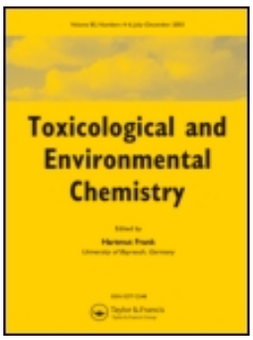

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Early postnatal ozone exposure alters rat nodose and jugular sensory neuron development

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Sensory neurons originating in nodose and jugular ganglia that innervate airway epithelium (airway neurons) play a role in inflammation observed following exposure to inhaled environmental irritants such as ozone (O_3). Airway neurons can mediate airway inflammation through the release of the neuropeptide substance P (SP). While susceptibility to airway irritants is increased in early life, the developmental dynamics of afferent airway neurons are not well characterized. The hypothesis of this study was that airway neuron number might increase with increasing age, and that an acute, early postnatal O_3 exposure might increase both the number of sensory airway neurons as well as the number SP-containing airway neurons. Studies using Fischer 344 rat pups were conducted to determine if age or acute O_3 exposure might alter airway neuron number. Airway neurons in nodose and jugular ganglia were retrogradely labeled, removed, dissociated, and counted by means of a novel technique employing flow cytometry. In Study 1, neuron counts were conducted on postnatal days (PD) 6, 10, 15, 21, and 28. Numbers of total and airway neurons increased significantly between PD6 and PD10, then generally stabilized. In Study 2, animals were exposed to O_3 (2 ppm) or filtered air (FA) on PD5 and neurons were counted on PD10, 15, 21, and 28. O_3-exposed animals displayed significantly less total neurons on PD21 than FA controls. This study shows that age-related changes in neuron number occur, and that an acute, early postnatal O_3 exposure significantly alters sensory neuron development.

Keywords: substance P; flow cytometry; airway neurons

Introduction

Sensory innervation in the airways is important in the pathogenesis of inflammation associated with asthma and other obstructive respiratory disorders (Carr et al. 2002; Lee et al. 2002; Maggi et al. 1995). However, few studies have been undertaken to describe the dynamics and development of airway innervation during early postnatal lung development. Furthermore, the lung continues to grow and develop throughout childhood (Harding, Pinkerton, and Plopper 2004) and early childhood is recognized as highly sensitive to the effects of airway irritants, including allergens, cigarette smoke, and air pollutants (Wang and Pinkerton 2007).

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There are several components of postnatal airway development that contribute to normal sensory innervation. One component originates from the sensory neurons in the nodose and jugular ganglia. These ganglia are largely responsible for the sensory innervation in the thoracic and abdominal viscera, including the heart, lung, and GI tract. Nerve-tracing studies demonstrated that airway afferent fibers have cell bodies located in the nodose and jugular ganglia (Kalia and Mesulam 1980a; Dey et al. 1990; Hunter and Undem 1999) whose projections include nociceptive C-fibers that mediate airway inflammation through the release of the neuropeptide, substance P (SP; Kalia and Mesulam 1980b; Lundberg et al. 1983; Helke and Hill 1988).

Many airway environmental irritants including cigarette smoke, allergens, and ozone (O_3) may affect the extent of neuropeptide expression in the airways (Fischer et al. 1996; Kwong et al. 2001; Larson et al. 2004; Kajekar et al. 2007) and early life may be a particularly susceptible period. Hunter, Wu, and Dey (2010) found that rats exposed to O_3 during early postnatal life (between postnatal days 2 and 6), and then re-exposed to O_3 later in life (postnatal day 28), have increased SP innervation compared to rats exposed only on PD28. However, the underlying mechanism generating this altered neural response to early-life O_3 exposure has not been established.

This investigation examined the possibility that neuron number changes in an age-specific manner and that O_3 exposures in early life increase the number of airway neurons as well as raising the number of SP-containing neurons in the nodose and jugular ganglia that innervate the airway. The approach involves a novel technique combining neural tracing of airway neurons, enzymatic dissociation of the nodose and jugular ganglia, and high-throughput cell quantification using flow cytometry.

Experimental design

In order to test the hypothesis, experiments were designed to quantify the number of neurons in the nodose and jugular ganglia innervating the airway epithelium that contain SP.

Study 1: age specific dynamics of sensory neurons

The first experiment was designed to determine the normal pattern of development for airway innervating sensory neurons in the nodose and jugular ganglia. Rat pups were instilled with fluorescent microspheres on postnatal days (PD) 2 or 5 to retrogradely label neurons in sensory ganglia innervating airway epithelium (airway neurons). On PD6, 10, 15, 21, or 28 ganglia were removed and dissociated; neurons were isolated, purified, and counted using flow cytometry (Figure 1A).

Study 2: ozone or filtered air exposure

The second experiment evaluated if a single acute O_3 exposure in early postnatal life might change the number of SP-containing airway neurons, or the number of airway neurons. Animals were instilled with fluorescent microspheres on PD5 to retrogradely label neurons in sensory ganglia innervating airway epithelium. On the same day (PD5), rat pups were exposed to O_3, 2 ppm or filtered air (FA) for 3 h. Five, 10, 16, and 23 days post treatment (corresponding to postnatal ages 10, 15, 21, and 28, respectively) ganglia were removed, dissociated and neurons were isolated, purified, and counted using flow cytometry (Figure 1B).

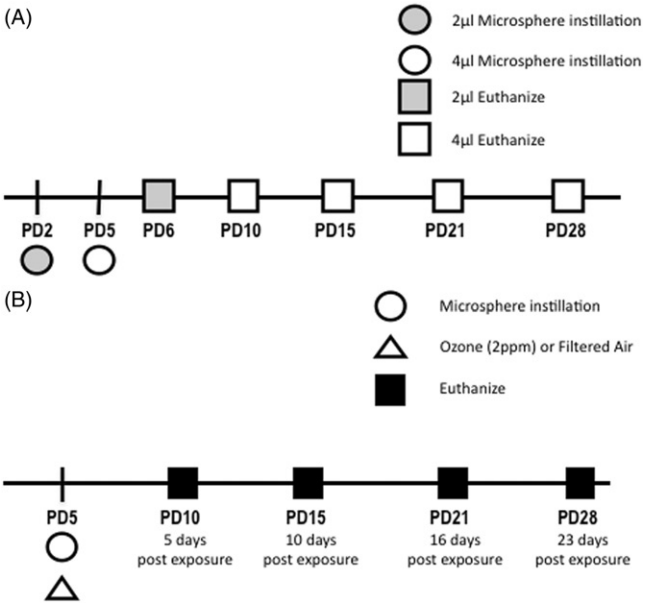

Figure 1. Experimental design for Study 1: age-specific dynamics of sensory neurons and Study 2: O_3 or FA exposure. (A) Study 1: To examine the normal pattern of development of nodose and jugular sensory neurons innervating the airways, tracheas of Fischer 344 rat pups were instilled with fluorescent microspheres on postnatal day (PD) 2 (gray circle) or 5 (white circle). On PD6, animals beaded on PD2 were sacrificed (gray square), while animals beaded on PD5 were euthanized on PD10, 15, or 28 (white square). (B) Study 2: To determine if neuron numbers were changing in response to an acute, early postnatal O_3 exposure, 5-day-old Fischer 344 rat pups were instilled with fluorescent microspheres to retrogradely label sensory neurons innervating airway epithelium (circle). On the same day, pups were exposed to either O_3 (2 ppm) or FA for 3 h (triangle). Rat pups were returned to their mother's cages, where they remained until sacrifice 5, 10, 16, or 23 days after the exposure, which corresponded to PD10, 15, 21, and 28, respectively (square).

Materials and methods

Animals

Late gestation pregnant female Fischer 344 rats were purchased from Harlan Laboratories and kept in filtered ventilated cages in an USDA approved, specific pathogen-free, and environmentally controlled animal facility. While in the vivarium, animals were provided with HEPA-filtered air, autoclaved diet and tap water *ad libitum*, and housed under controlled 12 h light–dark cycle and temperature (22–24°C) conditions. After rat pups were born, they were kept in the same cage as the mother. Rat pups were analyzed on postnatal days (PD) 6, 10, 15, 21, and 28. All procedures were done in accordance with IACUC regulations and approved IACUC protocols.

Fluorescent microsphere instillation

Retrograde tracers were instilled into the tracheal lumen in order to identify sensory neurons in the nodose and jugular ganglia that specifically innervate the airway epithelium. Briefly, on PD2 or 5 depending on the study, rat pups were anesthetized via ice immersion-induced hypothermia (3–5 min; Danneman and Mandrell 1997).

The pups were placed inside a latex sleeve, to protect their skin. When sufficiently anesthetized (unresponsive to foot pinch), trachea was exposed with a midline incision in the skin on the ventral surface of the neck. Next, 2 μL (PD2) or 4 μL (PD5) of Green Fluorescent RetroBeads™ (beads, LumaFluor Inc., Durham, NC) were instilled into the tracheal lumen using a 10 μL Hamilton syringe. Incisions were closed using surgical glue. Rat pups were revived using gentle warming and artificially ventilated if needed. When animals regained consciousness and were breathing normally, pups were returned to their mother. As the beads need a minimum of 4 days to travel from the epithelium to the cell body and it was shown that the beads reach maximal uptake within 7 days of instillation (Hunter and Dey 1998), animals being examined on PD6 received bead instillation on PD2, whereas all other age group rat pups received beads on PD5. Through preliminary studies it was determined that intratracheal bead instillations did not result in airway inflammation as evidenced by a lack of neutrophil infiltration in lung lavage fluid. Optimization of bead instillation volumes was also completed and the selected volumes were found to result in the best survival rates at the specific ages.

Ozone exposure

Technical details of our ozone exposure apparatus have been published (Wu, Satterfield, and Dey 2003). On PD5, following bead instillation, the rat pups were exposed for 3 h to either O_3 (2 ppm) or filtered air (FA) in a stainless steel-and-glass exposure chamber. Passing hospital-grade air through a drying and high-efficiency particle filter and then through an ultraviolet light source produced O_3. The O_3 concentration in the chamber was measured by chemiluminescence with a calibrated O_3 analyzer (model OA 350-2 R, Forney, Carrollton, TX). In the FA-exposed animals, procedures were identical to those described above except O_3 was not delivered to the exposure chamber.

Nodose and jugular ganglia neuron isolation

At PD 6, 10, 15, 21, or 28 pups were euthanized with an i.p. injection of sodium pentobarbital (50 mg mL^{-1}), both vagus nerves were exposed and the left and right nodose and jugular ganglia were removed. Neurons were isolated using a method previously described by Kwong and Lee (2002). Briefly, following removal, ganglia were desheathed, cut into smaller pieces, and placed in a 0.125% collagenase-type IV in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) media solution overnight at 37°C. To terminate collagenase activity, the suspension was pelleted (150 g, 5 min) and resuspended in a solution of 0.15% trypsin EDTA in Hank's Balanced Salt Solution (HBSS) (<1 min) repelleted (150 g, 5 min) and resuspended in modified DMEM/F12 solution. This final suspension, containing a mixture of cells and cellular connective tissue fragments, was layered on top of a solution of 15% bovine serum albumin (BSA) in DMEM/F12. Subsequent centrifugation (500 *g*, 8 min) through the BSA solution resulted in the trapping of myelinated debris such that the remaining pellet contained mostly neurons free of cellular and connective tissue fragments. The pelleted neurons were collected and then prepared for flow cytometry.

Flow cytometry cell preparation

Cell pellets containing isolated neurons were resuspended in 500 μL of DMEM/F12 media with 250 μL of BD Cytofix/Cytoperm Kit (BD Biosciences, San Jose, CA) for 20 min to fix and permeablize them. Cells were incubated with a guinea pig primary antibody (Chemicon/Millipore, Temecula, CA) against the pan-neuronal marker, protein gene product 9.5 (PGP-9.5) for 30 min at 4°C. After incubation, cells were washed twice to remove any unbound primary antibody, followed by the addition of an R-Phycoerythrin (RPE) conjugated donkey anti-guinea pig antibody (Jackson ImmunoResearch, West Grove, PA). Cells incubated with the secondary antibody for 30 min at 4°C, and were then washed twice to remove any unbound antibody. All cells were then incubated for 10 min at 4°C with 4′6-diamindino-2-phenylindole (DAPI) (Sigma Aldrich, St. Louis, MO) in order to label nuclei of cells, washed to remove any unbound DAPI, and resuspended in a final volume of 300 μL. Prior to analysis, samples were passed through a 70 μm cell strainer to remove any cellular debris that might remain. Cells were analyzed on a BD FACSAria (BD Biosciences, San Jose, CA) located in the WVU Flow Cytometry Core Facility using DIVA 6.1 software. For each sample, data were collected using a constant flow rate for 60 s. An event was considered an intact cell if it was $DAPI^+$. Whole cells were deemed neurons if they were both $DAPI^+PGP\text{-}9.5^+$. Neurons that innervate the airways were identified as being $FITC^+$ as an indicator of the retrograde transport of green RetroBeads™.

In the second set of experiments, cells from animals in the O_3/FA exposed groups were also labeled for tachykinin, SP. During the primary antibody incubation, a mouse monoclonal primary antibody (R&D Systems, Minneapolis, MN) against SP was added at the same time as the anti-PGP-9.5 antibody. In order to detect the SP, a Cy-5 conjugated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) was added along with the RPE-conjugated secondary antibody. Other than these additions, the same procedural steps were taken as described above. This allowed for neurons to be further identified as SP^+ neurons (non-airway SP-containing neurons, $DAPI^+PGP\text{-}9.5^+SP^+$) or SP^+ airway neurons (SP-containing airway neurons, $DAPI^+PGP\text{-}9.5^+FITC^+SP^+$).

To calculate the number of neurons in each sample, the following equation was used:

$$\frac{\#\text{ of events}}{\text{volume sampled}} = \frac{\text{neuron number}}{\text{total sample volume}}$$

For each sample set analyzed, a tube containing a known volume of buffer was run through the FASCAria for the same time and at the same flow rate used for the sample analysis. The volume sampled was determined by measuring the amount remaining in the tube.

Data analysis

Results of experiments are expressed as mean (±SE). For an individual result to be included in the study, it had to be within 2 standard deviations of the mean. Statistical analysis was evaluated by using a one- or two-way ANOVA. When an effect was considered significant, a pair-wise comparison was made with a *post hoc* analysis. A value of p less than 0.05 was considered significant for each endpoint and n represents the number of animals studied per experimental group. An $n = 6$ was used for each age group unless otherwise noted.

Results

Identification and confirmation of neurons

The number of neurons contained in the nodose and jugular ganglia was determined using flow cytometry. This method allows for unbiased, absolute counting, as well as the ability to identify multiple populations of neurons in a single run. Cells were first identified as being $DAPI^+$, which indicated the presence of an intact nucleus (Figure 2A: blue box). From the $DAPI^+$ population, the neuron specific marker, PGP-9.5, was used to determine the neurons cells, as shown in Figure 2(C): lower right quadrant (total neurons; $DAPI^+PGP^+$). Neurons projecting to the airway epithelium were neurons that contained Retrobeads^{TM} (airway neurons; $DAPI^+PGP^+FITC^+$), as seen in Figure 2(C): upper right quadrant. Samples prepared for flow cytometry were also observed by confocal microscopy to confirm the structural and labeling attributes of neurons. Figure 2(B) shows an example of a non-airway neuron. It is labeled for PGP-9.5, indicating a neuron, DAPI showing that it is a whole cell with a nucleus, and had no microspheres indicating that it was not a neuron projecting to the airway epithelium. The neuron in Figure 2(D) was labeled for both PGP-9.5 and DAPI, and was also labeled with green microspheres transported from the airway, indicating that this neuron projected to the airway epithelium.

In order to determine the number of SP-containing neurons in O_3/FA-exposed animals, total and airway neurons were first identified. From the $DAPI^+$ population, cells were classified as neurons ($PGP\text{-}9.5^+$) and were then categorized as either being non-airway SP-containing neurons (SP^+ neurons, $DAPI^+PGP^+SP^+$), or SP-containing airway neurons (SP^+ airway neurons, $DAPI^+PGP^+FITC^+SP^+$), as seen in Figure 2(E) (lower right quadrant and upper right quadrant, respectively). Figure 2(F) is a representative image of a SP-containing airway neuron, demonstrating labeling with DAPI, PGP-9.5, green beads (FITC), and SP.

Age specific dynamics of sensory neurons

Nodose and jugular ganglia sensory neurons were isolated from rat pups during early postnatal life (PD6, 10, 15, 21, and 28) in order to determine the pattern of normal sensory neuron development of all neurons in the nodose and jugular ganglia and of a subpopulation of neurons innervating the airway epithelium (refer to Figure 1A).

Total nodose and jugular ganglia neurons ($DAPI^+PGP\text{-}9.5^+$)

Using FACS, it was determined that the number of neurons at PD6 was 924($\pm$470; Figure 3A). There was a significant increase in total neurons between PD6 and PD10 (8197 ± 1170), which remained elevated at all subsequent ages examined ($PD15 = 9677 \pm 2596$; $PD21 = 6185 \pm 547$; $PD28 = 6765 \pm 1745$). Neuron numbers on PD10 through PD28 were not significantly different from each other. To confirm the quantitative and significant increase in neuron number between PD6 and PD10, ganglia from both ages were removed, weighed, processed for immunohistochemistry and visualized using confocal microscopy. Ganglia removed from PD10 animals ($n = 3$) weighed significantly more than ganglia from PD6 ($n = 3$) rats (0.0052 g and 0.0021 g, respectively; Figure 4A). Visual inspection of the ganglia revealed that PD10 ganglia have more tightly packed neurons compared to PD6 ganglia, as shown in Figure 4(C) and (B), respectively.

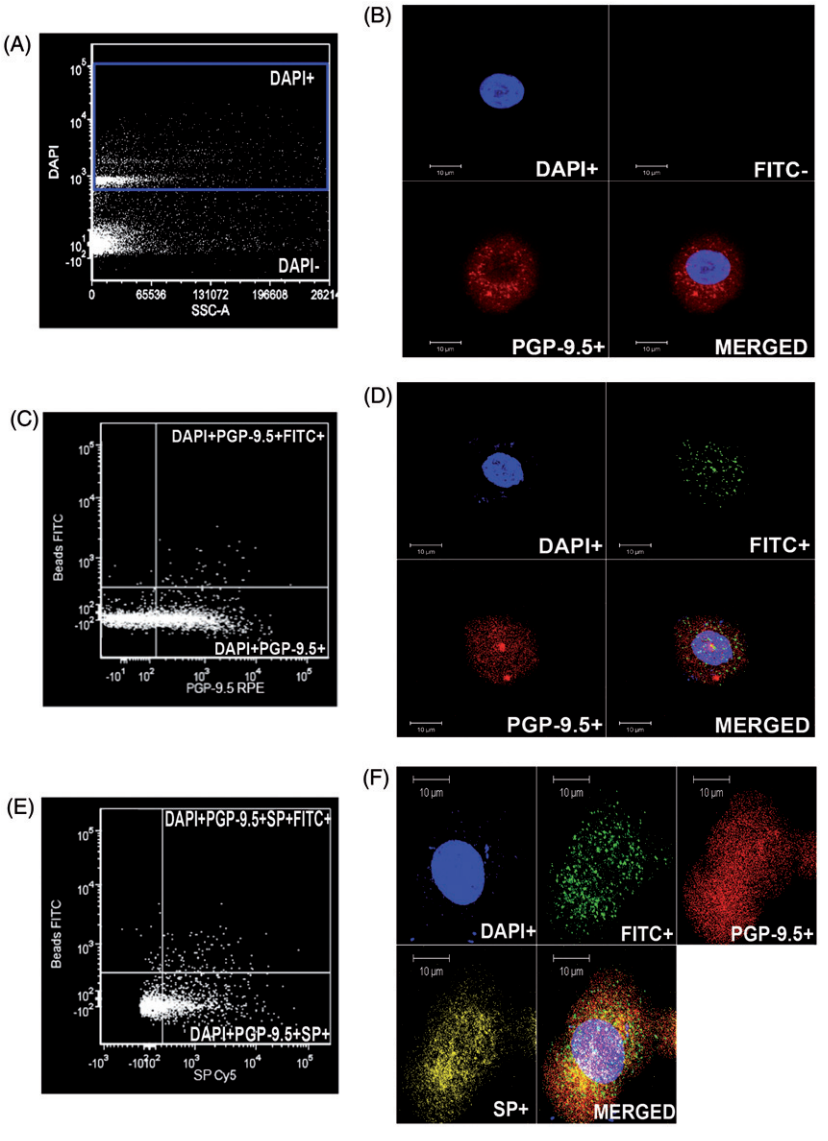

Figure 2. Identification and confirmation of nodose and jugular ganglia neuron populations. Neurons contained in the nodose and jugular ganglia were identified using flow cytometry as being (A) *whole cells*; $DAPI^+$, (C) neurons; $DAPI^+$ $PGP\text{-}9.5^+$, airway neurons; $DAPI^+$ $PGP\text{-}9.5^+$ $FITC^+$, (E) Substance P neurons; $DAPI^+$ $PGP\text{-}9.5^+$ SP^+, or SP^+ P airway neurons; $DAPI^+$ $PGP\text{-}9.5^+$ $FITC^+$ SP^+. Representative confocal microscopy images of (B) a nodose and jugular neuron, (D) an airway neuron, and (F) a SP airway neuron. Images were taken on a Zeiss LSM 510 confocal microscope using a $63\times/1.4$ oil immersion objective. A Z-stack (5 sections at 1 μm intervals) was taken of each neuron, flattened, and a median filter was applied using Zeiss AIM software. Contrast was adjusted to enhance detail.

Airway neurons ($DAPI^+PGP\text{-}9.5^+FITC^+$)

The number of airway neurons at PD6 was 83 (±35). Neuron numbers rose significantly to 439 (±51) on PD10. Airway neuron numbers remained significantly elevated relative to

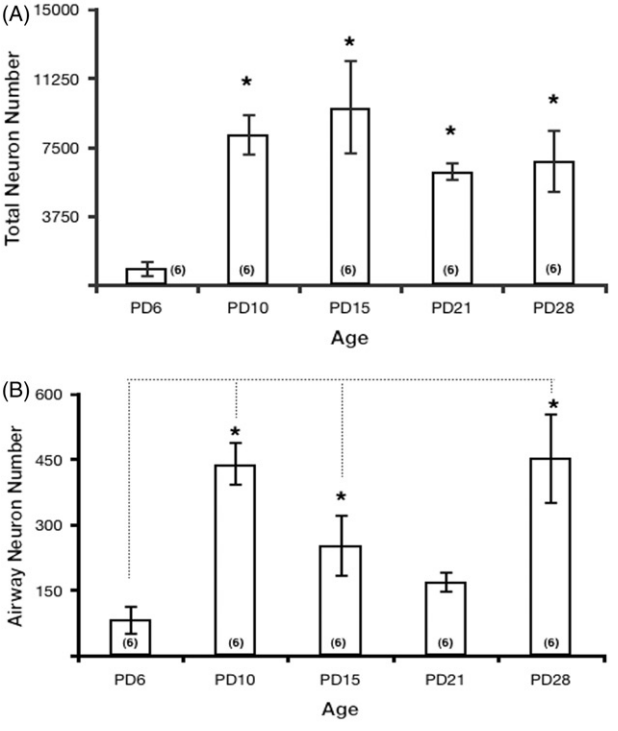

Figure 3. Age-specific dynamics of sensory nodose and jugular ganglia neurons in PD6, 10, 15, 21, and 28 rats. Two populations of nodose and jugular ganglia neurons were quantified in this study; total neurons and neurons that innervate the airway epithelium. All quantifications were done on the FASCSAria flow cytometer. Neuron numbers represent the average of all animals sampled for a particular age group. (A) Quantification of total nodose and jugular ganglia neurons ($DAPI^+$ $PGP\text{-}9.5^+$) found that PD6 rat pups significantly less total neurons than PD10, PD15, PD21, and PD28 animals ($^*p < 0.05$). (B) Neurons that innervated the airway were labeled using a green fluorescent retrograde tracer, identified as $DAPI^+PGP\text{-}9.5^+FITC^+$ and then quantified. PD10, PD15, and PD28 animals had significantly more neurons than PD6 ($^*p < 0.05$) rats. The number of airway neurons in PD21 animals was not significantly different from any other day examined. The *n* value for each age group is listed in parentheses.

PD6 on PD15 (253 ± 73) and PD28 (453 ± 105). PD21 (169 ± 26) was not significantly different from any other age (Figure 3B). Altogether, these results indicate that a substantial increase in total nodose and jugular neurons and in airway neurons occurs during the early periods between PD6 and 10, and that neuron numbers stabilize from PD10 through PD28.

Ozone or filtered air exposure

On PD5, rat pups were instilled with RetroBeads^{TM}, exposed to O_3 or FA, then nodose and jugular ganglia sensory neurons were isolated and counted 5, 10, 16, and 23 days after exposure to elucidate if a single acute O_3 exposure might affect sensory neuron development (Figure 1B).

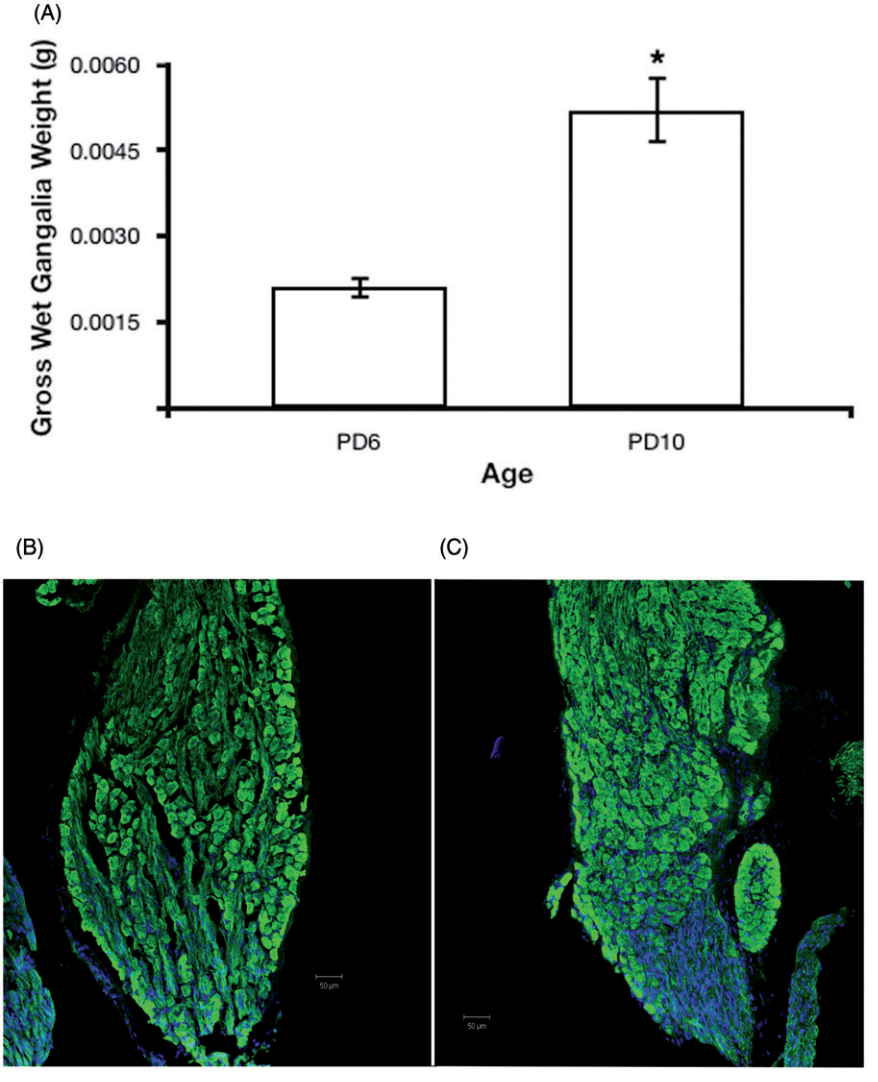

Figure 4. Differences in nodose and jugular ganglia gross wet weight and neuron distribution between PD6 and PD10 rats. Ganglia were removed from PD6 ($n = 3$) and PD10 ($n = 3$) animals, weighed, and immunohistochemically processed for PGP-9.5 and counterstained with DAPI. (A) Ganglia from PD10 rats weighed significantly more than ganglia from PD6 pups (*=$p < 0.05$). (B–C) The significant and quantitative increase in the number of total nodose and jugular ganglia neurons between PD6 and PD10 were visually confirmed in tissue sections. PD10 (C) rats had more densely packed neurons than PD6 (B) animals. Images were collected on a Zeiss LSM 510 confocal microscope using a 10×/0.30 objective. Z-stacks (5 sections at 1 μm intervals) were taken of representative sections, flattened, and a median filter was applied using Zeiss AIM software. Contrast was adjusted to enhance detail.

Total nodose and jugular ganglia neurons ($DAPI^{+}PGP\text{-}9.5^{+}$)

Total neuron numbers in PD21 rat pups exposed to O_3 (2845 ± 1132; $n = 9$) were significantly less than the number of neurons seen in PD21 FA-exposed rat pups (10542 ± 3150; $n = 7$). Although no other significant differences were seen between groups,

the total neuron numbers in O_3-exposed animals were less than the numbers noted in FA-exposed animals at PD15, 21, and 28 (Figure 5A). Ganglia removed from PD 21 animals exposed to both FA ($n = 3$) and O_3 ($n = 3$) revealed that ganglia from O_3-exposed animals weighed less than ganglia from FA controls (0.0055 g and 0.0095 g, respectively, Figure 6A). Through visual inspection of tissue sections it was noted that ganglia removed from O_3-exposed rats appeared to have fewer neurons in the central regions of the ganglia (Figure 6B and C). Thus, O_3 exposure resulted in a marked reduction in total neuron number in the entire nodose and jugular ganglia compared to FA controls.

SP^+ neurons ($DAPI^+PGP^+SP^+$)

The number of SP^+ neurons in the nodose and jugular ganglia of the O_3 group were less than the number of SP^+ neurons in the FA group at every time point except PD28 (Figure 5B). Although no significant differences were observed at any postnatal age, the difference at PD21 rose numerically, (FA-exposed, 1938 ± 793, $n = 7$ and O_3-exposed, 525 ± 129, $n = 8$), suggesting a possible reduction in total SP neuron numbers after O_3 exposure.

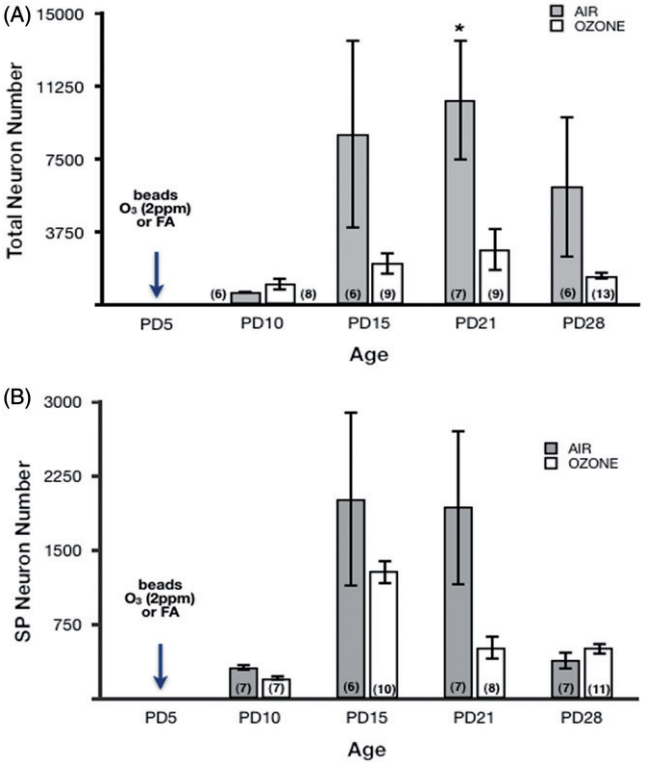

Figure 5. Effect of acute postnatal ozone exposure on total nodose and jugular neuron number and total SP containing neuron number in rats. Rat pups were exposed to O_3 (2 ppm) or FA for 3 h on PD5, and then euthanized on PD10, 15, 21, or 28. The number of nodose and jugular ganglia neurons and the number of SP-containing neurons were quantified. All quantifications were done on the FASCSAria flow cytometer. Neuron numbers represent the mean of all animals sampled for a particular age group. (A) On PD21, the number of total neurons in O_3-exposed animals on was significantly less than the neuron number in the FA group ($*p < 0.05$). (B) There were no significant differences between exposure groups in the number of SP neurons. The n value for each age group is listed in parentheses.

Airway neurons (DAPI$^+$PGP-9.5$^+$FITC$^+$)

Although no significant differences were found in airway neuron numbers between the O_3 and FA groups at any time point, the number of airway neurons in O_3-exposed animals was quantitatively lower than airway neuron numbers seen in FA controls at PD10, 15, and 21, (Figure 7A). While airway neuron numbers in both exposure groups were maximally increased at PD 21, as was the case with the number of total nodose and jugular

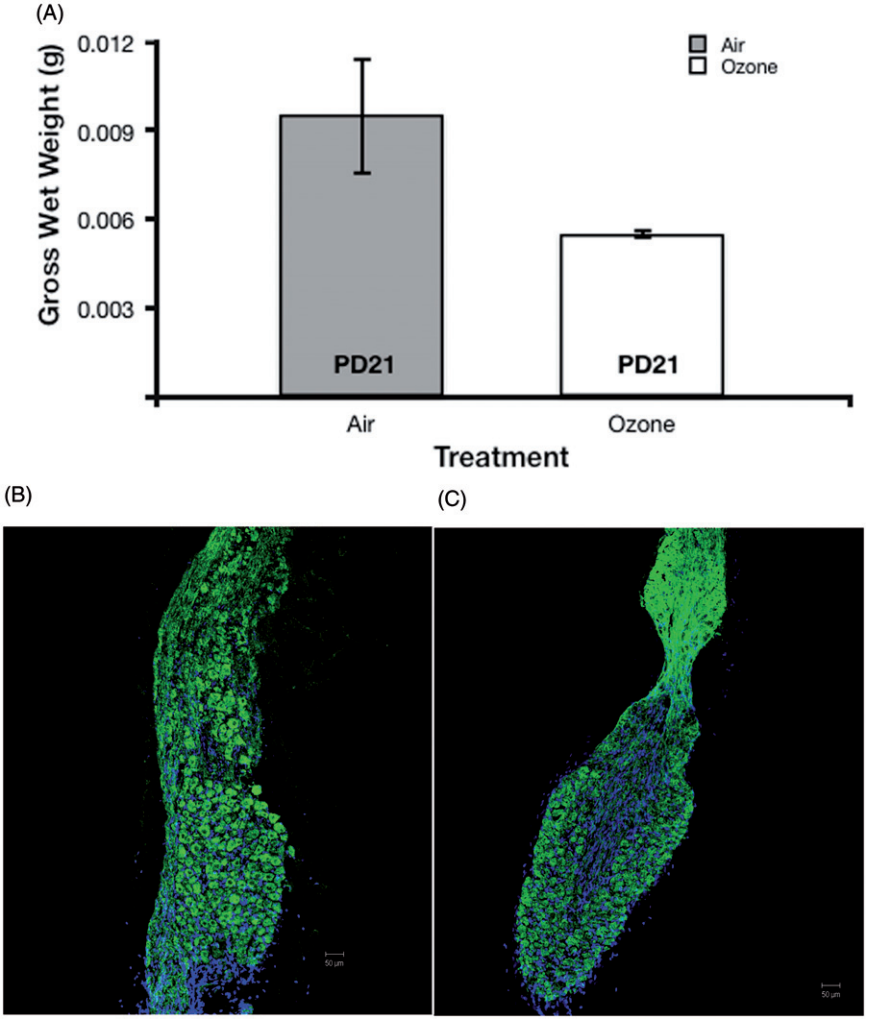

Figure 6. Differences in nodose and jugular ganglia gross wet weight and neuron distribution in PD21 rats following O_3- or FA-exposure on PD5. Rat pups were exposed to O_3 (2 ppm; $n = 3$) or FA ($n = 3$) for 3 h on PD5, and then euthanized on PD21. Nodose and jugular ganglia were removed, weighed, and immunohistochemically processed for PGP-9.5 and counterstained with DAPI. (A) Ganglia from PD21 animals exposed to FA weighed more than the ganglia removed from animals exposed to O_3. (B-C) PD21 animals exposed to O_3 on PD5 appeared to have fewer neurons in the central region of the ganglia when compared with FA-exposed rats. Images were collected on a Zeiss LSM 510 confocal microscope using a 10×/0.30 objective. Z-stacks (5 sections at 1 µm intervals) were taken of representative sections, flattened, and a median filter was applied using Zeiss AIM software. Contrast was adjusted to enhance detail.

neurons, a single early postnatal O_3 exposure did not produce a similarly large decrease in airway neuron number.

SP^+ airway neurons ($DAPI^+PGP^+FITC^+SP^+$)

The number of SP^+ airway neurons in O_3-exposed groups was not markedly different from the numbers found in FA groups (Figure 7B). However, at PD28, 23 days post exposure, the number of SP^+ airway neurons in the O_3 group (41 ± 9; $n = 14$) was quantitatively greater than in the FA-exposed group (18 ± 6). Data indicate that an acute, early postnatal O_3 exposure might play a role in sustaining a specific subpopulation of airway neurons known to play an important role in inflammatory responses in the airways.

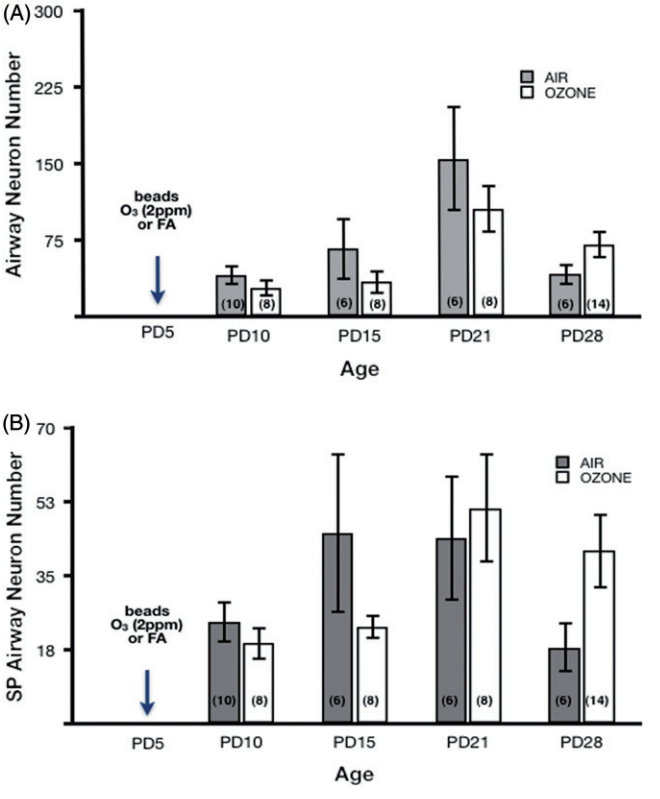

Figure 7. Effect of acute postnatal ozone exposure on airway neuron number and SP-containing airway neuron number in rats. On PD5, rat pups were intratracheally instilled with fluorescent microspheres, to identify neurons in the nodose and jugular ganglia that innervate the airway epithelium. On the same day, animals were exposed to O_3 (2 ppm) or FA for 3 h, and then euthanized on PD10, 15, 21, or 28. The number of airway neurons contained in the nodose and jugular ganglia and the number of SP airway neurons were quantified. All quantifications were done on the FASCSAria flow cytometer. Neuron numbers represent the mean of all animals sampled for a particular age group. (A) No significant difference in airway neuron number was found between O_3- and FA-exposed groups. (B) Quantification of SP airway neurons found no significant differences between exposure groups. The *n* value for each age group is listed in parentheses.

Discussion

The results of this study show that the number of labeled airway sensory neurons and the total number of neurons of the nodose and jugular ganglia during early postnatal development increase between PD6 and 10 and then generally stabilize. Animals exposed to O_3 early in postnatal life show a quantitative reduction in total nodose and jugular ganglia neurons on PD 15, 21, and 28, compared to FA controls. Airway neuron numbers in O_3-exposed animals were also quantitatively decreased compared to FA control animals. The number of total SP-containing neurons was no different from numbers found in FA controls. The number of SP-containing airway neurons in O_3 exposed rats was increased on PD28 (23 days post exposure), while in FA controls the number of SP^+ airway neurons at the same time point was decreased.

Use of flow cytometry for neuron counts

Traditionally neuron counts are conducted using immunohistochemistry in conjunction with confocal microscopy, followed by the analysis using stereology software. For this study, however, flow cytometry was chosen as the preferred counting method for several reasons. First, flow cytometry allowed us to count whole, three-dimensional (3-D) neurons, as opposed to the two-dimensional (2-D) counting that is accomplished with confocal microscopy. The advantage of counting neurons in 3-D is that the chances of counting the same neuron twice are virtually eliminated. Second, by counting neurons using flow cytometry, the sample throughput is increased exponentially. The process of preparing neurons for counting essentially requires the same amount of time as it would to prepare sections for counting, however 40 samples or more can be analyzed in a single day using flow cytometry as opposed to weeks it might take to count neurons from the same number of animals using tissue sections. A third advantage of flow cytometry is that human bias is virtually removed from counting. Instead of a person deciding if a neuron is positive, as is the case in counting by eye, neurons pass a highly sensitive detector that determines if fluorescence is higher than unstained and positive control samples. One final reason for employing flow cytometry in this study is because it allowed for multiple neuronal markers to be analyzed simultaneously in a single sample. However, possible limitations of this technique that could lead to potential variation in results are that 100% of airway neurons will neither be labeled with Retrobeads^{TM} nor will 100% of neurons be recovered following ganglia dissociation. Additional limitations include variation in dissecting techniques and in antibody labeling due to disparities in manufacturing. Nevertheless, the advantages far outweighed the positives of using traditional sectioning to determine neuron number in the nodose and jugular ganglia.

Age specific changes in total nodose and jugular ganglia neuron number

The total number of neurons determined in this study differ from the numbers found in studies by Bakal and Wright (1993) and Cooper (1984). Bakal and Wright (1993) found that total neuron number in the nodose ganglion decreased between PD1 and 14, while Cooper (1984) noted that nodose neuron numbers remained constant from birth. This study showed that neuron numbers rise between PD6 and PD10, and then stabilize (Figure 3A). Both of the previous studies used microscopy techniques to estimate total neuron number. In this study, neurons from the nodose and jugular ganglia were isolated and counted individually, using flow cytometry, allowing for more exact counting and

decreased stereological bias. Because neuron isolation was used in this study, no distinction was made between nodose and jugular ganglia, which is the most likely explanation for the overall larger neuronal number in our study.

Age specific changes in airway neuron number

The utilization of retrograde tracing to identify neurons innervating the airway epithelium in this study allowed for the examination of a specific neuronal population that is known to mediate defensive and inflammatory responses in the lung, including the release of SP and neuronal reflexes (Lindsay and Harmar 1989; Lee and Pisarri 2001). This is the first time the number of this subpopulation of neurons was examined *in vivo* in the context of postnatal development.

The significant increase in airway neuron number between PD6 and PD10 (Figure 3B) might be interpreted in several different ways. The first is that neuron proliferation is occurring and that new neurons send new axons to the airway epithelium where they are subsequently labeled with Green Retrobeads. However, this seems to be an unlikely explanation because the growth and development of a new, airway-innervating neuron that would be retrogradely labeled with beads would take longer than the maximum 4 days. Yet, given the quantitative increase in airway neuron numbers, this possibility should not be discounted and requires further study. A second, and more likely possibility is that the rise in neuron number may be attributed to neurons, present in the nodose and jugular ganglia at PD6, whose axons were destined to innervate the airway epithelium but had not yet reached that target until PD10. If the axons were not yet in the epithelium to take up the beads, the cell body would not be labeled when analyzed at PD6 but would be labeled when neuron counts were performed at PD10.

Changes in total neuron number following early postnatal acute O_3 exposure

This study determined that the total number of neurons in the nodose and jugular ganglia of animals exposed to O_3 on PD5 was markedly lower than the neuron number found in FA controls at all ages and significantly lower at PD21 (Figure 5A). Despite this sizeable reduction in neuron number, no marked differences were noted between FA- and O_3-exposed rats in either body weight or behavior (feeding, grooming, activity, etc.). One possible explanation for this significant decrease might be that neurons in the nodose and jugular ganglia also innervate other organs including the heart and stomach. The large fall in neuron number might be attributed to a reduction in neurons innervating those organs. While the studies conducted here focused on the effects of an inhaled O_3 exposure, as animals are placed in an exposure chamber, systemic O_3 exposure is unavoidable. O_3 entering the GI tract may directly interact with nodose and jugular afferent fibers, which might adversely affect neuronal survival. This systemic O_3 exposure might result in cell death, which may be a possible explanation for the significant fall in total neuron number found in this study.

Changes in SP^+ neuron number following early postnatal acute O_3 exposure

The number of SP^+ nodose and jugular neurons in O_3-exposed animals was quantitatively lower than the number seen in FA-exposed controls at PD 10, 15, and 21 (Figure 5B). The

lack of change in total number of SP^+ neurons between FA controls and O_3-exposed animals was expected, as the SP^+ neurons are a small subpopulation of the total neurons.

Changes in airway neuron number following early postnatal acute O_3 exposure

The number of nodose and jugular airway neurons in O_3-exposed rat pups were numerically lower than neuron numbers quantified in FA controls (Figure 7A). Although this result was unexpected, it is particularly interesting when taking into account the quantitative decrease in total nodose and jugular neuron number that was seen following O_3 exposure. Our finding that early postnatal O_3 exposure lowered total neuron number, which had no marked effect on airway neuron number, may indicate that airway neurons possess protective mechanisms allowing them to survive following O_3, while non-airway neurons are more adversely affected.

Changes in SP^+ airway neuron number following early postnatal O_3 exposure

No significant difference in SP^+ airway neuron number was found between FA controls and O_3-exposed rat pups (Figure 7B), however the quantitative increase on PD28 in SP^+ airway neurons 23 days post O_3 exposure is noteworthy. It may lend insight into why Hunter et al. (2011) found a rise in the % SP^+ airway neurons in rat pups administered Nerve Growth Factor (NGF) on PD6 and later exposed to O_3 on PD28. O_3 exposure increased both NGF measured in bronchoalveolar lavage fluid and relative levels of NGF mRNA in tracheal epithelial cells (Hunter et al. 2011). In sensory neurons, neuropeptide gene and peptide expression is upregulated by NGF (Lindsay and Harmar 1989). The quantitative changes in SP^+ airway neurons following acute early postnatal O_3 might be mediated by alterations in the amount of NGF present in the airway at developmentally critical periods. While experiments determining how instillation of NGF into the airways affects the number of both airway neurons and SP^+ airway neurons need to be conducted, the results of this study may play a role in helping to elucidate the intermediary steps in the mechanism of O_3-induced changes in sensory airway innervation that are occurring during postnatal airway development.

Overall, this study shows that the development of sensory neurons in nodose and jugular ganglia is a dynamic process that is not only occurring in an age-related manner, but might also be altered by acute O_3 exposure in early postnatal life.

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References

Bakal, R.S., and L.L. Wright. 1993. Postnatal neuron death in the nodose ganglia of the rat. *Developmental Neuroscience* 15: 22–6.

Carr, M.J., D.D. Hunter, D.B. Jacoby, and B.J. Undem. 2002. Expression of tachykinins in nonnociceptive vagal afferent neurons during respiratory viral infection in guinea pigs. *American Journal of Respiratory and Critical Care Medicine* 165: 1071–5.

Cooper, E. 1984. Synapse formation among developing sensory neurons from rat nodose ganglia grown in tissue culture. *Journal of Physiology* 351: 263–74.

Danneman, P.J., and T.D. Mandrell. 1997. Evaluation of five agents/methods for anesthesia of neonatal rats. *Laboratory Animal Science* 47: 386–95.

Dey, R.D., J.B. Altemus, I. Zervos, and J. Hoffpauir. 1990. Origin and colocalization of CGRP- and SP-reactive nerves in cat airway epithelium. *Journal of Applied Physiology* 68: 770–8.

Fischer, A., G.P. McGregor, A. Saria, B. Philippin, and W. Kummer. 1996. Induction of tachykinin gene and peptide expression in guinea pig nodose primary afferent neurons by allergic airway inflammation. *Journal of Clinical Investigation* 98: 2284–91.

Harding, R., K.E. Pinkerton, and C.G. Plopper, eds. 2004. *The lung: Development, aging and the environment*. London: Elsevier Academic Press.

Helke, C.J., and K.M. Hill. 1988. Immunohistochemical study of neuropeptides in vagal and glossopharyngeal afferent neurons in the rat. *Neuroscience* 26: 539–51.

Hunter, D.D., L.A. Carrell-Jacks, T.P. Batchelor, and R.D. Dey. 2011. Role of nerve growth factor in ozone-induced neural responses in early postnatal airway development. *American Journal of Respiratory Cell and Molecular Biology* 45(2): 359–65.

Hunter, D.D., and R.D. Dey. 1998. Identification and neuropeptide content of trigeminal neurons innervating the rat nasal epithelium. *Neuroscience* 83: 591–9.

Hunter, D.D., and B.J. Undem. 1999. Identification and substance p content of vagal afferent neurons innervating the epithelium of the guinea pig trachea. *American Journal of Respiratory and Critical Care Medicine* 159: 1943–8.

Hunter, D.D., Z. Wu, and R.D. Dey. 2010. Sensory neural responses to ozone exposure during early postnatal development in rat airways. *American Journal of Respiratory Cell and Molecular Biology* 43: 750–7.

Kajekar, R., E.M. Pieczarka, S.M. Smiley-Jewell, E.S. Schelegle, M.V. Fanucchi, and C.G. Plopper. 2007. Early postnatal exposure to allergen and ozone leads to hyperinnervation of the pulmonary epithelium. *Respiratory Physiology & Neurobiology* 155: 55–63.

Kalia, M., and M.M. Mesulam. 1980a. Brain stem projections of sensory and motor components of the vagus complex in the cat: I. The cervical vagus and nodose ganglion. *Journal of Comparative Neurology* 193: 435–65.

Kalia, M., and M.M. Mesulam. 1980b. Brain stem projections of sensory and motor components of the vagus complex in the cat: Ii. Laryngeal, tracheobronchial, pulmonary, cardiac, and gastrointestinal branches. *Journal of Comparative Neurology* 193: 467–508.

Kwong, K., and L.-Y. Lee. 2002. PGE(2) sensitizes cultured pulmonary vagal sensory neurons to chemical and electrical stimuli. *Journal of Applied Physiology* 93: 1419–28.

Kwong, K., Z.X. Wu, M.L. Kashon, K.M. Krajnak, P.M. Wise, and L.Y. Lee. 2001. Chronic smoking enhances tachykinin synthesis and airway responsiveness in guinea pigs. *American Journal of Respiratory Cell and Molecular Biology* 25: 299–305.

Larson, S.D., E.S. Schelegle, W.F. Walby, L.J. Gershwin, M.V. Fanuccihi, M.J. Evans, J.P. Joad, B.K. Tarkington, D.M. Hyde, and C.G. Plopper. 2004. Postnatal remodeling of the neural components of the epithelial-mesenchymal trophic unit in the proximal airways of infant rhesus monkeys exposed to ozone and allergen. *Toxicology and Applied Pharmacology* 194: 211–20.

Lee, L.Y., K. Kwong, Y.S. Lin, and Q. Gu. 2002. Hypersensitivity of bronchopulmonary c-fibers induced by airway mucosal inflammation: Cellular mechanisms. *Pulmonary Pharmacology and Therapeutics* 15: 199–204.

Lee, L.Y., and T.E. Pisarri. 2001. Afferent properties and reflex functions of bronchopulmonary c-fibers. *Respiratory Physiology* 125: 47–65.

Lindsay, R.M., and A.J. Harmar. 1989. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature* 337: 362–4.

Lundberg, J.M., A. Saria, E. Brodin, S. Rosell, and K. Folkers. 1983. A substance p antagonist inhibits vagally induced increase in vascular permeability and bronchial smooth muscle contraction in the guinea pig. *Proceedings of the National Academy of Sciences of USA* 80: 1120–4.

Maggi, C.A., A. Giachetti, R.D. Dey, and S.I. Said. 1995. Neuropeptides as regulators of airway function: Vasoactive intestinal peptide and the tachykinins. *Physiological Reviews* 75: 277–322.

Pham-Huy, L.A., H. Hua, and C. Pham-Huy. 2008. Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science* 4: 89–96.

Ueno, I., M. Hoshino, T. Miura, and N. Shinriki. 1998. Ozone exposure generates free radicals in the blood samples *in vitro*. Detection by the esr spin-trapping technique. *Free Radical Research* 29: 127–35.

Wang, L., and K.E. Pinkerton. 2007. Air pollutant effects on fetal and early postnatal development. *Birth Defects Research Part C: Embryo Today* 81: 144–54.

Wu, Z.X., B.E. Satterfield, and R.D. Dey. 2003. Substance p released from intrinsic airway neurons contributes to ozone-enhanced airway hyperresponsiveness in ferret trachea. *Journal of Applied Physiology* 95: 742–50.