Lung C-Fiber CNS Reflex: Role in the Respiratory Consequences of Extended Environmental Tobacco Smoke Exposure in Young Guinea Pigs

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Environmental tobacco smoke (ETS) exposure harms the respiratory health of children and is associated with an increased risk of asthma and sudden infant death syndrome (SIDS). The mechanisms by which ETS causes these effects are not understood. We hypothesized that one mechanism is an upregulation of the lung C-fiber central nervous system (CNS) reflex responses, which would result in exaggerated reflex responses of apnea, bronchoconstriction, and mucous hypersecretion. The purpose of this work is to highlight evidence obtained in an animal model of postnatal ETS exposure supporting the hypothesis and present data suggesting that actions of the neuropeptide substance P in the nucleus tractus solitarius (NTS) may contribute. Exposing young guinea pigs to sidestream smoke, the surrogate for ETS, for 5 weeks during the equivalent of human childhood, increased the excitability of afferent lung C fibers and NTS neurons in the CNS reflex pathway and prolonged the expiratory apnea. The findings suggest that an increased excitability of NTS neurons that can augment reflex output may contribute to respiratory symptoms in children exposed to ETS. Besides ETS exposure, substance P can also excite NTS neurons and augment lung C-fiber CNS reflex responses. Others have shown that substance P synthesis in lung C fibers is upregulated by another environmental stimulant, allergen. Thus, an upregulation of the substance P system at NTS synapses could contribute to the increased NTS excitability and enhanced reflex responses to lung C-fiber stimulation, providing a potential mechanism to help explain the association of ETS exposure with respiratory symptoms and SIDS. *Key words*: CNS, environmental tobacco smoke, lung C fibers, neuroplasticity. — *Environ Health Perspect* 109(suppl 4):573–578 (2001).
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Environmental Tobacco Smoke and Neural Control of the Lung

Extended exposure to environmental tobacco smoke (ETS) adversely affects the respiratory health of children. Those exposed to ETS have more coughs (*1*,*2*), wheeze (*1*), airway obstruction (*3*), increased airway reactivity (*2*,*4*), and increased sputum production (*1*). ETS exposure is also associated with an increased risk of lower respiratory tract illnesses (*5*), an increased rate and earlier onset of asthma (*6*), and an increased risk of sudden infant death syndrome (SIDS) (*7*). Although the association between extended ETS exposure and harmful effects on children's respiratory health has been established by epidemiologic studies (*1*–*3*,*5*–*7*), much less is known about the mechanisms by which ETS causes these effects (*8*,*9*). Identifying the mechanisms may help to discourage exposing children to ETS, perhaps preventing these adverse effects, and may also help in the development of novel therapeutic strategies for those exposed.

The purpose of this research is 2-fold: *a*) to highlight evidence obtained in an animal model of postnatal ETS exposure supporting the overall hypothesis that an upregulation of the lung C-fiber CNS reflex system may contribute to the asthmalike symptoms associated with extended postnatal ETS exposure in children, and *b*) to develop the proposal that the neuropeptide substance P (SP) may contribute to the upregulation.

Sensory nonmyelinated C fibers innervating the lung are vigorously stimulated by components of ETS including nicotine (*10*), acrolein (*11*), and oxidants (*12*) as well as by mainstream tobacco smoke (*13*–*17*). When stimulated, these lung C fibers can trigger profound respiratory responses through a local axon and central nervous system (CNS) reflex (*10*,*18*). Some of the reflex responses resemble the symptoms associated with extended ETS exposure: bronchoconstriction, mucous secretion, and increased microvascular leak (*18*). The responses evoked through the local axon reflex include bronchoconstriction, mucous secretion, and increased microvascular leak and are thought to be mediated by the local release of substance P and neurokinin A contained in the C fibers (*10*). The CNS reflex respiratory responses also include bronchoconstriction, mucous secretion, and increased microvascular leak but are not limited to the airways and include breathing changes composed of an expiratory apnea sometimes followed by rapid shallow breathing and cardiovascular changes such as decreases in blood pressure and heart rate. Figure 1 gives a simplified overview of the lung C-fiber CNS reflex pathway illustrating the reflex responses evoked by stimulation of the lung C fibers by an injection of capsaicin into the left atrium of a young guinea pig. As shown in Figure 1A, the first-order afferent lung C fibers course in the vagus nerve with their cell bodies located in the nodose or jugular ganglia. Upon entering the medulla, the central axons become part of the tractus solitarius (TS), then exit the tractus to make excitatory synapses onto second-order neurons in the caudomedial region of the nucleus of the tractus solitarius (NTS) as shown in the coronal section in the inset (*19*,*20*). The signals are conditioned in the NTS and then transmitted to distal synapses in the medulla to ultimately elicit the coordinated reflex responses illustrated in Figure 1B.

Our specific hypothesis is that extended exposure to ETS increases the excitability of the primary lung afferent C fibers and that this increase in afferent traffic to the CNS triggers a further increase in lung C-fiber signal transmission, ultimately augmenting the CNS reflex responses, which could contribute to the respiratory symptoms and SIDS associated with ETS exposure in children. We tested the hypothesis in young guinea pigs randomly assigned to a group exposed to either sidestream smoke, the surrogate for ETS, or filtered air for 6 hr/day, 5 days/week, from 1 to 6 weeks of life (a 5-week exposure period). Like the human, the guinea pig shows advanced development of lung function and morphology at birth (*21*). The age of puberty in guinea pigs is 7–10 weeks, and the maximum life span is approximately 7 years (*22*). Thus, these guinea pigs were

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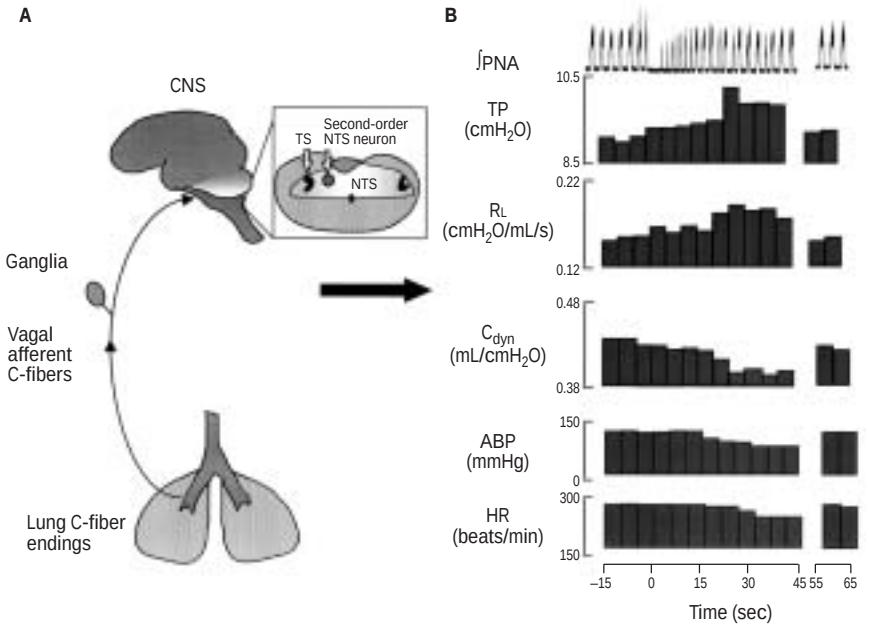

Figure 1. Lung C-fiber CNS reflex. Stimulation of lung C-fiber endings gives rise to afferent signals in C-fiber axons coursing in the vagus nerve, with cell bodies in the nodose and jugular sensory ganglia. The afferent fibers form the TS in the brainstem and synapse onto second-order neurons in the NTS (shown in cross-section in the inset). The signals are conditioned in the NTS and then transmitted to distal synapses to elicit the complex respiratory output: an increase in expiratory time (interval between the integrated [∫] phrenic nerve bursts), an increase in TP and total pulmonary resistance (R_L), a decrease in dynamic compliance (C_{dyn}), and decreases in ABP and HR. The reflex was evoked by injection of capsaicin into the left atrium of a guinea pig at time 0.

exposed during a period equivalent to human childhood and were tested during the period equivalent to human adolescence. Sidestream smoke was generated by an automated cigarette-smoking machine that smoked conditioned 1R4F cigarettes (1.2 mg nicotine/cigarette) from the University of Kentucky Tobacco and Health Research Institute in Lexington, Kentucky. The smoke was collected from the smoldering end of the cigarette, then aged and diluted to a particulate concentration of 1.00 ± 0.07 mg/m^3 (*23*). Sixteen hours after the last exposure, we anesthetized each guinea pig and recorded the spiking activity of individual primary lung C fibers in the vagus nerve. We compared the baseline activity and the increase in spiking activity evoked by mechanical stimulation with lung hyperinflation and by chemical stimulation with left atrial injections of the potent C-fiber stimulant, capsaicin. Using this model we showed that exposing young guinea pigs to sidestream smoke for an extended period of time (5 weeks) increased the excitability (number of action potentials evoked) from both mechanical and chemical stimulation (*23*).

The second specific hypothesis, illustrated in Figure 2A, was that if an increased responsiveness of the primary lung C fibers contributes to the respiratory symptoms associated with ETS via the CNS reflex pathway, the increased responsiveness should be manifest at NTS neurons and ultimately lead to an increase in at least some component of the reflex output. If, on the other hand, the increased excitability of the primary sensory fibers is extinguished by signal modulation in the NTS, the reflex output may be unchanged. We focused on electrophysiologic recordings of caudomedial NTS neurons, as it is at these synapses that the primary sensory information is first subject to neuronal modulation before it is ultimately transformed into a complex output to the lungs, airways, respiratory muscles, heart, and blood vessels (*24*). The studies were performed using the same exposure protocol as the study described previously. We simultaneously recorded extracellular action potentials of second-order NTS neurons in the lung C-fiber reflex pathway and phrenic nerve activity as an index of respiratory output. As shown in the two upper traces in Figure 2B, NTS neurons recorded from sidestream smoke-exposed guinea pigs were more excited by lung C-fiber activation than those neurons recorded from the filtered air-exposed control animals. The lower panel of Figure 2B shows the grouped data from the highest dose of capsaicin and confirms that sidestream smoke exposure increased both the magnitude and the duration of the synaptic responses, the excitability of NTS neurons (*23*). The peak increase in NTS activity in the sidestream smoke-exposed animals was significantly greater than that in the filtered air-exposed animals, as was the duration of the response. In addition, as shown in Figure 2C, the apnea (prolongation of the expiratory time, T_E) was significantly greater in the sidestream smoke-exposed animals compared to that in the filtered air-exposed control group (*25*).

These data, linking changes in NTS neuronal behavior to an augmentation of at least one respiratory motor output, suggest the possibility that one mechanism by which extended ETS exposure may worsen respiratory symptoms in children is by an upregulation of the lung C-fiber CNS reflex at synapses in the NTS. The question is what is the underlying mechanism(s). We propose substance P as one possibility, based on several lines of evidence: First, substance P is synthesized in the cell bodies of the vagal afferent C fibers located in the nodose and jugular ganglia, making the neuropeptide available for neuronal release (*26–29*). Most studies have focused on the local release of substance P in the axon reflex, documenting that neuropeptide can be transported peripherally (*26*) and released locally in the airways (*30*) where it has been implicated in causing allergen-induced asthma (*30,31*). What may have been under-appreciated is the considerable morphologic and physiologic evidence suggesting that substance P may also be released at central synapses in the NTS. Second, this NTS region contains a high density of substance P-containing nerve terminals (*26,27,32–37*), some of which have been shown to emanate from vagal afferent C fibers (*10,28*) as well as from axons and soma throughout the CNS (*38*). Third, there is a parallel distribution of substance P [neurokinin-1 (NK1)] receptors with respect to the nerve terminals, providing targets for substance P release in the nucleus (*29,39–41*). Fourth, there is a striking parallel between neural changes that can occur with extended ETS exposure and those that occur during extended neuropathic or inflammatory pain. Prolonged peripheral inflammation increases the sensitivity of pain sensory fiber endings to noxious stimuli (*42*) (analogous to extended ETS exposure increasing the sensitivity of the lung afferent C fibers) and also increases the synaptic excitation of spinal cord neurons (analogous to increases in synaptic excitation of NTS neurons in the lung C-fiber reflex pathway). The augmentation of synaptic excitation in the spinal cord has been characterized by Woolf and colleagues as an increase in the intrinsic postsynaptic excitability of spinal neurons (*42,43*) that may be triggered in part from an induced novel (substance P) input from peripheral Aβ afferent fibers and an increased substance P input from the peripheral nociceptive C fibers.

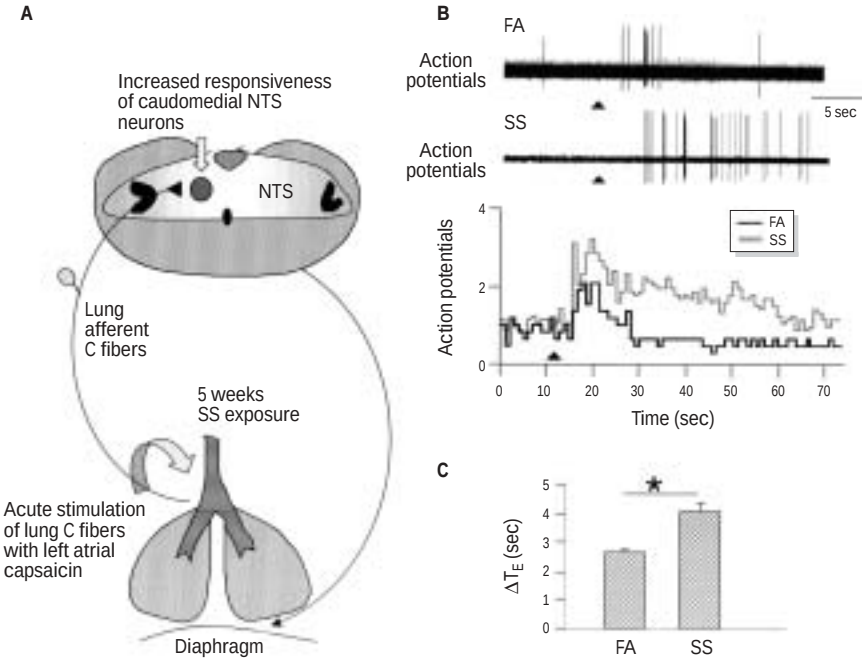

Figure 2. (*A*) Model showing hypothesized increase in the excitability of NTS neurons to lung C-fiber input from guinea pigs exposed for 5 weeks to sidestream smoke (SS). (*B*, two upper panels) Left atrial capsaicin [▲ (LA CAP), 2.0 µg/kg] evoked increases in action potential firing that were significantly greater in the NTS neurons recorded in guinea pigs exposed to SS compared to filtered air (FA). (*B*, lower panel) Grouped data showing the time course of the responses of the NTS neurons to LA CAP injections in guinea pigs exposed to FA (n = 6 neurons) or SS (n = 10 neurons). The magnitude and duration of the increases in NTS neuronal activity were significantly greater in the SS than in the FA-exposed guinea pigs (magnitude, p = 0.030, exposure effect, ANOVA; p = 0.02, Fisher's LSD) (duration, p = 0.01, Fishers LSD). Action-potential responses are plotted in 1-sec bins. (*C*) LA CAP-evoked prolongation of T_E in the same animals was significantly greater in the SS-exposed than the FA-exposed control group (p = 0.030, exposure effect). The grouped data are expressed as means ± SE. Modified from Mutoh et al. (*25*).

Finally, there is evidence that substance P expression can be upregulated in sensory ganglia of the lungs. Two laboratories have demonstrated that allergic inflammation increases substance P message and content in the vagal sensory ganglia (*31*,*44*). These findings raise the possibility that under conditions of extended ETS exposure, an increased substance P could be transported centrally and released in the NTS to augment lung C-fiber afferent signaling and ultimately reflex function.

The first step to determine the extent to which substance P can upregulate lung C-fiber afferent signal transmission with prolonged ETS exposure is to examine whether the neuropeptide has the capacity to modulate signal transmission at NTS synapses under control conditions. To make this determination, we asked the following questions: Does substance P in the NTS augment any component of the lung C-fiber reflex output? Does substance P applied at NTS synapses enhance synaptic transmission between vagal afferent C fibers and NTS neurons? Does substance P applied at NTS synapses increase the excitability of postsynaptic NTS neurons in the lung C-fiber afferent pathway?

Substance P in the NTS Augments Lung C-Fiber Reflex-Evoked Bronchoconstriction

The hypothesis that substance P excitatory actions at NTS synapses augments lung C-fiber CNS reflex output is illustrated in Figure 3A. In support of the hypothesis we previously showed that exogenous application of substance P in the NTS could augment the lung C-fiber reflex output (*45*). We performed these studies on anesthetized guinea pigs in which we made left atrial injections of capsaicin (0.5 µg/kg), a dose that modestly increases (< 15 %) primary and NTS lung C-fiber neuron-spiking activity (*23*,*25*). We measured the changes in the capsaicin-evoked increase in tracheal pressure (TP), apnea (increase in T_E), decrease in arterial blood pressure (ABP), and decrease in heart rate (HR) before and during substance P (200 µM, 25 nL) was stereotaxically injected in the NTS. To determine if the substance P-evoked augmentations of the responses were mediated by NTS NK1 receptors, we compared the effect of substance P on the reflex responses before and after NTS injections of the NK1 receptor antagonist, CP-96345 or its inactive (2*R*,3*R*) enantiomer (CP-96344) (1 mM, 50 nL).

The grouped data from 12 guinea pigs are shown in Figure 3B. Modest increases in TP (upper panel) and T_E (lower panel) evoked by left atrial capsaicin injections (LA CAP) were statistically significantly augmented following discrete injections of substance P in the caudomedial NTS (SP + LA CAP). The augmentative effects of substance P were abolished by prior injection of the NK1 receptor antagonist but not by the inactive enantiomer. The reflex-evoked decreases in ABP and HR were also statistically significantly augmented (data not shown).

The findings suggested that activation of substance P receptors in the NTS can have a physiologically relevant effect by augmenting the lung C-fiber reflex output; however, the data did not address the question of whether substance P can directly increase the baseline activity of lung C-fiber neurons (action potential discharge rate) or their response to synaptic activation by lung sensory input.

Does Substance P Enhance Synaptic Transmission at NTS Synapses?

These data were obtained from extracellular recordings of action-potential responses obtained from NTS neurons in the whole animal and from whole-cell patch-clamp recordings obtained from NTS neurons in a coronal brainstem slice preparation. The slice contained the caudomedial NTS, the same region studied *in vivo*, and the peripheral (including vagal) afferent fibers in the TS. To determine whether exogenous substance P can enhance the transmission of input from peripheral vagal afferent C fibers, we compared the action-potential responses of NTS neurons evoked by electrical stimulation of the vagal afferent C fibers before and during iontophoretic application of substance P *in vivo*. The findings are illustrated in the example in Figure 4A. Under control conditions, 30 sequential stimuli applied to the ipsilateral vagus nerve at intensities to activate C fibers evoked 16 action potentials for a 53% action-potential response rate. During the iontophoresis of substance P in amounts that did not change the baseline activity, the same stimulation protocol evoked 30 responses for a 100% response rate. The grouped data are shown in the right panel. Seven of 8 NTS neurons tested exhibited a statistically significant increased response rate (number of action potentials evoked divided by the number of stimuli delivered × 100) to vagal afferent C-fiber stimulation.

The *in vitro* studies performed in the NTS slice (Figure 4B) confirmed the *in vivo* findings. As shown in the example (left

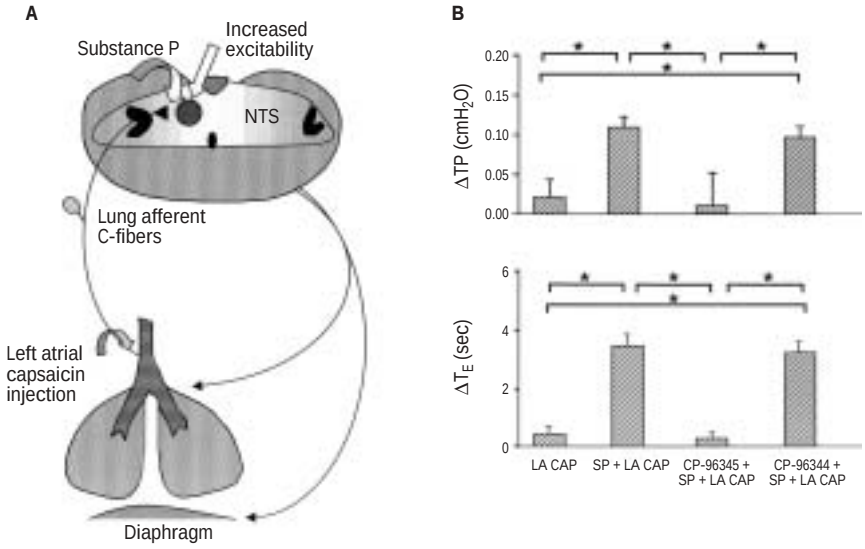

Figure 3. (*A*) Model illustrating the hypothesis that substance P injections in the NTS would increase neuronal responsiveness to lung C-fiber input and result in an augmented reflex output. (*B*) Substance P (SP) injections in the NTS augmented the C-fiber evoked increase in TP and T_E (n = 12). (*B*, upper panel) Left atrial capsaicin (LA CAP, 0.5 µg/kg) evoked small increases in TP that were not statistically significant. After bilateral NTS injections of SP, the LA-CAP–evoked increase in TP was increased (LA CAP vs SP + LA CAP; $p < 0.02$, Fisher's test). The augmented effects of SP were prevented by prior injection of the NK1 receptor antagonist, CP-96345 (SP + LA CAP vs CP-96345 + SP + LA CAP; $p < 0.02$ Fisher's test) but not of the inactive enantiomer, CP-96344 (SP + LA CAP vs CP-96344 + SP + LA CAP; $p > 0.49$, Fisher's test). (*B*, lower panel) LA CAP produced a slight increase in T_E that was significantly augmented by bilateral NTS injections of SP (LA CAP vs SP + LA CAP; $p < 0.0001$, Fisher's test). The augmented effects of SP were prevented by prior injection of CP-96345 (SP + LA CAP vs CP-96345 + SP + LA CAP; $p < 0.0001$, Fisher's test) but not of CP-96344 (SP + LA CAP vs CP-96344 + SP + LA CAP; $p = 0.99$, Fisher's test). The grouped data are expressed as means ± SE. Modified from Mutoh et al. (*45*).

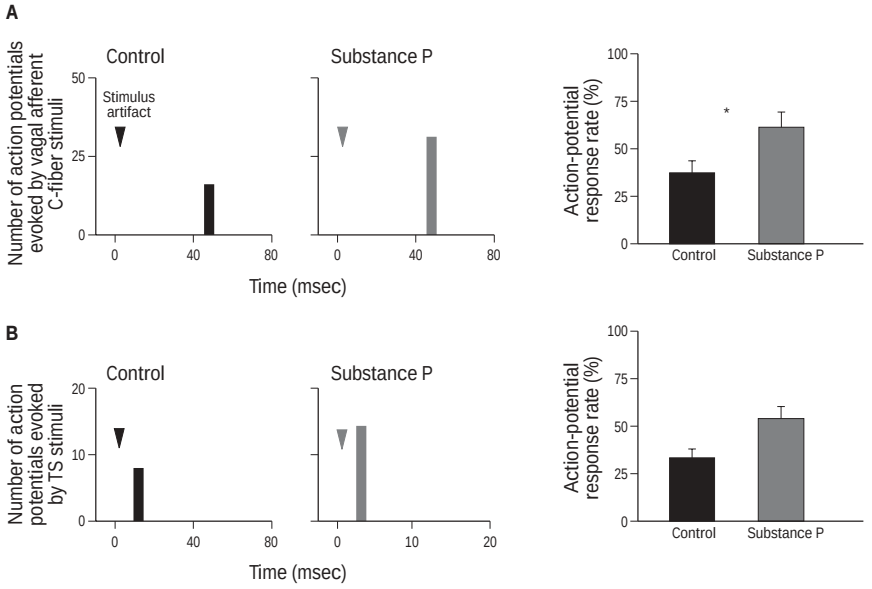

Figure 4. (*A*) Substance P applied at NTS synapses enhanced the postsynaptic responses to vagal afferent C-fiber input *in vivo*. (*A*, left panel) Peristimulus time histogram shows the number of action potentials evoked in NTS neurons by 30 sequential electrical stimuli applied to the vagus nerve under control conditions and during the iontophoresis of substance P onto the neuron. (*A*, right panel) The grouped data confirmed that application of substance P in the NTS augmented synaptic transmission between the vagal afferent C fibers and NTS neurons ($p = 0.02$, paired t-test). (*B*) Stimulation of sensory afferent fibers in the TS also evoked action potential responses in NTS neurons in an *in vitro* slice preparation. As shown in the example (left panel) and grouped data (right panel), perfusion of the slice with substance P statistically significantly augmented the action potential response rate to TS stimulation, confirming the results obtained *in vivo*. The grouped data are expressed as means ± SE.

panel), 20 stimuli in the TS evoked eight action potentials in an NTS neuron under control conditions for a 40% response rate. During perfusion of the slice with substance P (2 µM), the response rate increased to 70% (14 action potentials evoked). In the grouped data (right panel), substance P enhanced the response rate of evoked action potentials in three of five neurons tested. The augmentation was similar in magnitude to that observed *in vivo*. Substance P had no effect on signal transmission in the remaining two neurons. The data suggest that substance P acting in the NTS can augment peripheral sensory input to NTS neurons without changing the baseline NTS neuronal activity.

Does Substance P Increase Baseline NTS Neuronal Activity?

To determine whether substance P has excitatory effects on NTS neurons that receive vagal afferent C-fiber input, we performed extracellular recordings of the baseline spiking activity of NTS neurons before and during the local iontophoretic application of substance P in the *in vivo* studies. The *in vivo* data are shown in Figure 5A. In the example (left panel), substance P modestly increased the baseline action potential firing rate. As shown in the right panel, a statistically significant increase was confirmed in nine of ten neurons tested; the spiking activity increased from a mean resting activity of 11 ± 3 Hz in the preceding 30 sec to a peak 18 ± 3 Hz averaged over 60 sec.

Because all neurons may not display spiking responses to substance P, we further determined whether substance P could depolarize the membrane potential with or without concomitant spiking in the NTS in the brainstem slice *in vitro*. The *in vitro* data are shown in Figure 5B. An example of substance P-induced depolarization and spiking in one NTS neuron is shown in the left panel. The grouped data are shown in the middle panel; in 11 or 14 neurons, perfusion of the NTS with substance P (0.5–10 µM) modestly but statistically significantly depolarized the resting membrane potential from a holding potential of –60 mV. In a separate group of 14 neurons, we counted the number of action potentials evoked by substance P. The grouped data are shown in the right panel. In 7 of the 14 neurons, substance P modestly but statistically significantly increased the action-potential firing rate ($p = 0.04$). The data suggest that substance P has the capacity to modestly excite some but not all postsynaptic neurons in the NTS; thus, substance P acting in the NTS can facilitate synaptic transmission between vagal afferent C fibers and postsynaptic NTS neurons by augmenting the postsynaptic neuronal responsiveness to the sensory input.

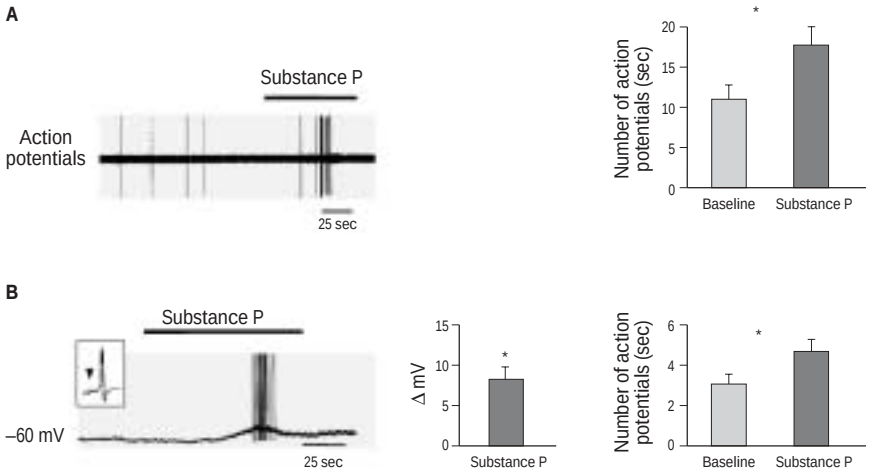

Figure 5. (*A*, left panel) Substance P iontophoretically applied onto a vagal afferent C-fiber NTS neuron in the whole animal evoked a modest increase in the action potential discharge rate. (*A*, right panel) The grouped data (n = 9) on the increase in spiking activity show a modest but statistically significant increase in the action potential firing rate (p = 0.002, paired t-test). (*B*, left panel) As shown in the example of an NTS neuron recorded in the brainstem slice, substance P perfusion (0.5 µM) evoked a depolarization of the resting membrane potential and subsequent action potentials. Inset, TS (▼) stimulation artifact and evoked action potentials of NTS neuron. (*B*, middle panel) The grouped data (n = 11 neurons) confirm a modest but statistically significant depolarization (8.4 ± 1.9 mV) from a holding potential of –60 mV (p = 0.03, paired t-test). (*B*, right panel) The grouped data on the increase in spiking activity (n = 7 neurons) show a modest but statistically significantly increase in action-potential firing rate in response to substance P (p = 0.04, paired t-test). The grouped data are expressed as means ± SE.

In summary, substance P can act in the NTS to augment synaptic transmission between sensory afferent fibers and NTS neurons and can also increase the baseline excitability of NTS neurons. Either or both effects could contribute to the ability of substance P acting in the NTS to augment lung C-fiber reflex output.

Conclusions and Speculation

The increased responsiveness of NTS neurons in the lung C-fiber reflex pathway and prolonged expiratory times suggest that extended ETS exposure can change respiratory function via the CNS. The results may provide a mechanism to explain some of the respiratory symptoms in children exposed to ETS. If extended exposure to ETS increases some aspect of the substance P system—either production or release in the lung C-fiber CNS reflex pathway, the neuropeptide may act in the NTS to augment lung C-fiber reflex output to increase the apnea and also the bronchoconstriction, mucous secretion, and microvascular leak—responses that resemble asthmalike symptoms associated with ETS exposure in children. Future studies will be required to determine if ETS increases the expression of substance P and if such increases affect the lung C-fiber CNS reflex. Understanding the mechanisms will underscore efforts to discourage exposing children to ETS and direct attention to new therapeutic strategies.

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