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Prenatal Nicotinic Exposure Upregulates Pulmonary C-fiber NK1R Expression to Prolong Pulmonary C-fiber-Mediated Apneic Response

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Abstract

Prenatal nicotinic exposure (PNE) prolongs bronchopulmonary C-fiber (PCF)-mediated apneic response to intra-atrial bolus injection of capsaicin in rat pups. The relevant mechanisms remain unclear. Pulmonary substance P and adenosine and their receptors (neurokinin-A receptor, NK1R and ADA_1 receptor, ADA_1R) and transient receptor potential cation channel subfamily V member 1 (TRPV1) expressed on PCFs are critical for PCF sensitization and/or activation. Here, we compared substance P and adenosine in BALF and NK1R, ADA_1R, and TRPV1 expression in the nodose/jugular (N/J) ganglia (vagal pulmonary C-neurons retrogradely labeled) between Ctrl and PNE pups. We found that PNE failed to change BALF substance P and adenosine content, but significantly upregulated both mRNA and protein TRPV1 and NK1R in the N/J ganglia and only NK1R mRNA in pulmonary C-neurons. To define the role of NK1R in the PNE-induced PCF sensitization, the apneic response to capsaicin (i.v.) without or with pretreatment of SR140333 (a peripheral and selective NK1R antagonist) was compared and the prolonged apnea by PNE significantly shortened by SR140333. To clarify if the PNE-evoked responses depended on action of nicotinic acetylcholine receptors (nAChRs), particularly α7nAChR, mecamylamine or methyllycaconitine (a general nAChRs or a selective α7nAChR antagonist) was administrated via another mini-pump over the PNE period. Mecamylamine or methyllycaconitine eliminated the PNE-evoked mRNA and protein responses. Our data suggest that PNE is able to elevate PCF NK1R expression via activation of nAChRs, especially α7nAChR, which likely contributes to sensitize PCFs and prolong the PCF-mediated apneic response to capsaicin.

Keywords

SIDS; NK1R; TRPV1; Adenosine; nAChRs; Nodose/Jugular Ganglion

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Introduction

Maternal smoking during pregnancy is closely associated with the risk of sudden infant death syndrome (SIDS) (Shea and Steiner, 2008) characterized by cardiorespiratory failure including lethal apnea (Harper *et al.*, 2000; Hafstrom *et al.*, 2002). Cigarette smoke contains numerous compounds emitted as gases and condensed tar particles, in which nicotine is the major neurotoxic chemical (Kalra *et al.*, 2002). More importantly, prenatal nicotinic exposure (PNE) has been extensively used to reveal the adverse impacts of cigarette smoke on cardiorespiratory functions and its potential linkage with SIDS pathogenesis (Fregosi and Pilarski, 2008; Campos *et al.*, 2009; Stephan-Blanchard *et al.*, 2013). Our recent study showed a plasticity of bronchopulmonary C-fibers (PCFs) in PNE rat pups (Zhuang *et al.*, 2015). This plasticity is characterized by a remarkable prolongation of the apneic response and augmentation of pulmonary C-neural response to right atrial injection of capsaicin, a selective stimulant to PCFs. However, to date, it remains unclear how PNE prolongs the PCF-mediated apnea.

Cigarette smoke (maternal) could increase synthesis and release of pulmonary substance P and adenosine in mice and mouse pups (Wu *et al.*, 2009; Lu *et al.*, 2013). Both substance P and adenosine are potent in sensitizing/activating PCFs via binding with corresponding PCF receptors, neurokinin-1 receptor (NK1R) and ADA_1 receptor (ADA_1R), respectively (Hong *et al.*, 1998; Lee and Pisarri, 2001; Bergren, 2006). In addition, PCFs abundantly express capsaicin receptor, transient receptor potential vanilloid type 1 (TRPV1) that is responsible for the PCF-mediated cardiorespiratory responses (Gu *et al.*, 2005). These, along with pulmonary inflammation induced by maternal cigarette smoke (Czekaj *et al.*, 2002), induce our hypothesis that PNE would promote pulmonary substance P and adenosine release and upregulate TRPV1, NK1R, and ADA_1R expression in PCFs associated with elevation of pulmonary inflammation in rat pups. An upregulation of NK1R, but not TRPV1 and ADA_1R, gene expression in vagal pulmonary C-neurons was observed in our preliminary study. Owing to the potentiating effect of substance P via activation of NK1R on the capsaicin-induced currents in these neurons (Zhang *et al.*, 2007), we tested the role of PCF NK1R in prolonging the capsaicin-induced apnea in PNE pups by using a peripheral NK1R blocker.

Recent reports have shown that nicotinic effect is either dependent on or independent of activating neuronal nicotinic acetylcholine receptors (nAChRs). For example, nicotine induced a several-fold increase in the capsaicin-activated currents in rat trigeminal ganglion neurons via facilitating TRPV1 currents, which was independent of the activation of neuronal nAChRs (Liu *et al.*, 2004). On the other hand, nicotine increased calcitonin gene related peptide release in response to capsaicin in rat buccal mucosa, which was blocked by the nAChR antagonist (Dussor *et al.*, 2003). Because α7nAChR among nAChRs is the most abundantly expressed in the lungs and plays a key role in the PNE-induced impairment of pulmonary development (Sekhon *et al.*, 1999; Wongtrakool *et al.*, 2012), we hypothesized that nAChRs, especially α7nAChRs, are critical for the PNE-induced mRNA and protein changes as proposed above.

Materials and Methods

Pathogen-free Sprague-Dawley rats (250–350 g) were purchased from Charles River Laboratories, Inc. (Wilmington, MA); housed in the animal facility at Lovelace Respiratory Research Institute in filter top cages; and provided with water and food ad libitum. The room was constantly ventilated and the temperature was kept at 23°C. The animals were quarantined for 2 weeks before experiments. The experimental protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, USA.

Animal Preparation

PNE—The PNE pretreatment was the same as previously reported (Zhuang *et al.*, 2015). Briefly, the adult females were randomly designated to receive vehicle (n = 25) and nicotine (n = 44) respectively via a mini-osmotic pump (0.25 μL/hr for 28 days, Alza Corp., Palo Alto, CA). The latter was subcutaneously implanted in the females to deliver vehicle or nicotine tartrate (6 mg/kg/day) that produces nicotine blood levels approximately equivalent to those that occur in moderate to heavy smokers (Slotkin *et al.*, 1997). Ten days after the implantation, each female rat was placed in a breeding cage with a male rat for up to 4 days. Those with vaginal plugs were considered pregnant and separated from the male. On the 7^{th} day of gestation, the pump was replaced with a new one filled accordingly with vehicle or nicotine.

Pretreatment with nAChRs or α7nAChR blockade—Of 44 females with nicotinic exposure, 18 were treated with mecamylamine (MM, n = 9) or methyllycaconitine (MLA, n = 9). MM is a noncompetitive antagonist for all known nAChR subunits (Singh *et al.*, 2013). Although MLA also interacts with α4β2 and α6β2 receptors at high concentration, the dose (3 mg/kg/d) we used is reported to selectively antagonize a7nAChR subunit (Northrop *et al.*, 2011; Rezvani *et al.*, 2012). In these females, besides the nicotinic pump, another mini-osmotic pump containing either MM (0.03 mg/kg/d) or MLA (3 mg/kg/d) was subcutaneously implanted.

Usage of rat pups—Rat pups born by spontaneous vaginal delivery were housed with their mother and siblings (24–25°C, and 12:12 h light/dark cycle). In all experiments, no more than three male pups from each litter with a similar overall litter size were used in each study to minimize the possible effect of genetic difference between litters on the results. Males at postnatal day 11 to day 14 (P11–14) were chosen in this study because males are much more vulnerable than females in human SIDS (Adams *et al.*, 1998) and pups' brain development at this period is equivalent to newborn infants at 2–4 month (Klaus, 2004), the highest risk time window for SIDS. All studies were performed during 9:00 and 17:00 hours to avoid any influence from the circadian rhythm (Stephenson *et al.*, 2001). Pups from vehicle-and nicotine-treated dams, and the latter coupled with MM or MLA pretreatment were grouped as Ctrl, PNE, MM+PNE, and MLA+PNE that were randomly assigned to the following six *Study Series*.

Experimental protocols

Study Series I was performed to test whether PNE altered substance P and adenosine in BALF (n = 10 for Ctrl and PNE, respectively), mRNA and protein levels of NK1R, ADA_1R, and TRPV1 in N/J ganglionic neurons (n = 20 per group). To this end, substance P was detected by duplicate use of ELISA kit (Phoenix, EK-061–05), while adenosine was measured by amperometric measurements with needle-shaped biosensors (Bekar *et al.*, 2008). The mRNA and protein levels of NK1R, ADA_1R, and TRPV1 in N/J ganglionic neurons were analyzed by TaqMan real time PCR and Western blot, respectively.

Study Series II was designed to define PNE effect on NK1R, ADA_1R, and TRPV1 expression in vagal pulmonary C-neurons. Ctrl and PNE pups (n = 12/group) at P3 were anesthetized by isoflurane (3–5%)to sufficiently suppress corneal and withdrawal reflexes. 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, 0.25 mg/ml; 1% ethanol concentration) was applied via intratracheal instillation (0.05 ml X 2) to retrogradely trace vagal pulmonary C-neurons within the nodose/jugular (N/J) ganglia. As previously reported (Hu *et al.*, 2010), ten to twelve days later, the animals were euthanized to collect the N/J ganglia and then pulmonary C-neurons. mRNA levels of NK1R, ADA_1R, and TRPV1 in vagal pulmonary C-neurons were detected by TaqMan real time PCR, while Beta-actin (NM_031144.2) was used as the endogenous control.

Study Series III was conducted to examine the effects of MM (a general antagonist of nAChR) or MLA (a selective antagonist of α7nAChR) pretreatment on the PNE-induced changes in gene and protein levels of NK1R, ADA_1R, and TRPV1 in N/J ganglionic neurons, and pulmonary inflammatory cells. Four groups of pups (Ctrl, PNE, MM+PNE, MLA+PNE; N = 20, 20, 20, and 25) were used. After euthanasia, BLAF was collected from 10 pups in each group for counting the total cells and cells differentiation (macrophages, neutrophils, lymphocytes, or eosinophils). Subsequently, N/J ganglia were harvested in all pups used in this series. The procedures and methods to measure the mRNA and protein levels were the same as described in *Series I – II*. Owing to the lack of change in substance P and adenosine in BALF in our preliminary study, we did not measure substance P and adenosine here.

Study Series IV was performed to clarify if MLA was able to block the PNE-induced elevation of NK1R mRNA in pulmonary C-neuron as our preliminary results showed a unique PNE effect on elevating NK1R mRNA in pulmonary C-neurons and the similarity of blocking effects of MM or MLA in *Study Series III*. The procedures were the same as described in *Study Series II* with the exception that only NK1R mRNA in pulmonary C-neurons was compared among Ctrl, PNE, and MLA+PNE pups (N = 5/group).

Study Series V was aimed at confirming the role of peripheral blockade of NK1R in the PNE-induced prolongation of apneic response to capsaicin. We targeted peripheral NK1R because PNE only increased NK1R expression in pulmonary C-neurons in our preliminary study. Ctrl (n = 6) and PNE pups (n = 10) were anesthetized and the right jugular vein was cannulated as reported before (Zhuang J, 2014). The ventilatory responses to bolus injection of capsaicin (10 μg/kg) into the right atrium were compared without and with right atrial injection of SR140333 (100 μg/kg), a peripheral and selective NK1R antagonist that

completely blocked neurogenic inflammation (Emonds-Alt *et al.*, 1993; Amann *et al.*, 1995; Rupniak *et al.*, 2003). SR140333 pretreatment was made 10 min before capsaicin injection. After completion of the ventilatory test, the animals were euthanized and the caudal nucleus tractus solitaries of the medulla harvested to detect local substance P and NK1R expression. A previous report has demonstrated that microinjection of substance P into the caudal nucleus tractus solitaries prolongs the capsaicin-induced apnea in guinea pigs (Mutoh *et al.*, 2000). Thus, we investigated if PNE could elevate substance P and NK1R expression in the caudal nucleus tractus solitaries to centrally contribute to the prolongation of the apneic response to capsaicin.

Tissues collection and analysis/measurement

Collection of bronchoalveolar lavage fluid (BALF)—Briefly (Xu *et al.*, 2008), pups were sacrificed by an overdose of Euthasol, the trachea cannulated and BALF collected following 2 times lavage washes with 0.5 ml ice-cold PBS. Then, the total cells in BALF were counted. BALF was then centrifuged (200 g × 5min) and supernatants stored at −70°C for substance P and adenosine measurement and the cell pellet used for different cell analysis by cytospin. Slides were fixed and stained with Giemsa for leucocyte analysis and the count of monocytes, lymphocytes, neutrophils, and eosinophils in total of 200 cells determined per slide.

Collection of the caudal nucleus tractus solitaries—Briefly (Gozal *et al.*, 1998), the caudal nucleus tractus solitaries will be identified in the extracted brainstem under a surgical microscope according to the stereotaxic coordinate of the rat brain (Paxinos and Watson, 1998). the caudal nucleus tractus solitaries tissues were carefully collected with a 17-gauge, thin-walled hypodermic needle by punch sampling and stored at −80°C for following substance P and NK1R protein tests.

Pulmonary C neurons' collection and Single cell real-time PCR—Harvest of the N/J ganglia was the same as previously reported (Hu *et al.*, 2010). Immediately after euthanasia, the N/J ganglia were extracted and placed in ice-cold DMEM solution containing 0.1% type IV collagenase and 0.1% Trypsin. Then the N/J ganglia were cut into ~10 pieces and incubated in a CO_2 incubator containing 5% CO_2 at 37°C for 1 h. The tissue digestion was stopped by adding DMEM solution containing trypsin inhibitor (2 mg/ml). Subsequently, the cell suspension was centrifuged at 150 g for 5 min, the supernatant was aspirated, and the cells were resuspended in modified Neurobasal medium containing 10% FBS, 1% Glucose, 1 mM L-Glutamine, 2% B-27, and 100 U/ml penicillin-streptomycin. The suspended cells were plated on poly-L-lysine coated 13 mm cover slips and in 5% CO_2 incubated until cells were attached (overnight).

Vagal pulmonary C-neurons were identified by retrograde-labeling and by the neural size (< 25 μm) (Lee *et al.*, 2003) with fluorescence microscopy (Potenzieri *et al.*, 2012). With a negative pressure, single cells were harvested into a glass-pipette (tip diameter: 25 μm) that was pulled by a DMZ-universal (Dagan Corporation, MN, USA). The pipette tip was then broken in a PCR tube containing 9 ul single cell lysis solution and 1 μl DNAase I (Single cell-to-CT™ kit, 4458237, Ambion), and the PCR tube was immediately snap frozen. A

sample of the bath solution from the vicinity of a labelled neuron was collected from each coverslip for no-template experiments (bath negative control). As kit's manual instructions, after reverse transcription, TRPV1 (NM_031982.1), NK1R (NM_012667.1), ADA_1R (NM_017155.2) and beta-actin (NM_031144.3) primers were mixed for preamplification based on the instructions. The products from the preamplification stage were firstly used to detect the TRPV1 and then analyze NK1R and ADA_1R expression by TaqMan gene assay in those neurons with positive signal of TRPV1.

Total mRNA isolation from N/J ganglia—Total mRNA in the N/J ganglia was isolated using RNeasy Mini Kit (Qiagen 74104, German). cDNA was synthesized by reverse transcription using Sensiscript RT Kit (Qiagen 205211, German).

Real-time analysis—TaqMan real time PCR was conducted on ABI PRISM 7900 HT system (Applied Biosystems, USA) to measure TRPV1, NK1R, and ADA_1R in cDNA using the ΔΔCT method. GAPDH (NM_017008.3) and beta-actin were used as the endogenous control for N/J ganglia and pulmonary C-neurons, respectively. Reaction conditions were carried out as follows: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 72°C for 30 s.

Western blot—For N/J ganglia, seven pups' tissues were pooled as one trial, and for the caudal nucleus tractus solitaries tissue, one pup's tissue was as one trial. The tissue were homogenized with M2 buffer (20 mM Tris-HCl, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM-glycerophosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin). 100 μg of each protein homogenates was run in 12% SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane and probed with goat anti-NK1R polyclonal antibody (1:100, Santa Cruz), goat anti-TRPV1 polyclonal antibody (1:100, Santa Cruz), and rabbit anti-ADA_1R polyclonal antibody (1:200, Santa Cruz), respectively, overnight at 4°C. After incubation with respective horseradish peroxidase–conjugated second antibody (1:2,000), the signals were detected by enhanced chemiluminescence according to the manuals (Millipore, Billerica, MA). The band density was quantified with ImageJ software (NIH, Bethesda, MD, USA) and normalized to corresponding loading control. Then all values were normalized to the mean of Ctrl group.

Measurement of ventilation—The animals were anesthetized (urethane, 1400 mg/kg, i.p.) to record the PCF-mediated apnea. Supplemental doses of anesthetics (120–240 mg/kg, urethane, i.v.) were provided as needed to suppress corneal and withdrawal reflexes. As previously reported (Zhang *et al.*, 2013), the trachea was cannulated and connected to a pneumotachograph (Frank's Mfg. Co., Albuquerque, NM) to record airflow. The airflow signals were integrated to generate tidal volume (V_T), respiratory frequency (fR), and minute ventilatory volume (V_E). The right femoral artery and jugular vein were cannulated for monitoring and recording arterial blood pressure/heart rate and delivering drug into the pulmonary circulation, respectively. The animal was exposed to 30% O_2 throughout the experiment to prevent hypoxia. The core temperature of the animal was monitored with a rectal probe and maintained at ~37.5°C with a heating pad and radiant heat.

Data Acquisition and Statistical Analysis

All variables were expressed as absolute values with the exception that the apneic duration evoked by capsaicin was represented as Δ% of baseline expiratory duration (T_E). Group data were reported as means ± SE. Student's t-test was used to analyze the significant differences between the two groups (Ctrl vs. PNE), while one-way ANOVA was used to analyze the significant difference of PNE-evoked mRNA and protein responses before and after MM or MLA pretreatment. Two-way ANOVA was used to analyze the significant difference of apneic responses to capsaicin without and with SR140333 in Ctrl and PNE pups. If an overall test was significant, Tukey's test was utilized for specific comparisons between individual groups. P-values < 0.05 were considered significant.

Results

PNE has little effect on pups' behaviors—The pregnant rats undergoing nicotinic alone or coupled with MM or MLA exposure showed no discernible behavior abnormalities, such as agitation, loss of appetite, or shortness of breath. There was no significant difference in birth numbers among Ctrl, PNE, MM+PNE, and MLA+PNE groups (10.3 ± 1.9; 12.7 ± 2.2; 10.9 ± 1.7; and 11.2 ± 2.1; $P > 0.05$). All of the pups in the four groups were delivered vaginally at full term of gestational day 21 with no dead fetuses found and no differences in body weight (29.3 ± 3.0 g; 31.2 ± 4.2 g; 29.7 ± 2.8 g; and 30.4 ± 4.4 g; $P > 0.05$).

PNE fails to significantly change substance P and adenosine in BALF—We compared substance P and adenosine protein in BALF between Ctrl and PNE pups. PNE did not markedly alter substance P and adenosine content in BALF (Fig. 1), supporting a lack of PNE effect on pulmonary release of SP and adenosine.

PNE elevates TRPV1 and NK1R in the N/J ganglia, and NK1R mRNA in vagal pulmonary C-neuron—To reveal PNE impact on vagal expression of TRPV1, NK1R, and ADA_1R, we compared their mRNA and protein expressions in the N/J ganglia between Ctrl and PNE pups. As exhibited in Fig. 2, PNE significantly increased mRNA level of TRPV1, NK1R, and ADA_1R and protein levels of NK1R and TRPV1 in N/J ganglia. The N/J ganglia contain different types of neurons, including, but not being limited to, vagal pulmonary C-neurons (the cell bodies of PCFs). To further define whether these changes were reflected in vagal pulmonary C-neurons, we collected vagal pulmonary C-neurons retrogradely labeled by DiI and compared gene expression of TRPV1, NK1R, and ADA_1R using signal cell RT-PCR. As presented in Fig. 3, of 24 vagal pulmonary C-neurons, TRPV1 positive neurons were found in 8/12 Ctrl and 10/12 PNE pups. The majority (≥ 80%) of TRPV1 positive neurons co-expressed NK1R in both groups and NK1R mRNA was significantly higher in PNE than Ctrl pups. In sharp contrast, only 2 pulmonary C-neurons showing TRPV1 co-presented ADA_1R and NK1R mRNA (triple expression) and the levels of ADA_1R mRNA appeared similar between Ctrl and PNE pups.

PNE increases inflammatory cells in BALF—We evaluated the impact of PNE on pulmonary inflammatory cells. As illustrated in Fig. 4, total cells were significantly

increased by PNE with remarkable elevation in macrophages, lymphocytes, and neutrophils without significant change in eosinophils.

MM or MLA blocks the PNE-induced changes in mRNA and protein and inflammatory cells—Our above studies showed that PNE upregulated TRPV1, NK1R, and ADA_1R in the N/J ganglia and inflammatory cells in the airways/lungs. The goal of this experiment was to verify if these PNE-induced changes were dependent on nAChRs, particularly α7nAChR. MM and MLA were used to block nAChRs and α7 nAChR respectively. As shown in Fig. 5, the upregulation of TRPV1, NK1R, and ADA_1R mRNA in the N/J ganglia by PNE was abolished by MM or MLA. Interestingly, a significantly lower level of ADA_1R mRNA in the N/J ganglia was found after MM pretreatment in PNE pups compared to Ctrl pups, implying that MM not only blocks the ADA_1R mRNA response to PNE but also reduces its expression in Ctrl pups. Because of the similarity of MM and MLA effects on the mRNA expressions (Fig. 5A–C), we focused on MLA impact on the protein changes. MLA blocked the upregulation of NK1R and TRPV1 protein expression by PNE with the typical examples presented in Fig. 5D and the quantified data in Fig. 5E–G. Moreover, the responses of inflammatory cells to PNE were blocked after blockade of nAChRs or α7 nAChR (Fig. 4). The values of total cells and macrophages in MLA+PNE were significantly lower than MM+PNE group, pointing to α7nAChR involvement in maintaining the normal level of macrophages in the airways/lungs.

MLA eliminates the PNE-induced elevation of NK1R mRNA in pulmonary C-neurons—Our above results showed that PNE only elevated NK1R mRNA in pulmonary C-neurons and MLA (α7 nAChR antagonist) blocked the PNE-induced responses similarly to MM (nAChRs antagonist). Thus, we tested if the PNE-induced elevation of NK1R mRNA in pulmonary C-neurons was dependent on activation of α7nAChR and found that the pulmonary C-neural NK1R mRNA response to PNE was abolished after blockade of α7nAChR (Fig. 6).

SR140333 shortens the PNE-induced prolongation of the apneic response to capsaicin—The fact that PNE induces a remarkable elevation of only NK1R expression in vagal pulmonary C-neurons raised a fundamental question as to whether PCF NK1R plays an important role in the PNE-induced prolongation of the apneic response to capsaicin. To address this issue, we compared the apneic response to right atrial bolus injection of capsaicin (10 μg/kg) before and 10 min after right atrial injection of SR140333, a peripheral NK1R antagonist, in Ctrl and PNE pups. As illustrated in Fig. 7, capsaicin-induced apnea (baseline T_E = 0.34 sec ± 0.04 sec vs. apneic T_E = 2.50 ± 0.32 sec) was prolonged by 1.5-fold by PNE (baseline T_E values = 0.35 ± 0.05 sec vs. apneic T_E = 6.33 ± 0.84 sec). Pretreatment with SR140333 did not strikingly change the capsaicin-induced apnea in Ctrl pups, but significantly shortened the PNE-induced prolongation of the apneic response to capsaicin by ~30%. It should be noted that similar to conscious pups (Zhuang *et al.*, 2014), PNE did not change baseline ventilation in anesthetized pups and SR140333 did not alter baseline respiratory variables in both Ctrl and PNE pups (Table 1). Considering that substance P in the caudal nucleus tractus solitaries is capable of amplifying the PCF-mediated apneic response (Mutoh *et al.*, 2000), we asked whether PNE affected substance P

and NK1R expression in the caudal nucleus tractus solitaries. Surprisingly, both substance P and NK1R protein expression in the caudal nucleus tractus solitaries were significantly lower in PNE than Ctrl pups (Fig. 8).

Discussion

Our recent studies have shown that PNE sensitizes PCFs and prolongs the apneic response to right atrial bolus injection of capsaicin (Zhuang J, 2014). However, the mechanisms underlying this PNE modulation is unknown. Considering the potent sensitization and activation of PCFs by substance P/NK1R, adenosine/ADA_1R, and TRPV1, we tested PNE effect on expression of substance P and adenosine in BALF and NK1R, ADA_1R, and TRPV1 in the N/J ganglia, particularly vagal pulmonary C-neurons. One of our major findings in this study is that PNE upregulates mRNA of the three receptors and protein expression of TRPV1 and NK1R in N/J ganglionic neurons, and NK1R mRNA in vagal pulmonary C-neurons. Although lack of direct protein evidence, our data showing that the prolonged apneic response to capsaicin by PNE is shortened by blockade of PCFs' NK1R support our assumption that PNE upregulates NK1R expression in vagal pulmonary C-neurons. It is surprising to us that TRPV1 mRNA is elevated by PNE in N/J ganglionic neurons but not in vagal pulmonary C-neurons. This discrepancy may be due to the fact that the single-cell RT-PCR used in this study cannot fully reflect TRPV1 response of pulmonary C-neurons to PNE. Alternatively, the enhanced TRPV1 and ADA_1R in N/J ganglionic neurons could result from other sources of C-type neurons, such as those innervate gastrointestinal organs. Synthesis and release of pulmonary substance P and adenosine are increased by cigarette smoke (maternal) in mice and mouse pups (Wu *et al.*, 2009; Lu *et al.*, 2013). However, both substance P and adenosine content in BALF were not significantly increased in our study. The different exposure (PNE vs. maternal cigarette smoke) and species (rat pups vs. mouse pups) used in our lab and other investigators may have contributed to this difference. On the other hand, this negative result may be due to that the evoked release of substance P and adenosine, if it occurs, is trapped in the tissue with limited release into the BALF. Additionally, we found in this study that PNE increased inflammation cells in BALF. This result indicates a deleterious effect of PNE on the lungs, consistent with other reports (Singh *et al.*, 2013; Huang *et al.*, 2014).

NK1R is able to sensitize and activate C-fibers (Xu *et al.*, 1992; Ustinova *et al.*, 2006), especially PCFs (Bergren, 2006), consistent with our mRNA finding that the majority of TRPV1 positive neurons (≥80%) co-expressed NK1R and PNE only elevated NK1R mRNA in pulmonary C-neurons. Thus, we tested the possible NK1R involvement in the prolongation of the PCF-mediated apnea and found that this prolongation was significantly shortened by peripheral blockade of NK1R with SR140333. We are aware that SR140333 is a peripheral NK1R antagonist that does not only block PCFs' NK1R. However, it is generally accepted that right atrial bolus injection of capsaicin could stimulate PCFs via selective activation on TRPV1 (Lee and Pisarri, 2001) and NK1R expressed on PCFs is capable of sensitizing TRPV1 (Zhang *et al.*, 2007; Hazari *et al.*, 2008). Thus, our results of peripheral NK1R blockade suggest that PCF NK1R likely upregulated by PNE contributes to the prolongation of the PCF-mediated apneic response. Previous study has shown that microinjection of substance P into the caudal nucleus tractus solitaries where PCFs project

to could prolong the PCF-mediated apnea (Mutoh *et al.*, 2000). Moreover, a recent study indicates an upregulation of substance P in the brainstem after PNE (Berner *et al.*, 2008). These results encourage us to explore PNE effect on substance P and NK1R expression in the caudal nucleus tractus solitaries. Unexpectedly, we found a higher substance P and NK1R expression of the caudal nucleus tractus solitaries in Ctrl than PNE pups. We reason that PNE enhances PCF input partially via increasing PCF NK1R that overcomes the downregulation of substance P and NK1R expression in the caudal nucleus tractus solitaries that could inhibit local neurons receiving PCF afferent inputs. The observation that SR140333 diminished but did not block the prolongation of the apneic response to capsaicin by PNE suggests involvements of other peripheral and central mechanisms, which is pending further investigation. One may ask whether PNE effect on PCFs' plasticity and the capsaicin-induced apnea are age-dependent. A previous study has reported that PNE-induced blunted heart rate response to hypoxia only occurs in P13 but not in P26 rat pups (Boychuk and Hayward, 2011), suggesting an age-dependent impact of PNE on cardiac response to hypoxia. Clearly, further studies are needed to determine if PNE effect on PCFs in the pups persists through their adult life.

The mechanism by which PNE upregulates PCF NK1R to potentiate the capsaicin-induced apnea is unclear. Two lines of recent studies suggest dual impacts of NK1R on C-fibers including PCFs. First, substance P has been reported to depolarize dorsal root ganglionic neurons in rats and cats (Dray and Pinnock, 1982; Inoue *et al.*, 1995) and trigeminal ganglion neurons of guinea pigs by intracellular or whole-cell patch recordings (Li and Zhao, 1998) via action on NK1R. Second, NK1R could interact with TRPV1 to amplify PCF response to capsaicin. *In vitro*, NK1Rs were found to be co-expressed with TRPV1 in dorsal root ganglionic neurons and substance P significantly potentiates the capsaicin-induced currents in these neurons via action of NK1R (Zhang *et al.*, 2007). *In vivo*, the apneic response to capsaicin was significantly prolonged after whole-body exposure to acrolein in rats, and this prolongation was abolished by pretreatment with TRPV1 antagonist or NK1R antagonist (Hazari *et al.*, 2008). Intrarectal trinitrobenzenesulfonic acid substantially increased single-unit pelvic C-fiber firing response to intravesical infusion of capsaicin and this response was greatly blunted after depletion of substance P (Ustinova *et al.*, 2007). Collectively, it is possible that PNE upregulation of PCF NK1R expression potentiates TRPV1 and thereby contributes to the prolongation of the PCF-mediated apneic response to capsaicin.

Logically, the PNE-induced changes should be achieved by action on nAChRs. However, this conception has been challenged by a recent report showing an independence of nicotine-induced increase in capsaicin-activated currents in rat trigeminal ganglion neurons of nAChRs (Liu *et al.*, 2004). Thus, we asked if nAChRs, especially α7nAChRs, were essential for the PNE-induced changes in NK1R, ADA_1R, and TRPV1 expressions in N/J ganglionic neurons and pulmonary C-neurons and pulmonary inflammation. Our data showed that the PNE-induced elevation of these receptors in N/J ganglionic neurons, and only NK1R mRNA in pulmonary C-neurons, and pulmonary inflammatory cells were eliminated after blocking nAChRs or α7nAChRs. In agreement with our observation, it was reported that nicotine-induced increase of calcitonin gene related peptide release from rat buccal mucosa in

response to capsaicin relied on nAChRs (Dussor *et al.*, 2003). Our results lead to the conclusion that α7nAChRs are necessary for PNE impacts on NK1R, ADA_1R, and TRPV1 expression in N/J ganglionic neurons, NK1R mRNA in pulmonary C-neurons, and pulmonary inflammatory cells. In fact, the role of α7nAChR in impairing the airways/lung in pups with maternal exposure to cigarette smoke or nicotine has been reported. For example, the maternal exposure could increase the susceptibilities for the development of pediatric lung disorders (Schuller *et al.*, 2000) and decrease forced expiratory flows in offspring largely through the action of α7nAChRs (Wongtrakool *et al.*, 2012). Additionally, the effects of MM and MLA on the PNE-induced responses were not the same. MM not only eliminated ADA_1R response to PNE, but also led to a significantly lower ADA_1R compared to Ctrl (see Fig. 5). Similarly, MLA not only blocked macrophage response to PNE, but also reduced it to a level lower than Ctrl (see Fig. 4). These data point to a contribution of nAChRs to maintain the normal level of ADA_1R expression in N/J ganglionic neurons and α7nAChRs to keep normal pulmonary macrophage level.

Perspective

The risk of exposure to cigarette smoke is highest during fetal and early postnatal life (DiFranza *et al.*, 2004), but nearly one third of mothers still keep smoking during pregnancy (Hylkema and Blacquiere, 2009). The typical adverse consequence in these maternal cigarette smokers is that their offspring has the highest vulnerability of suffering from SIDS featured by cardiorespiratory failure (Trachtenberg *et al.*, 2012). Nicotine is the major neurotoxic compound of cigarette smoke and responsible for pathogenesis of SIDS (Harper *et al.*, 2000; Hafstrom *et al.*, 2002). We previously showed that PNE significantly prolonged the apneic response and augmented pulmonary C-neural response to right atrial injection of capsaicin, proving the PNE-induced PCF plasticity (Zhuang J, 2014). Because PCF activation depresses hypoxic and hypercapnic ventilation that could be responsible for respiratory failure (see discussion in ref. (Zhuang J, 2014)), it is important to investigate how PNE sensitizes and activates PCFs. Our results provide the first evidence to show that PCF NK1R upregulation by PNE is likely responsible for prolongation of the PCF-mediated apneic response. These results not only allow us to better understand how PNE augments the PCF-mediated apneic response, but also gain insight into PCF cardiorespiratory pathophysiology. Most importantly, these results are relevant to pathogenesis of SIDS as an overexpression of C-fibers has been observed in SIDS victims (Becker *et al.*, 1993). Our previous study has shown that PNE-induced lethal apnea during hypoxia is causal to animal death (Zhuang *et al.*, 2014). However, questions remain as to whether upregulation of PCF NK1R by PNE is uniquely involved in the respiratory failure in our study, and if so, how.

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Abbreviations

AD	Adenosine
ADA$_1$R	Adenosine A1 receptor
BALF	Bronchoalveolar lavage fluid
fR	Respiratory frequency
MLA	Methyllycaconitine (a selective antagonist of α7nAChR)
MM	Mecamylamine (a general antagonist of nAChR)
nAChRs	Nicotine acetylcholine receptors
NK1R	Neurokinin-A receptor
N/J	Nodose/jugular
PCF	Bronchopulmonary C-fiber
PNE	Prenatal nicotinic exposure
SIDS	Sudden infant death syndrome
SP	Substance P
TRPV1	Transient receptor potential cation channel subfamily V member 1
V_E	Minute ventilatory volume
V_T	Tidal volume

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Research Highlights

- PNE upregulated NK1R and TRPV1 gene and protein expression in the N/J ganglia.
- PNE only elevated NK1R mRNA in vagal pulmonary C-neurons.
- Blockage of peripheral NK1R reduced the PNE-induced PCF sensitization.
- PNE induced gene and protein changes in NK1R and TRPV1 dependent on action of α7nAChR.

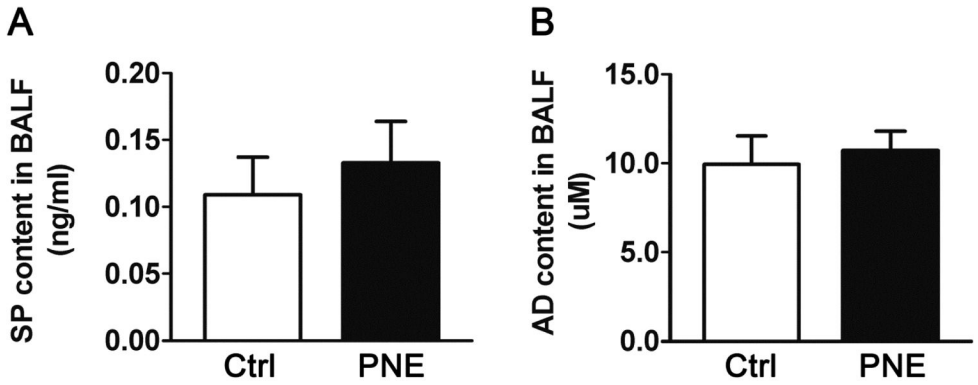


Figure 1.

PNE impacts on SP (A) and AD (B) concentration in BALF. PNE fails to alter SP and AD content (n = 10/group). Data present as Mean ± SE.

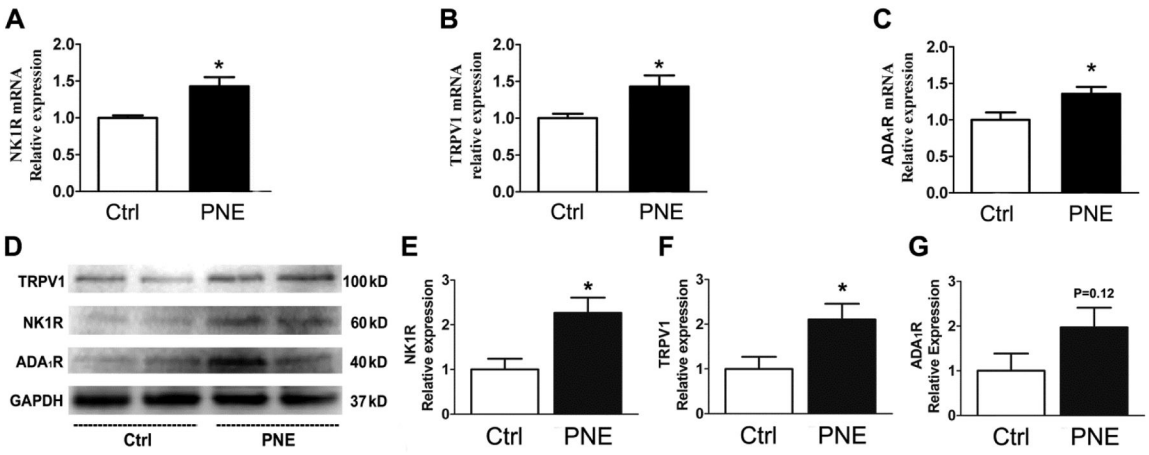
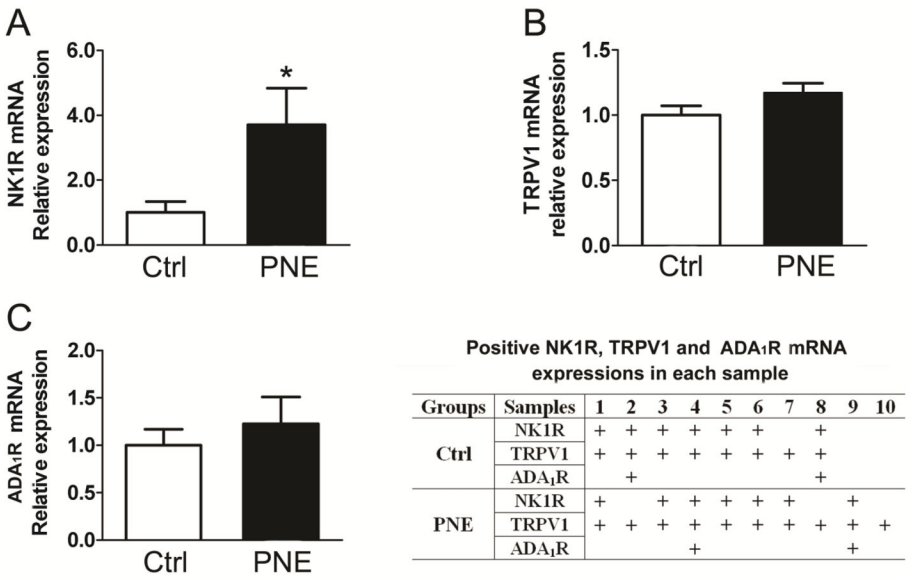


Figure 2.

Comparison of gene (A–C) and protein expression (D, examples and E–G quantified data) of NK1R, ADA1R, and TRPV1 in the N/J ganglia between Ctrl and PNE pups. PNE significantly upregulates gene of NK1R, ADA1R, and TRPV1 in the N/J ganglia (n = 6 in each group for gene detection). In D, the samples from both groups showed an equal amount of protein homogenates. GAPDH (37kD) functioned as a loading control. After quantification (E–G), all values were normalized to the mean of control groups. PNE significantly upregulated NK1R and TRPV1 protein expression in N/J ganglia (4 trial samples for each group). Data present as Mean ± SE; * $P < 0.05$ compared with Ctrl group.



Positive NK1R, TRPV1 and ADA_1R mRNA expressions in each sample

Groups	Samples	1	2	3	4	5	6	7	8	9	10
Ctrl	NK1R	+	+	+	+	+	+		+		
	TRPV1	+	+	+	+	+	+	+	+		
	ADA_1R		+						+		
PNE	NK1R	+		+	+	+	+	+		+	
	TRPV1	+	+	+	+	+	+	+	+	+	+
	ADA_1R				+					+	

Figure 3.

The effects of PNE on gene NK1R (A), TRPV1 (B), and ADA_1R (C) in vagal pulmonary C-neurons in Ctrl and PNE groups by single cell PCR. Data present as Mean ± SE; PNE upregulates NK1R mRNA expression in vagal pulmonary C-neurons. The table lists 8/12 neurons in Ctrl group and 10/12 neurons in PNE group showing TRPV1 mRNA and their co-expression of NK1R and/or ADA_1R mRNA. "+" represents the positive result. Majority of pulmonary C neurons expressing TRPV1 are NK1R positive (n = 7 and 8 for Ctrl and PNE group respectively), while ADA_1R mRNA only expresses in 2 TRPV1+NK1R co-expression cells in both group (n = 2 in each group). Statistical analysis was not performed for ADA_1R mRNA.

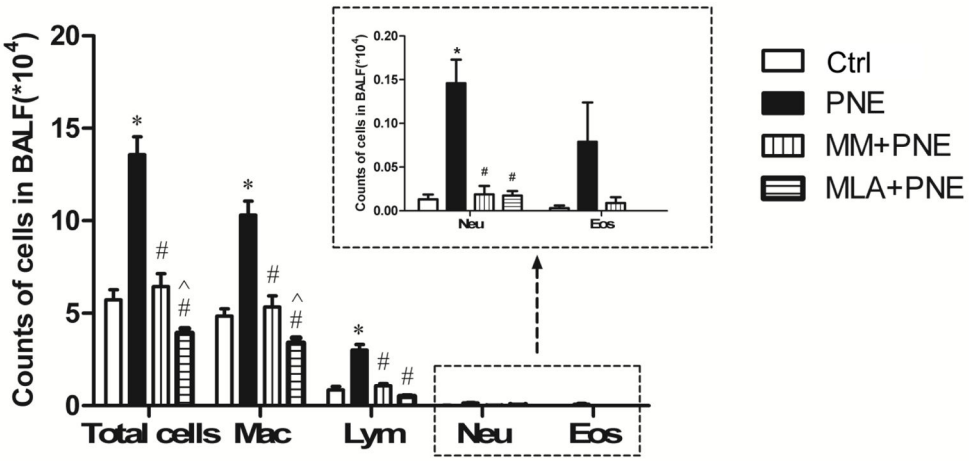


Figure 4.

Inflammatory cells in BALF of Ctrl and PNE pups. PNE significantly increases the total inflammatory cells, especially macrophages, lymphocytes, and neutrophils, while MM and MLA eliminates these responses. N = 10/group. Data present as Mean ± SE; *, #, and ^ $P < 0.05$ compared with Ctrl, PNE, and MM+PNE group, respectively. Mac = macrophages; Lym = lymphocytes; Neu = neutrophils; and Eos = eosinophils.

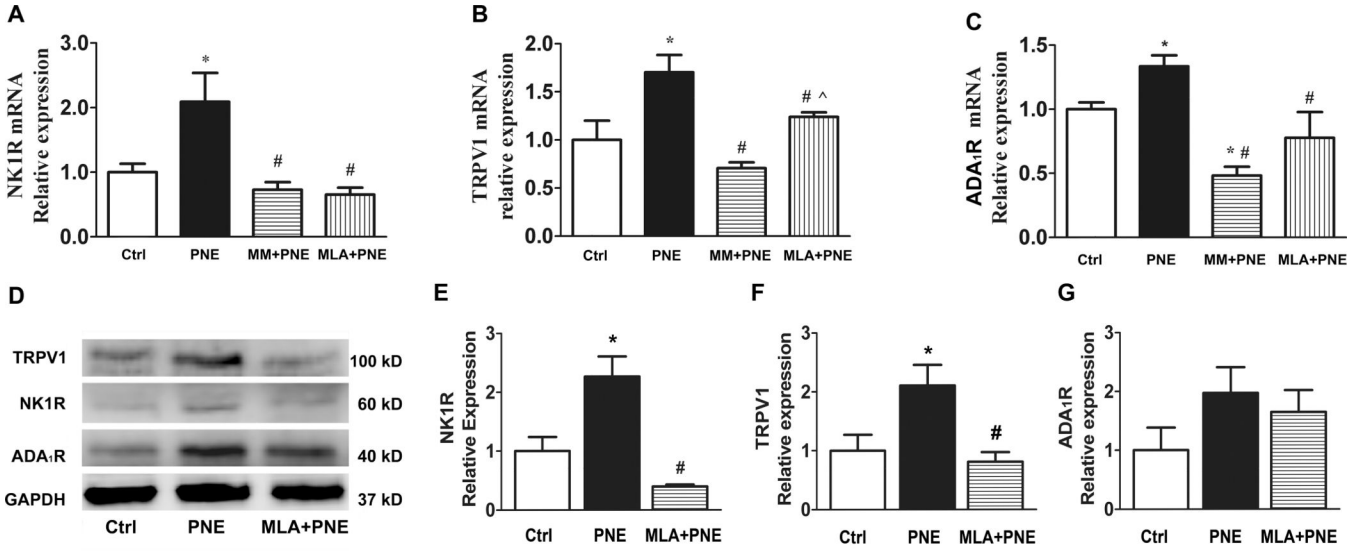


Figure 5.

Effects of MM or MLA on the PNE-induced changes in gene (A–C) and protein expression (D for the examples and E–G for quantified data) of NK1R, ADA_1R, and TRPV1 in the N/J ganglia. MM or MLA blocks the PNE-induced gene upregulation of NK1R, ADA_1R, and TRPV1 in the N/J ganglia (n = 6 in each group for gene detection). PNE also increased the protein levels of TRPV1 (100 kD) and NK1R (60 kD) by Western blot with equal amount of protein homogenates of samples from the pups' N/J ganglia. GAPDH (37 kD) functioned as a loading control. After quantification (E–G), all values were normalized to the mean of control groups (n = 4, 4, and 2 trial samples for Ctrl, PNE, and MLA+PNE group). MLA blocks the protein upregulation of NK1R and TRPV1 by PNE. Data present as Mean ± SE; *, #, and ^ $P < 0.05$ compared with Ctrl, PNE, and MM+PNE group respectively.

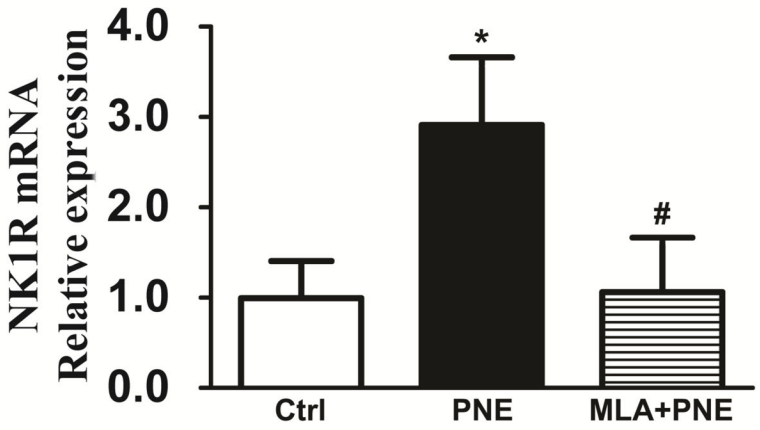


Figure 6.

The effects of MLA treatment on NK1R mRNA expression in pulmonary C-neurons in Ctrl and PNE pups. MLA blocks the PNE-induced NK1R upregulation in vagal pulmonary C-neurons. Data present as Mean ± SE; * and # $P < 0.05$ compared to Ctrl and PNE, respectively. N = 5 in Ctrl, PNE, and MLA+PNE group.

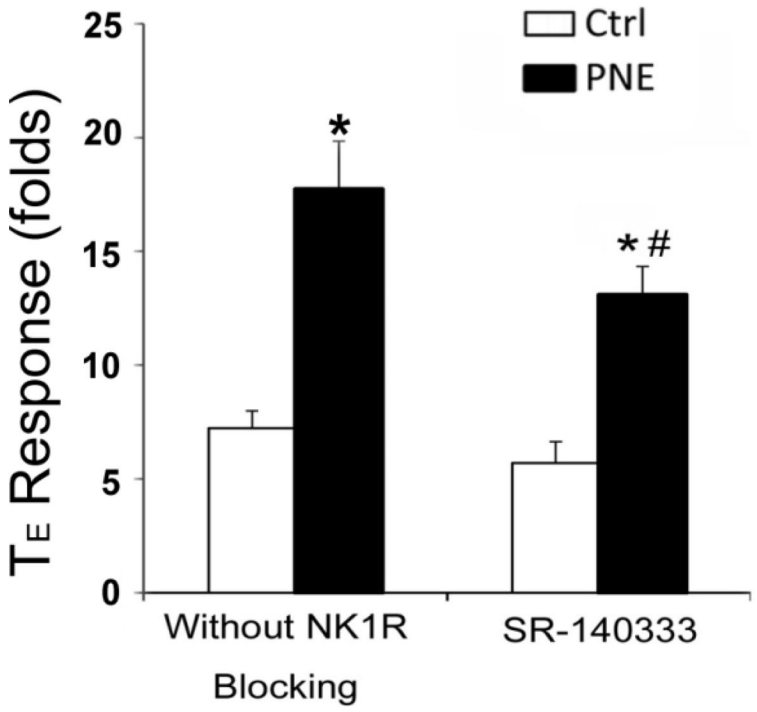


Figure 7.

The effects of treatment with SR140333, a peripheral NK1R antagonist (100 μg/kg) on the apneic response to capsaicin (CAP, 10 μg/kg) in Ctrl and PNE pups. SR140333 significantly shortens the PNE-induced prolongation of the apneic response to CAP with no effect on the CAP-induced apnea in Ctrl pups. Data present as Mean ± SE; N = 6 and 10 for Ctrl and PNE group. * and # $P < 0.05$ compared to Ctrl and PNE before the blockade respectively.

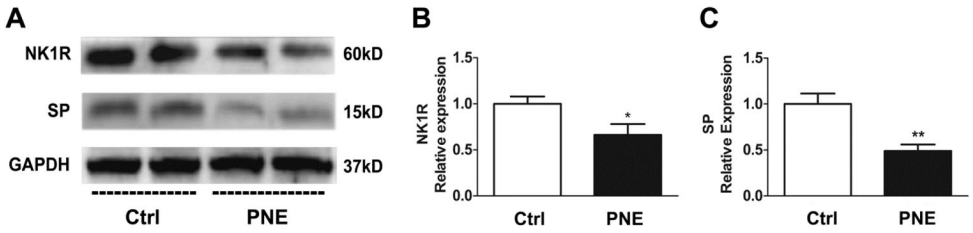


Figure 8.

Comparison of NTS SP and NK1R protein expression between Ctrl and PNE pups. A: Gel images of western blotting between Ctrl and PNE groups. GAPDH (37kD) functioned as a loading control. B and C: group data showing that PNE downregulates protein expressions of SP (15 kD) and NK1R (60 kD) in the NTS. Data present as Mean ± SE; with N = 8 and 12 for Ctrl and PNE. * $P < 0.05$ and ** $P < 0.01$ compared with Ctrl group.

Table 1

Effects of PNE on baseline VE, fR, and VT without and with peripheral NK1R blocker (SR140333) in anesthetized rats' pups

Treatment	Animal Group	V_E (ml/min)	fR (breaths/min)	V_T (ml)
Without blocker	Ctrl	25.2 ± 1.7	108.1 ± 6.7	0.23 ± 0.03
	PNE	24.8 ± 1.2	111.6 ± 5.0	0.22 ± 0.02
SR140333	Ctrl	24.8 ± 1.3	102.0 ± 5.2	0.22 ± 0.02
	PNE	25.0 ± 0.7	116.0 ± 4.7	0.21 ± 0.01