral afferents make their first central synapses (Mifflin, 1992), has intense anatomical connections with central noradrenergic neural structures (Loewy,

1990). The cNTS also contains noradrenergic neurons, i.e., the A2 cell group. Both α_1- and α_2-adrenoreceptors exist throughout the NTS (Dashwood et al., 1985; Day et al., 1997; Jones et al., 1985; Young and Kuhar, 1980). These data suggest that NE, acting through various adrenoreceptor subtypes, could function as an important neuromodulator of synaptic transmission of peripheral chemoreceptor inputs in cNTS. However, the synaptic mechanisms whereby NE might modulate cardiorespiratory afferent integration by NTS neurons remains to be clarified.

A few studies have examined the involvement of NTS α_2-adrenoreceptors in peripheral chemoreflexes. Microinjection of an α_2-adrenoreceptor antagonist in cNTS attenuated the cardiorespiratory responses to peripheral chemoreceptor stimulation with potassium cyanide (Hayward, 2001). Stimulating locus coeruleus inhibits NTS neuronal discharge evoked by peripheral chemoreceptor stimulation via α_2-adrenoreceptors (Perez et al., 1998). To our knowledge, no studies have investigated modulation of synaptic transmission of peripheral chemoreceptor inputs in cNTS by α_1-adrenoreceptors. Activation of α_1-adrenoreceptors inhibited neuronal discharge in medial NTS, which primarily receives baroreceptor inputs (Feldman and Moises, 1988; Feldman and Felder, 1989). It is therefore hypothesized that activation of α_1-adrenoreceptors will inhibit synaptic transmission of peripheral chemoreceptor inputs in cNTS. Using *in vitro* whole-cell recording, we studied the effect of α_1-adrenoreceptor activation on synaptic transmission of both excitatory glutamatergic and inhibitory GABAergic synaptic inputs to second-order peripheral chemoreceptor neurons in cNTS. We further investigated whether α_1-adrenoreceptor modulation of synaptic transmission occurs via a pre- and/or post-synaptic mechanism.

METHODS

All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio.

Surgical preparation for labeling carotid body. Male Sprague-Dawley rats (100–125 g) were anaesthetized with a combination of ketamine (75 mg/kg, i.p., Fort Dodge) and medetomidine (0.5 mg/kg, i.p., Pfizer). Under aseptic conditions, crystals of anterograde fluorescent dye DiA were gently applied unilaterally to the carotid body region. DiA dissolves in the nerve axons and diffuse centrally permitting visualization of chemoreceptor synaptic terminals and neurons receiving these synaptic contacts as previously described for an aortic nerve study (Mendelowitz et al., 1992). The area was then embedded with silicone adhesive (Kwik-Sil, WPI, Sarasota, FL). Anesthesia was terminated by atipamezole (1 mg/kg i.p., Pfizer) at the conclusion of the surgical procedures. Post-operative analgesics (nubaine, i.m.) were available as needed. The rats were allowed to recover for 7-10 days before the experimental protocols.

Brain slice preparation. Rats were anesthetized with isoflurane and the brainstem rapidly removed and placed in ice-cold, high-sucrose, artificial cerebrospinal fluid (aCSF) that contained (in mM): 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, and 206 sucrose, pH 7.4 when continuously bubbled with 95% O_2/5% CO_2. Brainstem horizontal slices (250 μm thickness) were cut with a sapphire knife (Delaware Diamond Knives, Wilmington, DE) and mounted in a vibrating microtome (VT1000E, Leica Microsystems, Bannockburn, IL). Then the slices were incubated for at least one hour in normal aCSF that contained (in mM): 124 NaCl, 3 KCl, 2 $MgSO_4$, 1.25 NaH_2PO_4,

26 $NaHCO_3$, 10 glucose and 2 $CaCl_2$, pH 7.4 when continuously bubbled with 95% O_2/5% CO_2.

Electrophysiological Recording. A single slice was transferred to the recording chamber on an upright epifluorescent microscope (Olympus BX50WI, Tokyo) equipped with infrared differential interference contrast (IR-DIC) and an optical filter set for visualization of DiA. The slice was held in place with a nylon mesh, submerged in normal aCSF equilibrated with 95% O_2/5% CO_2 and perfused at a rate of approximately 2 ml/min. All images were captured with a charge-coupled device (CCD) camera (IR-1000, CCD-100; Dage-MTI, Michigan City, IN) displayed on a TV monitor and stored in a PC computer. Patch pipettes were pulled from borosilicate glass capillaries with an inner filament (0.90 mm ID, 1.2 mm OD, WPI, Sarasota, FL) on a pipette puller (Model P-2000, Sutter Instrument Company, Novato, CA) and were filled with a solution of the following composition (in mM): 145 K-gluconate (replaced with KCl when recording IPSCs), l $MgCl_2$, 10 *N*-2-hydroxy-ethylpiperazine-*N*'-2-ethanesulphonic acid (HEPEs), 1.1 ethylene glycol-bis(ß-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), 2 Mg_2ATP, and 0.3 Na_3GTP. The pH was adjusted to 7.3 with KOH. With this pipette solution, the junction potential was 15.5 mV at 24°C (3.6 mV for KCl based pipette solution) and was not corrected in subsequent analysis. The pipette resistance ranged from 3 to 6 MΩ. A seal resistance of at least 1 GΩ or above, and an access resistance < 20 MΩ which changed <15% during recording were considered acceptable. Series resistance was optimally compensated. Cells were clamped at a membrane potential of -60 mV. Input resistances of cells were monitored by frequently applying a 10 mV hyperpolarizing voltage step (100 msec duration) from a holding potential of -60 mV.

Recordings of postsynaptic currents began 5 min later, after the whole cell access was established and the holding current reached a steady state. Recordings were made with an AxoPatch 200B patch-clamp amplifier and pClamp software version 8 (Axon Instruments, Union City, CA). Whole-cell currents were filtered at 2 kHz, digitized at 10 kHz with the DigiData 1200 Interface (Axon Instruments) and stored in a PC computer for offline analysis. All experiments were performed at room temperature.

Whole-cell voltage-clamp recordings were performed on second-order NTS peripheral chemoreceptor neurons labeled with fluorescent DiA. Evoked EPSCs (eEPSCs) were elicited by electrical stimulation of the ipsilateral solitary tract (ST) using concentric bipolar electrodes (FHC, Bowdoinham, ME) with a tip diameter of 200 μm. Square electric pulses of 0.1 msec duration with a frequency of 0.2 Hz were delivered through a stimulus isolator A360 (WPI, Sarasota, FL), in series with a programmable stimulator (Master8, AMPI, Jerusalem, Israel). When recording evoked IPSCs (eIPSCs), the electrode was positioned in the NTS ipsilateral to the recording neuron and medial to the ST. Electrical stimuli were delivered at 0.1 Hz. Stimulus intensity was 50-300 μA. To determine the effect of PE on paired-pulse stimulation, two synaptic responses (A1 and A2) were evoked by a pair of stimuli given at short intervals (40 msec for eEPSCs and 50 msec for eIPSCs). Paired-pulse ratio (PPR) was expressed as the amplitude ratio of the second synaptic response to the first synaptic response (A2/A1). Bath application of drugs typically lasted about 3-5 min before beginning electrophysiological recordings.

Recordings of glutamatergic EPSCs were performed in the presence of the $GABA_A$ receptor antagonist (-)-bicuculline methiodide (BIC, 30 μM). Recordings of the GABAergic IPSCs were performed in the presence of the non-NMDA receptor

antagonist 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM). Miniature EPSCs (mEPSCs) and IPSCs (mIPSCs) were recorded in the presence of the sodium channel blocker tetrodotoxin (TTX, 1 μM), and 30 μM BIC or 10 μM CNQX respectively.

To test whether PE has a post-synaptic effect and changes current-voltage relationships of second-order peripheral chemoreceptor neurons, a voltage step protocol was performed in the presence of 1μM TTX, 30 μM BIC, 50 μM DL-2-amino-5-phosphonopentanoic acid (AP-5), and 10 μM CNQX. Membrane potential was changed from -130 mV to -30 mV in 10 mV steps. The duration of each step was 500 msec and voltage steps were applied every 2 sec.

Data analysis. Data are presented as mean ± SEM. Peak amplitudes of averaged evoked post-synaptic currents (≥10 sweeps) were calculated as the difference from the baseline measured several milliseconds before the stimulation artifacts. Differences in drug effects were tested by one-way repeated-measures ANOVA or paired *t*-test. The threshold value for detecting miniature IPSCs/EPSCs was set as four times the root-mean-square baseline noise and all miniature events detected by the software were visually checked to minimize errors. Cumulative distributions of miniature synaptic current amplitudes and frequencies were averaged over the 5 min period during control, during PE application, and after 15 min washout. All miniature events were detected with MiniAnalysis software (v6.0, Synaptosoft Inc., Fort Lee, NJ). Cumulative distributions of miniature synaptic current amplitudes and frequencies were compared using Kolmogorov-Smirnov (K-S test) nonparametric analysis. Averaged data were compared with paired *t*-test. Statistics were performed using SigmaStat (v2.03, SPSS software,

Chicago, IL), and graphs were made in SigmaPlot (v8.0, SPSS software, Chicago, IL). Values of $p<0.05$ were considered significant.

Drugs. DiA was obtained from Molecular Probes (Eugene, OR). SCH-50911 was obtained from Tocris (Ballwin, MO). Bicuculline, phenylephrine, CNQX, AP-5, prazosin and other chemicals were obtained from Sigma (St Louis, MO).

RESULTS

Data were obtained from second-order neurons in cNTS, identified by presence of DiA labeled somatic appositions as shown in Fig. 1A. These fluorescently labeled boutons usually formed along the outline of the soma and proximal processes of the neurons while leaving the center largely empty. Whole-cell patch clamp recordings were performed on 135 DiA labeled NTS cells from 59 rats (weight 282±5 g). Labeled NTS neurons displayed an average resting membrane potential of 57.5 ± 0.5 mV and input resistance of 705.6 ± 31.0 MΩ.

Direct Effect of PE on labeled NTS neurons. Activation of post-synaptic α_1-adrenoreceptors decreases potassium conductance and depolarizes neurons in CNS (Aghajanian, 1985). Therefore, a post-synaptic effect of PE would be expressed as increased input resistance and/or inward current at a holding potential of -60 mV, thus the change of current-voltage (I-V) relationship. In the presence of 1 μM TTX, PE (20 μM) did not significantly alter the I-V relationship (curve slope, 0.405±0.057 vs 0.410±0.061, n=17, $p>0.05$), indicating no significant change in potassium conductance. There was no significant change in input resistance after bath application of 20 μM PE (688.9 ± 45.3 MΩ vs 693.7 ± 52.0 MΩ, n=60, $p>0.05$). PE did not cause discernable alteration in holding current, except that in one cell bath application of 20 μM PE caused a 93.7 pA inward current. To further confirm the lack of direct post-synaptic effect, we tested responses to 100 μM PE. This concentration of PE elicited inward current in only 1 out of 15 cells. PE did not significantly change action potential discharge when applied to cells with spontaneous discharge (n=2). These data suggest that activation of α_1-

adrenoreceptors does not have a post-synaptic effect on second-order neurons of peripheral chemoreceptors in cNTS.

Effect of PE on evoked EPSCs in labeled NTS neurons. To examine the effect of PE on glutamatergic synaptic inputs to second-order neurons in cNTS, eEPSCs were isolated at a holding potential of -60 mV and in the presence of 30 μM BIC. As previously described (Doyle and Andresen, 2001), eEPSCs elicited from ST stimulation were all-or-none responses with little recruitment at suprathreshold levels (Fig. 1B). The average latency of eEPSCs was 4.6 ± 0.2 msec (n=61). As calculated from response latencies of 10 eEPSCs sweeps, the standard deviation of onset latency ranged from 30.2 μsec to 175.0 μsec with a median value of 96.6 μsec, further suggesting these neurons receive mono-synaptic inputs from the tractus (Doyle and Andresen, 2001). The eEPSCs were completely eliminated in the presence of 10 μM CNQX (Fig. 1B*).* PE (10-40 μM) decreased the peak amplitudes of eEPSCs in a concentration-dependent manner without significant effect on response onset latencies (Fig. 1C, D and E). At the same time, application of PE did not cause discernable change of holding current. The eEPSCs in 2 out of 29 labeled NTS cells were not altered by PE (20 or 40 μM).

The PE effect was mediated by α_1-adrenoreceptors. In 3 DiA labeled cells, 20 μM PE decreased the amplitude of eEPSCs to 80.8±3.4% of control, confirming the previous observation of α_1-adrenoreceptors inhibition of eEPSCs. After washout of PE, application of the α_1-adrenoreceptor selective antagonist prazosin (10 μM) did not significantly change the amplitudes of eEPSCs (104.5±8.9% of control), suggesting α_1-adrenoreceptors were not tonically active in our preparation. During co-application of PE

and prazosin there was no significant change in the amplitudes of ePESCs (101.4±5.9% of control).

We further tested pre-synaptic mechanisms of α_1-adrenoreceptor activation on eEPSCs. The PPR of evoked post-synaptic currents was examined during the application of PE (Fig. 1F and G). PE at 20 μM significantly increased the PPR of eEPSCs (p<0.01, n=6).

Effect of PE on mEPSCs of labeled NTS neurons. The pre-synaptic effect of PE on glutamatergic synaptic inputs to labeled NTS neurons was further examined by analysis of mEPSCs (Fig. 2A). The mEPSCs were recorded in the presence of 1 μM TTX and 30 μM BIC. PE at a concentration of 20 μM significantly decreased the frequency of mEPSCs (p<0.001, n=5, Fig. 2B and C) without altering amplitude (Fig. 2B and D). K-S testing of records from individual neurons also showed significant decreases in frequencies (p<0.001), but not amplitudes (p>0.05). Bath application of 10 μM CNQX completely abolished mEPSCs (not illustrated).

Effect of PE on evoked IPSCs in labeled NTS neurons. Second-order neurons in the NTS receive both glutamatergic and GABAergic inputs. The effect of PE on eIPSCs on labeled NTS neurons was examined. The eIPSCs were isolated at a holding potential of -60 mV and in the presence of 10 μM CNQX. The eIPSCs were completely abolished by 30 μM BIC (Fig. 3A). PE at 20 μM significantly increased the peak amplitude of eIPSCs (p<0.001, n=7, Fig. 3B and C), with no significant effect on response latencies (2.9±0.3 vs 3.0±0.3 msec) and holding current. In two labeled NTS neurons, prazosin at 10 μM did not significantly change the amplitudes of eIPSCs (93.4±1.7% of control), but abolished the effect of 20 μM PE on eIPSCs (95.3±12.8% of control).

The pre-synaptic mechanism of α_1-adrenoreceptor activation on eIPSCs was examined by testing PPR of eIPSCs during the application of PE. PE at 20 μM significantly decreased the PPR of eIPSCs ($p<0.05$, n=6) in labeled NTS neurons (Fig. 3D and E).

Effect of PE on mIPSCs of labeled NTS neurons. To further determine the pre-synaptic effect of PE on GABAergic synaptic inputs, we examined the effect of PE on mIPSCs in DiA labeled NTS neurons (Fig. 4A). The mIPSCs were recorded in the presence of 1 μM TTX and 10 μM CNQX. PE at 20 μM significantly increased the frequency of mIPSCs ($p<0.05$, n=6) without significantly altering the amplitude (Fig. 4B, C and D). K-S testing of records from individual neurons also showed significant increases in frequencies in 6/6 neurons ($p<0.001$), and amplitudes in 2/6 neurons ($p<0.01$). Bath application of 30 μM BIC completely abolished mIPSCs (not illustrated).

The role of $GABA_B$ receptors in PE induced inhibition on eEPSCs. The $GABA_B$ receptor selective antagonist SCH-50911 (20 μM) did not significantly change the amplitudes of eEPSCs (-190.3±25.1 vs -193.5±26.6 pA, $p>0.05$, n=10), suggesting no tonic effect of $GABA_B$ receptor activation on excitatory synaptic transmission in cNTS in our preparation. Previous work in our lab has shown this concentration blocked outward currents evoked by 10 μM $GABA_B$ receptor agonist baclofen (Zhang and Mifflin, unpublished observation). Co-application of 20 μM SCH-50911 did not significantly alter 40 μM PE inhibition of the amplitude of eEPSCs (39±3% vs 34±5%, $p>0.05$, n=6).

The role of NMDA receptors in PE induced inhibition on eEPSCs. NMDA receptors did not mediate the eEPSCs during low frequency tractus stimulation in our preparation. The NMDA receptor selective antagonist AP-5 (50 μM) did not significantly

change the amplitudes of eEPSCs (-189.4±19.6 vs -185.5±17.2 pA, $p>0.05$, n=10). Previous work in our lab has shown this concentration blocked currents evoked by NMDA (De Paula and Mifflin, unpublished observation). Furthermore, this concentration of AP-5 abolished NMDA receptor-mediated neuronal responses evoked by ST stimulation (Aylwin et al., 1997). During co-application of 50 μM AP-5 and 40 μM PE, the amplitude of eEPSCs was not significantly different from that measured during application of PE alone (32±4% vs 33±5%, $p>0.05$, n=5).

DISCUSSION

The effects of activation of α_1-adrenoreceptors on synaptic transmission to cNTS neurons were examined. Second-order neurons in cNTS relay chemoreceptor inputs to other neural structures throughout the CNS and modulate autonomic, respiratory and hormonal responses to hypoxia. The results showed that activation of α_1-adrenoreceptors inhibits glutamatergic excitatory inputs and increases GABAergic inhibitory inputs to the second-order neurons in cNTS. Both effects of PE were blocked by the α_1-adrenoreceptor antagonist prazosin. The effect of PE on synaptic transmission appears to be mediated primarily via pre-synaptic mechanisms. Alterations in α_1-adrenoreceptor inhibition of excitatory glutamatergic synaptic transmission were independent of $GABA_B$ and NMDA receptors. The results suggest that the overall effect of activation of α_1-adrenoreceptors is the inhibition of peripheral chemoreceptor inputs to cNTS neurons by a combined decrease in excitatory inputs and increase in inhibitory inputs.

This is the first study of the role of α_1-adrenoreceptors in synaptic transmission of peripheral chemoreceptor inputs in cNTS. In many central neural structures, activation of α_1-adrenoreceptors has been shown to cause a depolarization through the inhibition of a resting potassium conductance (Aghajanian, 1985). The α_1-adrenoreceptor is a G protein-coupled receptor. Activation of α_1-adrenoreceptors promotes phospholipase C activation and increases the level of biologically available calcium in the cytoplasm. Therefore, a direct effect of α_1-adrenoreceptor activation would be expected to increase neuronal excitability in cNTS. However, the present results strongly suggest that in second-order neurons in cNTS the primary target of α_1-adrenoreceptor activation is at a pre-synaptic

site. This is supported by the lack of any significant changes in input resistance, holding current, or the I-V relationship after PE application. The effect of α_1-adrenoreceptor activation in cNTS is mediated by modulating the balance between excitatory and inhibitory inputs to the second-order neurons, rather than directly affecting membrane properties of the neurons.

The neuronal circuits that mediate peripheral chemoreflexes rely on glutamatergic transmission from afferent terminals to second-order neurons in cNTS (Sapru, 1996; Zhang and Mifflin, 1993). Our results suggest that activation of α_1-adrenoreceptors reduces excitatory inputs to second-order neurons by decreasing the release of glutamate from afferent terminals as demonstrated by reduced eEPSCs amplitudes after PE application. This result suggests that α_1-adrenoreceptors are localized on primary afferent terminal since activation of α_1-adrenoreceptors decreased mono-synaptic eEPSCs. The pre-synaptic mechanism was further confirmed by the findings of a reduced PPR of eEPSCs and reduced frequency of mEPSCs. There are reports of pre-synaptic inhibition of glutamate release by α_1-adrenoreceptors (Kirkwood et al., 1999; Scanziani et al., 1993). The neural mechanisms are still not clear.

Several studies suggest that indirect mechanisms may be involved in PE-mediated inhibition of excitatory inputs. In hypothalamic hypocretin neurons, PE inhibited spontaneous discharge without affecting resting membrane potential, which was suggested to be due to increased bicuculline-sensitive inhibition (Li and van den Pol, 2005). In the current study, we demonstrated that activation of α_1-adrenoreceptors increased release of GABA from GABAergic terminals in cNTS. However, decreased excitatory synaptic inputs were observed in the presence of BIC, indicating that

noradrenergic modulation of $GABA_A$ receptor-mediated transmission was not involved in the reduction of excitatory inputs. Increased extracellular GABA in the NTS could spread to afferent terminals and activate pre-synaptic $GABA_B$ receptors thus inhibiting the release of glutamate (Isaacson et al., 1993). Our data with the $GABA_B$ receptor antagonist did not support this possibility and further demonstrated that peripheral chemoreceptor synaptic transmission in cNTS was not under the tonic influence of $GABA_B$ receptors in our preparation. Activation of α_1-adrenoreceptors in visual cortex elicited a long-term synaptic depression which was completely blocked by NMDA receptor blocker AP-5 (Kirkwood et al., 1999). We found that NMDA receptors did not mediate PE-induced inhibition of eEPSCs. A few studies reported that activation of α_1-adrenoreceptors can attenuate Ca^{2+} currents (Calcagnotto and Baraban, 2003; Li and van den Pol, 2005). Since release of neurotransmitters relies on pre-synaptic Ca^{2+} entry, this may explain PE-mediated inhibition on EPSCs in our preparation. We examined receptor systems most likely to mediate indirect PE effects based on work in other systems; GABA and NMDA. It is well beyond the scope of this study to analyze every potential neurotransmitter and neuromodulator receptor system that could alter synaptic inputs to NTS neurons (e.g. adenosine, metabotropic glutamate, serotonin, tachykinin, vasopressin, oxytocin, etc.). Future studies will be needed to elucidate the mechanisms of the PE-mediated inhibition observed in current project.

We further demonstrated that in cNTS activation of α_1-adrenoreceptors increased amplitudes of eIPSCs and the frequency of mIPSCs and decreased the PPR of eIPSCs, suggesting the activation of local GABAergic neurons and inhibitory GABAergic inputs to second-order neurons. This PE-induced increase in GABA release was not action

potential-dependent since mIPSCs recordings were observed in the presence of TTX. This increase in synaptic inhibition has been observed in other sites in CNS (McCormick and Wang, 1991; Alreja and Liu, 1996; Li and van den Pol, 2005). Activation of α_1-adrenoreceptors usually depolarizes neurons and increases neuronal excitability (Aghajanian, 1985; Summers and McMartin, 1993; Stevens et al., 1994). These GABAergic terminals could originate from local interneurons or from inputs to NTS from other brain structures. Therefore, α_1-adrenoreceptor-mediated activation of GABAergic neurons could provide increased inhibitory inputs to second-order neurons, and damp excitatory inputs from peripheral chemoreceptors.

This study focused on the role of α_1-adrenoreceptors in synaptic modulation of peripheral chemoreceptor afferent input integration in the NTS. It is not yet clear where the exact source of NE that activates NTS adrenoreceptors originates. Various central noradrenergic neural structures in the brain, including A1, A2, A5 and A6 noradrenergic cell groups, are activated by peripheral chemoreceptor stimulation (Buller et al., 1999; Erickson and Millhorn, 1994; Smith et al., 1995; Teppema et al., 1997). The noradrenergic projections from these neural structures or other brain sites including paraventricular nucleus could target primary afferent localized adrenoreceptors and modulate chemoreceptor afferent input integration in the NTS.

The results of this study place the NTS α_1-adrenoreceptors in a unique position. Unlike α_1-adrenoreceptors, activation of α_2-adrenoreceptors inhibits both excitatory and inhibitory inputs to second-order peripheral chemoreceptors neurons in cNTS (Zhang and Mifflin, unpublished observation). Thus the final effect of α_2-adrenoreceptor activation on synaptic transmission depends on the balance between these two inputs to a given

neuron at any point in time. Activation of α_1-adrenoreceptors appears capable of biasing this balance towards inhibition. Therefore, we would predict activation of α_1-adrenoreceptors in cNTS should attenuate chemoreflex responses, similar to the effect of PE in medial NTS (Feldman and Moises, 1988; Feldman and Felder, 1989). The physiological roles of NTS α_1-adrenoreceptors in peripheral chemoreflexes have yet to be explored. Long-term activation of peripheral chemoreceptors could change the role of different subtypes of adrenoreceptors in NE-induced responses. Chronic intermittent cold exposure significantly increased α_1-adrenoreceptor mediated responses in the paraventricular nucleus of the hypothalamus without significant changes in extracellular NE levels, suggesting enhanced α_1-adrenoreceptor sensitivity (Ma and Morilak, 2005). Chronic sustained hypoxia increases the activity and expression of tyrosine hydroxylase in the NTS (Schmitt et al., 1994; Soulier et al., 1992). If this results in increased NE release within the NTS, α_1-adrenoreceptor-mediated inhibition could provide neuroprotection to second-order neurons receiving increased excitatory afferent inputs from peripheral chemoreceptors. However, a reduced sensitivity of α_1-adrenoreceptor or receptor number in cNTS could enhance chemoreflexes. Further studies will be needed to investigate the long-term effects of chronic hypoxia on NTS α_1-adrenoreceptor function.

In summary, activation of α_1-adrenoreceptors inhibits synaptic transmission of peripheral chemoreceptor inputs in cNTS. This inhibitory effect is achieved by reducing glutamatergic excitatory inputs and increasing GABAergic inhibitory inputs to second-order peripheral chemoreceptor neurons in cNTS. These results suggest that activation of α_1-adrenoreceptors acts via pre-synaptic mechanisms to bias afferent inputs to NTS neurons receiving arterial chemoreceptor inputs towards inhibition by decreasing

excitation and increasing inhibition. This inhibitory biasing could lead to reduced or normalized reflex responses to hypoxia. The modulation of α_1-adrenoreceptor function in pathological conditions could impact the physiological responses to hypoxia.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Effect of phenylephrine (PE) on eEPSCs. *A:* Whole-cell patch clamp recording of second-order peripheral chemoreceptor neurons in caudal NTS (cNTS). Left photograph shows the position of stimulating electrode (arrow) covering the solitary tract (ST) and recording site at the tip of a recording pipette (*) in cNTS. Two cells are shown in right photograph with infrared differential contrast, but only one of them is labeled with DiA when viewed with fluorescence set (indicated by arrows); *B:* Example of 10 sweeps of eEPSCs. The response was completely abolished by 10 μM CNQX. Notice little variability of response latency; *C:* Dose-dependent inhibition of eEPSC amplitude by PE application. Each tracing represents the average of 10 sweeps; *D:* Group data showing PE dose-dependent inhibition of the amplitudes of eEPSCs in 4 NTS neurons which received all three doses of PE; *E:* Group data showing PE dose-dependently inhibits the amplitudes of eEPSCs in NTS neurons. Numbers by each point indicate the number of cells treated at each concentration; *F:* Effect of 20 μM PE on the paired-pulse ratio (PPR) of eEPSCs; *G:* Group data showing that 20 μM PE significantly increased the PPR of eEPSCs (n=6). All experiments were performed in the presence of 30 μM bicuculline (BIC). Stimulus artifacts were truncated for clear view. Data are presented as means ± SE. *: $p < 0.05$, vs control.

Figure 2. Effect of PE on mEPSCs. *A:* Recordings of mEPSCs before (control) and during 20 μM PE perfusion; *B:* Cumulative probabilities of the inter-event interval (left) and amplitude (right) before and during PE in the same neuron as illustrated in *A; C and*

D: Group data (*n*= 5) confirm that PE significantly decreased the mEPSC frequency (*C*), but not the amplitude (*D*), suggesting a pre-synaptic mechanism. All experiments were performed in the presence of 1 μM TTX and 30 μM BIC. Data are presented as means ± SE. *: $p < 0.05$, vs control.

Figure 3. Effect of PE on eIPSCs. *A:* Example of 10 sweeps of eIPSCs. The response was completely abolished by 30 μM BIC. *B*: Example showing that 20 μM PE increased amplitudes of eIPSCs; *C:* Group data showing that 20 μM PE significantly increased the amplitude of eIPSCs (n=7); *D:* Effect of 20 μM PE on the PPR of eIPSCs; *E:* Group data showing that 20 μM PE significantly decreased the PPR of eIPSCs (n=6). All experiments were performed in the presence of 10 μM CNQX. The tracing represents the average of 10 sweeps. Stimulus artifacts were truncated for clear view. Data are presented as means ± SE. *: $p < 0.05$, vs control.

Figure 4. Effect of PE on mIPSCs. *A:* Recordings of mIPSCs before (control) and during 20 μM PE perfusion; *B:* Cumulative probabilities of the inter-event interval (left) and amplitude (right) before and during PE in the same neuron as illustrated in *A; C and D:* Group data (*n*= 6) confirm that PE significantly increased the mIPSC frequency (*C*), but not the amplitude (*D*), suggesting a pre-synaptic mechanism. All experiments were performed in the presence of 1 μM TTX and 10 μM CNQX. Data are presented as means ± SE. *: $p < 0.05$, vs control.

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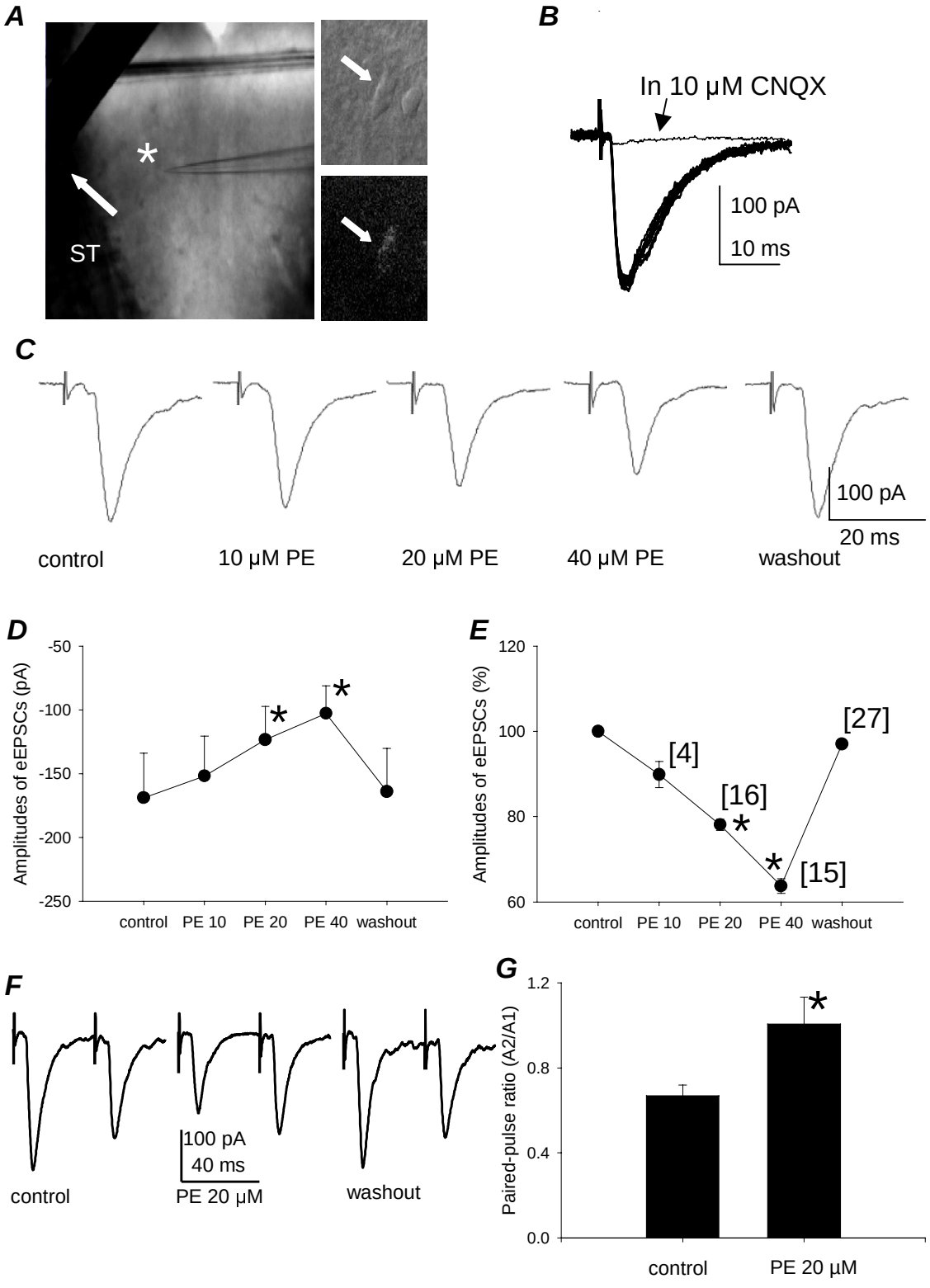

Figure 1

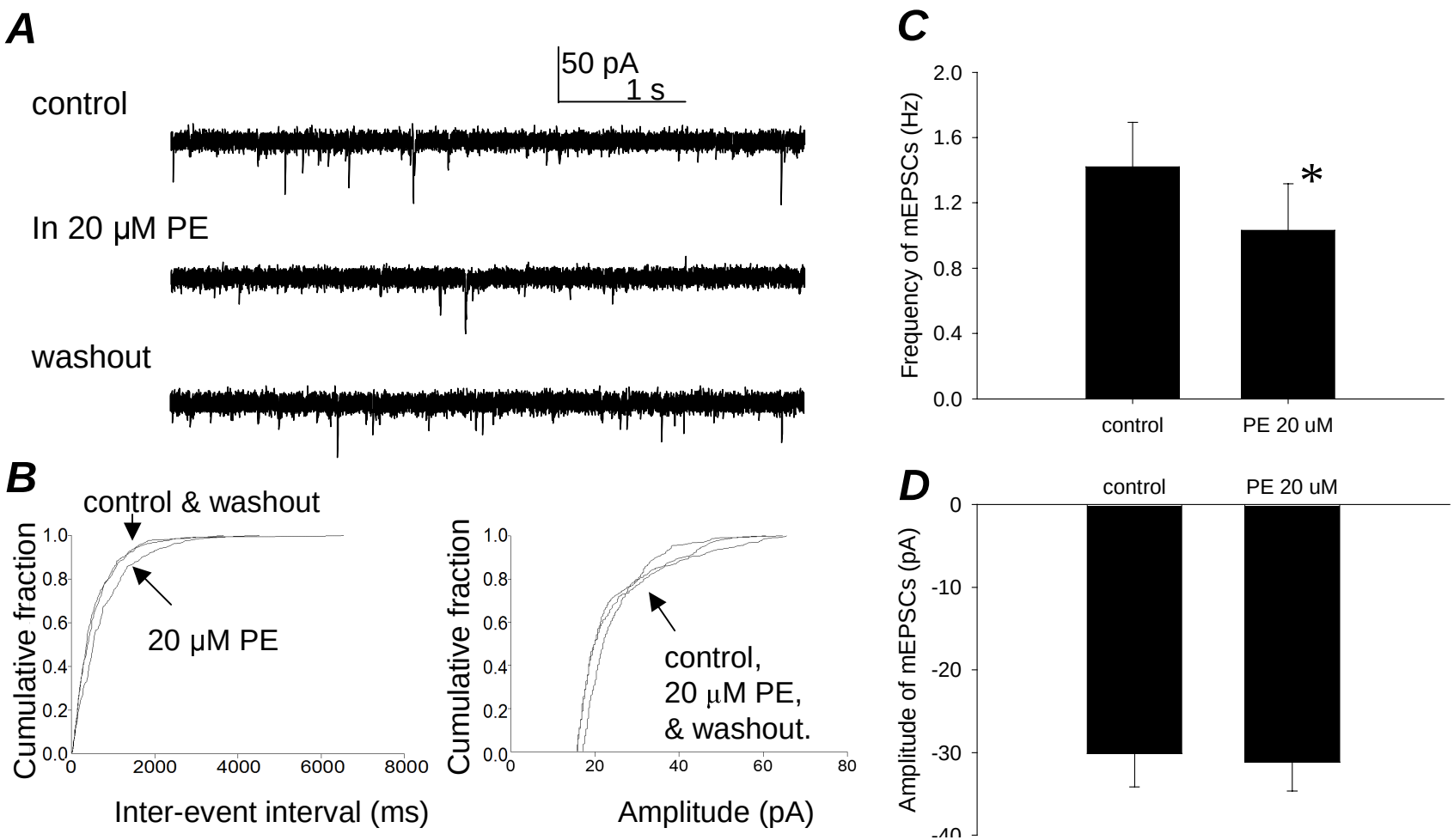

Figure 2

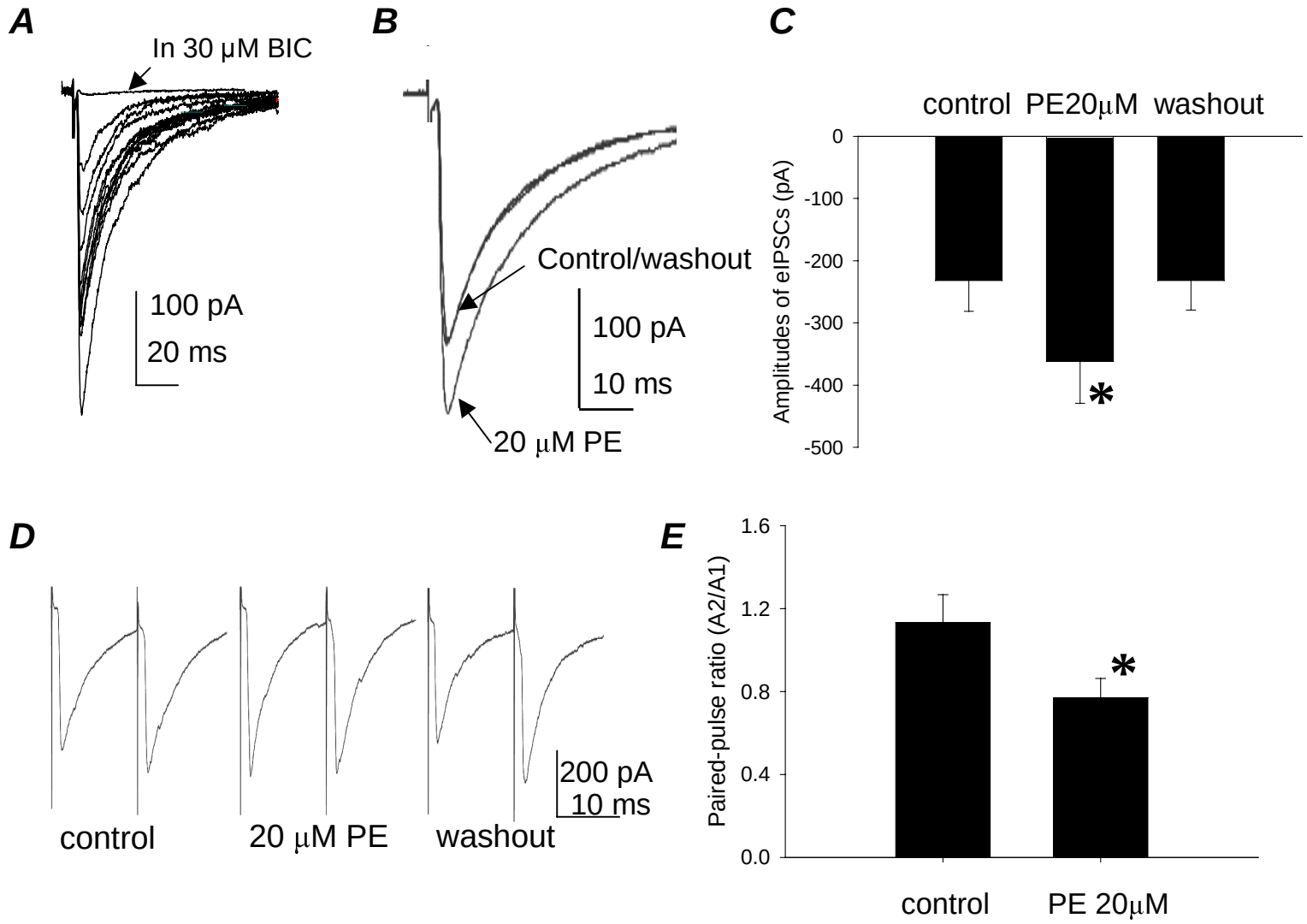

Figure 3

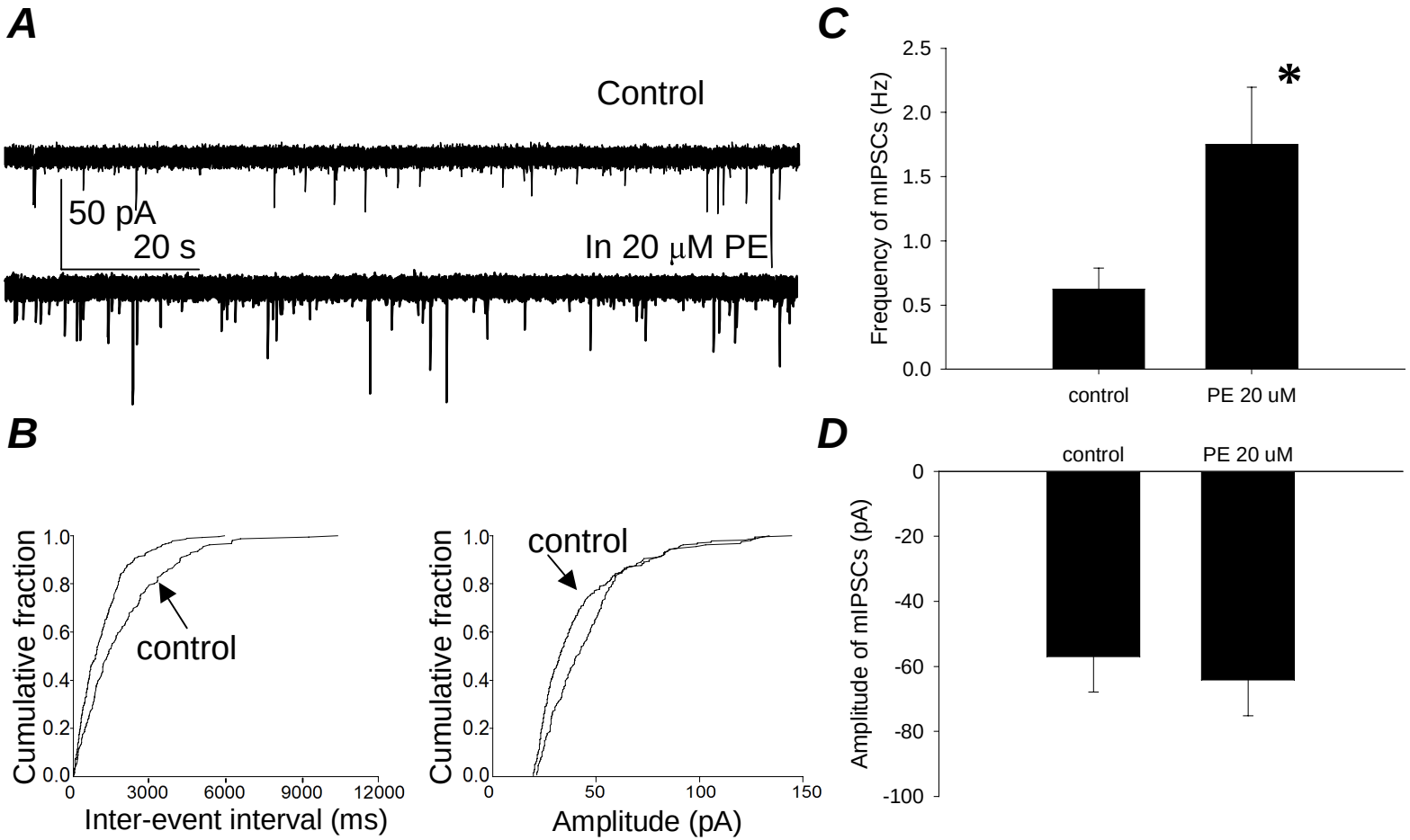

Figure 4