VENTILATORY EFFECTS OF SUBSTANCE P-SAPORIN LESIONS IN THE NUCLEUS TRACTUS SOLITARII OF CHRONICALLY HYPOXIC RATS

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RUNNING HEAD: Ventilatory effect of NK1R Lesion in the NTS

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Abstract

During ventilatory acclimatization to hypoxia (VAH), time-dependent increases in ventilation lower $P_{CO_2}$ levels and this persists upon return to normoxia. We hypothesized that plasticity in the caudal Nucleus Tractus Solitarii (NTS) contributes to VAH, as the NTS receives the first synapse from the carotid body chemoreceptor afferents and also contains $CO_2$-sensitive neurons. We lesioned cells in the caudal NTS containing the Neurokinin 1 Receptor (NK1R) by microinjecting the neurotoxin saporin conjugated to Substance P and measured ventilatory responses in awake, unrestrained rats 18 days later. Lesions did not affect hypoxic or hypercapnic ventilatory responses in normoxic control rats, in contrast to published reports for similar lesions in other central chemosensitive areas. Also, lesions did not affect the hypercapnic ventilatory response in chronically hypoxic rats ($P_{O_2} = 90$ Torr for 7 days). These results suggest functional differences between central chemoreceptor sites. However, lesions significantly increased ventilation in normoxia or acute hypoxia in chronically hypoxic rats. Hence, chronic hypoxia increases an inhibitory effect of NK1R neurons in the NTS on ventilatory drive, indicating that these neurons contribute to plasticity during chronic hypoxia although such plasticity does not explain VAH.

Keywords: chemosensitivity, Substance P conjugated saporin, ventilatory acclimatization to hypoxia
1. **Introduction**

Multiple areas of the brainstem have been established as CO₂-sensitive by both stimulation and lesion experiments (3-5, 11, 29, 36, 42-44, 47). The organization and significance of these multiple chemoreceptor sites is a major question in this field, and different sites may play different roles in controlling ventilation or the cardiovascular system during different states (19, 38, 41). We hypothesized that CO₂-sensitive chemoreceptors in the Nucleus Tractus Solitarii (NTS) play a role in ventilatory acclimatization to chronic hypoxia.

This hypothesis is based on several published observations. First, chronic hypoxia changes the ventilatory response to CO₂ (60) and the NTS contains CO₂-sensitive cells (11, 14, 43). Also, the NTS receives the first synapse from O₂-sensitive afferents from the carotid bodies (15, 21, 22, 33, 59) and this could lead to hypoxic or activity-dependent plasticity that has been demonstrated in the central nervous system with chronic hypoxia (16, 49, 62). The NTS is therefore well suited to integrate the increased hypoxic drive to breathe from the arterial chemoreceptors with the decreased drive to breathe from hypocapnia in chronic hypoxia.

To test the role of CO₂-sensitive chemoreceptors in the NTS to ventilatory acclimatization to hypoxia, we studied the effects of NTS lesions. Large lesions to the NTS are problematic because of the many sensory systems that synapse in the NTS, including not only the peripheral chemoreceptors, but also baroreceptors and pulmonary vagal afferents (15, 27). To quantify the importance of the NTS central chemoreceptor cells during chronic hypoxia, a lesion of only the CO₂-sensitive cells would ideally be performed. No unequivocal marker of chemoreceptive cells has been identified yet, but other groups have used the Neurokinin 1 Receptor (NK1R) as a candidate marker to lesion central chemoreceptors in the brainstem. All the putative central chemoreceptor areas contain cells expressing the NK1R, which is the receptor for Substance P (SP) (37).
The role of NK1R cells in CO₂-sensitivity is not known, but lesions specifically targeting the NK1R cells in other chemosensitive areas of the brainstem decrease the HCVR in rats and goats (20, 45, 46). Moreover, it has been shown that NK1R cell lesions in the NTS do not kill the animal (52). We made lesions to the NK1R-expressing cells in the NTS of rats and measured the effects on ventilatory responses to O₂ and CO₂ in conscious rats before or after acclimatization to hypoxia.

2. Methods

2.1 Experimental animals

Male Sprague-Dawley rats (290 – 400 g; Charles River) were housed in standard rat cages in a vivarium and fed ad libitum a standard rat diet. A 12:12-h light-dark cycle was maintained within the vivarium. All experiments were approved by the University of California, San Diego, Animal Care and Use Committee. The experiments conformed to national standards for the care and use of experimental animals as well as the American Physiological Society's "Guiding Principles in the Care and Use of Animals."

2.2 Experimental Groups

Animals were placed into one of four groups, depending on whether they were chemically lesioned or unlesioned and maintained in normoxia (N) or chronic hypoxia (CH). A stable form of Substance P conjugated to the neurotoxin saporin (SP-SAP, Advanced Targeting Systems, San Diego) was used to lesion NK1R expressing cells in the NTS (61). An 11 amino acid nonsense peptide conjugated to saporin (Blank-SAP, Advanced Targeting Systems, San Diego) was used as the control drug. For both N and CH, 11 Blank-SAP and 5 SP-SAP animals were used. A subset of these animals was measured in both N and CH (Blank n = 4; SP-SAP n =2). No significant differences in body weights between the groups were observed (Blank-SAP = 346.0 ± 1.65 g; SP-SAP = 360.4 ± 12.9 g).

2.3 Exposure to Chronic Hypoxia
Rats in the CH group were placed for 7 days in a hypobaric chamber maintained at 0.5 atm (380 mmHg), which approximates exposure to 10% inspired O2 in normobaric conditions at sea level and has been used extensively to study acclimatization in rats (e.g. 1). Animals, within individual cages, were placed into the hypobaric chamber, and the pressure was lowered from 1.0 to 0.5 atm over a 5-min period. The chamber was opened once daily for about 10 min for regular cage maintenance or when it was necessary to remove animals for experimentation. The hypobaric chamber was maintained in the same vivarium that housed the control animals.

2.4 Experimental Drugs

The NK1R is internalized following the binding of NK1R ligand (32), meaning that if the neurotoxin saporin is bound to SP it can enter and kill the cell (SP-SAP group). The Blank-SAP served as the control drug for these experiments, as the nonsense peptide should not bind to any receptors and the saporin will be unable to enter the cells. All animals received 200 nL injections of drug (0.013ng/nL), either SP-SAP or Blank-SAP, into the caudal NTS.

2.5 Surgical Preparation

Animals were initially anesthetized with 5% isoflurane in O2 and maintained under anesthesia with 2–2.5% isoflurane in O2. The skull was shaved and the skin sterilized with betadine and alcohol. The head was placed into a Kopf stereotaxic holder. To visualize the dorsal brainstem, a midline incision was made and the muscle retracted to expose the edge of the skull bone. The head was angled 45° nose-down and the dura cut at the point it connects with the skull. The calamus scriptorius (which corresponds to -14.3 mm caudal from Bregma) was then visible and used as a landmark for the injection sites (50). Microinjections were made (Nanopump, World Precision Instruments) with a glass micropipette with a 10-μm tip diameter. A total of 8 injections (25 nL each) were made, with the patency of the micropipette tested after each injection. The first set of injections was made 0.1 mm rostral to the calamus scriptorius bilaterally and the second
set 0.4 mm rostral. Injections were made at two depths (0.2 mm and 0.4 mm deep) at every injection site.

Following the injections, rats were maintained in normoxia for 11 days to allow for maximal cell killing, as determined by other groups (45) and confirmed by us in pilot studies. The CH group was then placed into the hypobaric chamber for an additional 7 days of hypoxia, while the N group remained in normoxia for 7 days. Most ventilatory measurements were taken as described below 18 days following the injection, but a subset of animals was measured in normoxia at 11 days before being placed in chronic hypoxia (Blank-SAP n = 4; SP-SAP n = 2).

Two days prior to ventilatory measurements, recovery surgery was performed under isoflurane to implant a temperature telemetry probe into the abdomen (G-2 Emitter, Respironics). Additionally, the femoral artery was cannulated with a custom catheter made of polyethylene tubing (PE-10 joined to PE-50). The catheter was tunneled under the skin to the shoulder area to allow access for blood gas sampling and to prevent the rat from damaging the tubing.

2.6 Ventilatory and Blood Gas Measurements

All ventilatory data was collected on awake, unrestrained animals using barometric plethysmography modified for continuous flow (1, 25). Briefly, barometric plethysmography records the pressure swings caused by the warming and expansion of air when it enters the lungs.

On the experimental day, rats were placed into a 7L, sealed Plexiglass chamber. A thermometer and humidity probe were sealed in the box (Physitemp Thermalert TH-5). An electronic gas-mixer (MFC-4, Sable) was used to regulate the inspired gas concentrations and provide high input impedance. The chamber gas concentrations were measured using a mass spectrometer (MGA 1100, Perkin-Elmer) with the gas exiting the chamber via a vacuum valve (m series, Nupro) to a vacuum pump. We did not use a reference chamber and compensated for changes in the pressure signal baseline by
adjusting the vacuum valve to maintain a chamber pressure near atmospheric level, monitored by water manometer after each change of inspired gases. Respiratory frequency (fR) was calculated directly from the ventilation-induced pressure swings (Validyne, MP45). Tidal volume (VT) was calculated using calibration pulses (1 mL) generated by using a gas-tight syringe and injecting air pulses into the chamber at a rate similar to the rats’ frequency following the experimental measurements. Ventilation (V\text{L}) was calculated as the product of fR and VT and normalized for the animal’s body weight. The protocol commenced following an acclimation period to the box which consisted of 35 minutes in either 21% O\text{2} for N rats and 10% O\text{2} for CH rats. Animals were exposed to 10 minutes of each experimental gas mixture before respiratory and arterial blood gas measurements were taken. Each animal was challenged with hypoxia (FIO\text{2} = 0.10) and 2 levels of hypercapnia (FICO\text{2} = 0.05 and 0.07) in addition to normoxia (FIO\text{2} = 0.21). After each gas mixture, the animal was returned to their baseline inspired O\text{2} concentration (21% O\text{2} for N rats and 10% O\text{2} for CH rats).

Arterial blood gases were measured immediately following 1-minute collection of ventilatory data, approximately 11 min into the gas exposure. Blood samples were taken no more than 4 times during the protocol, with a 0.2 mL sample taken each time. The samples were immediately analyzed on an Instrument Laboratory Synthesis GEM Premier 3000 blood gas machine (San Jose, CA), which returned arterial O\text{2} (P\text{aO2}), CO\text{2} (P\text{aCO2}), pH, and hematocrit (Hct) values.

2.7 Lesion Quantification

Following the last experimental measure, rats were transcardially perfused with 4% paraformaldehyde and the brainstems removed and post-fixed overnight in paraformaldehyde and then transferred to 30% sucrose and cryoprotected overnight. The brainstems were frozen and sectioned at 30 µm using a cryostat (Reichert Jung, Cryocut 1800). The area containing the NTS was then stained using the floating sections method
of immunohistochemistry for the NK1R. Primary antibody was applied overnight in a concentration of 1:1000 (Advanced Targeting Systems), followed by 2 hours of secondary antibody application (goat anti-rabbit conjugated to Cy-3; Jackson Immunoresearch). All incubations were done at room temperature on a lab shaker. The sections were then placed on slides and coverslipped using Vectashield mounting media plus DAPI to counterstain the nuclei (Vector Laboratories).

NK1R positive cells were counted in the NTS at four levels (-14.08, -13.8, -13.5, and -13.3 mm referenced to bregma). The most rostral section was outside of the targeted caudal NTS area. A subset of the sections was counted using a confocal microscope (Olympus FV-1000) where a 3D stack of images was collected and could be used to verify that NK1R immunoreactivity was surrounding a DAPI stained cell nucleus. These sections were also counted using a non-confocal fluorescent microscope (Nikon Eclipse E400). The cell counts were not significantly different between the two methods so the non-confocal microscope was used for quantification in the majority of the animals. To be considered an NK1R neuron, the cell had to be at least 10 μm large, have at least two processes, and the NK1 staining must surround a DAPI stained nuclei (criteria modified from (52)). Any rat that had less than 30% of the NK1R positive neurons than the average control rat was included as a lesioned animal. No differences were observed between the number of NK1R positive cells in the control N and CH rats, so all control rats were pooled for analysis. All pictures shown of the staining were taken using the Olympus FV-1000 confocal microscope.

2.9 Statistics

For each ventilatory (fR, VT, and V̇l) and blood gas (PaO₂, PaCO₂, pHₐ, Hct) variable a Mixed Factors ANOVA was performed. The between subjects variables were the presence or absence of NK1R cell lesion and chronic hypoxia. When testing the effect on the hypercapnic ventilatory response, percent inspired CO₂ (3 levels: 0, 5, and 7%) was the within subjects variable while percent inspired O₂ (2 levels: 10 and 21%) for
the hypoxic ventilatory response. All averages are expressed ± the standard error of the mean and p < 0.05 was considered significant. All statistical analysis was done using SPSS statistics software.

3. Results

3.1 Lesion Verification

In total, very few cell bodies in the NTS were stained for the NK1R. Most of the immunoreactivity was located on cell processes as illustrated in Fig. 1D-E. In the control animals, there was an average of 38.0 ± 1.7 cells in the three sections encompassing the target area of the caudal NTS. N and CH Blank-SAP injected animals were pooled as no significant differences in NK1R cell number was observed. NK1R cell number at all levels of the caudal NTS decreased with SP-SAP as shown in Fig. 1A. Only animals in which NK1R positive cell counts were ≤ 30% of control counts, i.e. less than 11 cells total, were included into the SP-SAP group (mean of 7.3 ± 1.1 cells). Representative staining is shown in Fig. 1D-F. Rostral to the obex, no difference in NK1R cell number was observed, indicating the lesion was localized to the caudal NTS where SP-SAP was microinjected (Fig. 1B, D-E).

3.2 Ventilatory Acclimatization in Chronically Hypoxic Rats

Ventilatory acclimatization was observed in the CH groups as fR, VT, and V̇t were all significantly increased in the CH groups (Figs. 2-3). Additionally PaCO₂ decreased significantly (Table 1) and hematocrit increased in the CH rats (N = 36.2 ± 4.4%; CH = 56.3 ± 3.1%) as reported previously (1).

3.3 Effect of SP-SAP Lesions on Ventilatory Sensitivity to CO₂

Contrary to our hypothesis, NTS lesions did not decrease ventilatory CO₂ sensitivity (Fig. 2). This conclusion is the same if the data is analyzed in terms of inspired or arterial P_{CO₂}, although there is a non-significant tendency for a decreased response after lesions in CH animals between 0% and 5% inhaled CO₂ when the data is analyzed versus arterial P_{CO₂} (cf. slopes on Fig. 2). There was a tendency for NTS lesions to
decrease \( \dot{V}t \) at the highest level of \( CO_2 \) in the normoxic control rats but in chronically hypoxic rats, NTS lesions tended to increase ventilation at all \( CO_2 \) levels (Fig. 2). The differences were primarily because of differences in \( fR \). Arterial \( P_{CO_2} \) was significantly decreased with lesions after chronic hypoxia breathing room air but differences were not significant with elevated \( CO_2 \) (Table 1).

3.4 Effect of SP-SAP Lesions on Ventilatory Sensitivity to \( O_2 \).

There was no effect of NTS lesions on the hypoxic ventilatory response (HVR) in normoxic rats (Fig. 3). However in chronically hypoxic rats, NTS lesions increased \( \dot{V}t \) significantly in room air and 10% \( O_2 \) (Fig. 3A). This difference was driven by an increased \( fR \) during chronic hypoxia in the lesioned animals (Fig. 3C) with no effect on \( VT \) (Fig. 3B). The results are similar when ventilatory data is plotted against \( PaO_2 \) instead of inspired \( P_{O_2} \) also (not shown). Arterial \( P_{CO_2} \) decreased significantly in these conditions too (Table 1), as expected for hyperventilation and considering that metabolic rate returns to control levels after 7 days of chronic hypoxia (1). Arterial \( P_{O_2} \) was not significantly different with NTS lesions although it tended to be lower in the lesioned animals with significantly lower \( PaCO_2 \) values (Table 1).

4. Discussion

4.1 Summary of Results

NK1R cell lesions in the caudal NTS had no effect on \( \dot{V}t \) in normoxic animals in room air, hypercapnic, or hypoxic gases. In contrast, lesion of the NK1R cells in the caudal NTS in chronically hypoxic rats resulted in hyperventilation during normoxic and hypoxic conditions. This hyperventilation was caused by an increased \( fR \) with no effect on \( VT \). There was a non-significant trend for \( \dot{V}t \) in hypercapnia to be higher with lesions in chronically hypoxic animals as well.

4.2 Critique of Method

The targeted cell killing method in this study has been used by several other laboratories (2, 18, 20, 41, 45, 46, 52, 57) and has been shown to specifically kill the
NK1R positive cells while not significantly decreasing the total number of cells in the injection site. We could not detect any differences in total cell number based on counts of DAPI stained nuclei and have no evidence of any effects of SP-SAP other than destroying NK1R positive cells.

We targeted the caudal NTS located under the area postrema as the location where the majority of CO₂ sensitive neurons in the NTS complex have been identified in vitro (13, 14). However, about 20% of chemoreceptors identified in medullary slices studied in vitro were located in the rostral NTS (13) and these would have been spared by our lesions. Hence, we cannot rule out that killing these other cells too would cause CO₂ sensitivity to decrease in the normoxic animals. On the other hand, NK1R immunoreactivity decreased in the Dorsal Motor Nucleus of the Vagus with SP-SAP lesions in the NTS of some animals, and this area contains CO₂-sensitive neurons also (48). Hence, our results show the effects of killing NK1R neurons primarily in the caudal NTS, which includes both CO₂-sensitive neurons and secondary neurons receiving afferent input from the arterial chemoreceptors that respond to O₂ and CO₂ (15, 21, 22).

The SP-SAP method of killing has been used by multiple lab groups specifically in the NTS, but to date only our group and one other (52) have quantified the number NK1R positive cells before and after lesion in this location. Potts et al. (47) found a greater absolute number of NK1R positive cells than we did but we used different antibodies, which may explain the differences. They had appropriate controls showing no fluorescence without the primary antibody, but the sensitivity of our primary antibodies could differ. Perhaps the most relevant observation is that they found significant physiological effects with lesions sparing only 10% to 25% of the NK1R positive neurons they identified (47). In contrast, we found no physiological effect with lesions sparing 19% of our neurons and have no evidence or reason to expect that the proportion
of neurons killed by our SP-SAP lesions is any different than the proportion we quantified with NK1R immunoreactivity. Our method gave reproducible results for counting NK1R positive neurons (cf. control animals had an average of 38.0 ± 1.7 cells) and was able to detect significant differences following our SP-SAP treatment (7.3 ± 1.1 cells, p > 0.05). Finally, we observed no correlations between the percentage deletion of NK1R positive neurons and changes in the hypercapnic ventilatory response when it was examined across individual animals.

Further evidence that the lesions we made should cause a significant decrease in the hypercapnic ventilatory response if these NK1R neurons are necessary is provided by studies using the same method in other areas of central CO₂-chemosensitivty. Nattie and Li (37) counted a similar number of NK1R positive neurons in the RTN (45 ± 12), and found significant effects with SP-SAP lesions that decreased NK1R positive cells to 21% of control. In contrast, we saw no effect with lesions leaving only 19% of control NK1R positive neurons in the NTS.

Although PaO₂ was not significantly different, it tended to be lower in the chronically hypoxic lesioned animals (Table 1). Also, PaCO₂ was significantly lower in these animals, suggesting that the lesions in chronically hypoxic rats may impair gas exchange. We observed no other evidence of adverse effects from the lesions, such as abnormal body temperature, grooming and appearance, or stools. However, it is important to consider if the lower PaO₂ may have provided a significantly greater stimulus for acclimatization to chronic hypoxia that could explain results between the groups.

Based on predictions from previous studies quantifying ventilation as a function of PaO₂ in conscious rats (1), the lower PaO₂ we measured in chronically hypoxic lesioned rats (5-6 Torr) would not increase ventilation as much as we observed. Extrapolating
between animals with and without lesions to make this prediction is reasonable considering that we observed no effect of the lesions on the slopes of the hypoxic ventilatory response (Fig. 3). Additionally, we have no evidence that the lesions affect metabolic responses to hypoxia, which could also influence the level of ventilation. In normoxic rats there were no differences in ventilation or PaCO\textsubscript{2} (Fig. 2 and Table 1, respectively), as expected if metabolic rate is similar with and without lesions. In chronically hypoxic rats, ventilation tends to be greater with lesions (Fig. 2) and PaCO\textsubscript{2} is less (Table 1), consistent with the expected change in PaCO\textsubscript{2} for increased ventilation and a constant metabolic rate.

### 4.3 Effect of the Lesion on the Ventilatory Sensitivity to CO\textsubscript{2}

The fact that NK1R cell destruction in the NTS does not diminish the hypercapnic ventilatory response (HCVR) in normoxia was unexpected. Lesions of the NK1R cells in both the RTN and medullary raphe, which are other sites of CO\textsubscript{2}-sensitivity, have been shown to decrease the HCVR (20, 41, 45). While the role of the NK1R cells in central CO\textsubscript{2}-sensitivity is unknown, the NK1R is present in every site of central chemosensitivity (37). The functional evidence for CO\textsubscript{2}-sensitivity in the NTS is the same as it is for other sites. CO\textsubscript{2}-sensitive cells have been identified in vitro (14) and stimulating the CO\textsubscript{2}-sensitive cells with either microinjected acetazolamide (11) or microdialized CO\textsubscript{2} (43) increases ventilation in rats. The lack of effect of our lesion could indicate that the NK1R positive cells are not involved in CO\textsubscript{2}-sensing in the NTS, they comprise only a portion of the chemoreceptor cells in the NTS (see Critique above), or they are redundant to CO\textsubscript{2}-sensitivity at other sites.

It is becoming increasingly clear that many cells in the brainstem have intrinsic chemosensitivity that can be modified by synaptic and/or gap junction connections with other cells. In fact the number of cells excited by CO\textsubscript{2} in the NTS increases in the presence of synaptic blockade, indicating the importance of synaptic input under normal
conditions (17). Potentially the NK1R positive cells could still be chemoreceptors but play a more important role during a different state that was not tested in these experiments. We tested our animals only in the awake state and there is some evidence for state-dependence of chemoreception. For instance, micordialysis of CO₂/H⁺ in the RTN and caudal ventrolateral medulla (Loeschcke’s area) increases ventilation in unanesthetized rats during wakefulness but not sleep (12, 30) while in the medullary raphe ventilation is increased only during sleep (44). We note that in similar experiments, microdialysis of CO₂/H⁺ into the NTS did increase ventilation in both sleep and wakefulness (43).

As we only targeted a small population of cells in the NTS, another possible explanation for the lack of effect of NK1R cell lesions in the NTS on the HCVR is that we may not have killed enough chemosensitive cells. Takakura et al (2008) found that 70% of the NK1R-Phox2B-positive cells in the RTN had to be destroyed to significantly decrease the HCVR. Other studies have found significant decreases in the HCVR with only 36 to 59% of the NK1R-positive cells in the RTN destroyed (45), but we used the more stringent criteria of 70% decreases in NK1R-positive cells for a successful lesion in the NTS (see Critique above). Focal inhibition with microinjections of muscimol in the NTS decreases the HCVR (40) and this treatment may be more effective because there are more GABAergic neurons that can be affected by this treatment compared to NK1R-positive cells being lesioned by our protocol. We do note that many processes were NK1R positive and if their cell bodies were in other brainstem areas they may not have been affected by our treatment. Additionally, other central chemosensitive sites and/or neurotransmitter mechanisms in the arterial chemoreflex pathway may compensate for the effects of NK1R lesions in the caudal NTS on the HCVR.

In chronically hypoxic rats, NK1R lesions in the caudal NTS caused a significant increase in \( \dot{V}_{l} \) and decrease in \( P_{aCO_2} \) while breathing room air. Also, there was a trend for these lesions to increase \( \dot{V}_{l} \) at all CO₂ levels (Fig. 2). These results are opposite of our
prediction that chronic hypoxia would increase ventilatory drive from NK1 cells in the NTS but they do indicate a role for NK1R cells in the NTS in ventilatory acclimatization to hypoxia.

4.4 Effects of lesions on the hypoxic ventilatory response (HVR)

Lesions had no effect on the HVR in normoxic rats (Fig. 3). This was surprising as the carotid sinus nerve afferents from carotid body chemoreceptors release SP in the caudal NTS in response to hypoxia (31, 56). However, after exposure to chronic hypoxia, NK1R lesions in the caudal NTS increased ventilation in both normoxia and hypoxia (Fig. 3). We note that the significant effects of the lesions in chronically hypoxic rats breathing room air are revealed in the HVR analysis using the very same normoxic control data (21% O2 and 0% CO2) that was used for the HCVR analysis. We observed this trend in the HCVR analysis, although it was not significant because variability in the 5% and 7% CO2 data decreased the interaction in ANOVA between chronic O2 level and drug effects. The significant increase in ventilation in chronically hypoxic, lesioned rats breathing room air, relative to control rats, is opposite what we predicted. As discussed above (4.2 Critique of Methods), we cannot completely rule out that the increased ventilation in the chronically hypoxic rats with lesions resulted from additional hypoxemia with gas exchange impairments from lesions, causing greater acclimatization to deeper hypoxia. However, we repeat that the magnitude of decrease in PaCO2 we observed (Table 1) is not easily explained by what is known about acclimatization in rats and our data here (cf. 4.2 Critique of Methods).

In CH rats, lesions increased both \( \dot{V}t \) and \( fR \) in room air and acute hypoxia. This is somewhat surprising because microinjection of SP into the caudal NTS of rats and rabbits increases \( \dot{V}t \) (9, 34, 35, 63, 64). Such an excitatory effect of SP is presumably acting by the carotid body chemoreflex pathway, as discussed above. Given the increased sensitivity to hypoxia of carotid bodies and afferent traffic in the carotid sinus nerve after
acclimatization to hypoxia (reviewed by reference (55)), one would have predicted a greater inhibitory effect on ventilation by blocking SP effects in chronically hypoxic rats. In contrast, we observed increased $\dot{V}_l$ with NK1R lesions.

4.5 Mechanisms of lesion effects

There is evidence for NK1R cells located in the caudal NTS being GABAergic inhibitory interneurons that might contribute to our results. NK1R and GABA$_A$ receptors co-localize in the NTS (8, 51, 53) and the inhibitory effect of somatosensory inputs on the baroreflex are blocked by GABA$_A$ or NK1 receptor antagonists in the caudal NTS (6, 54). NK1R cell lesions in the caudal NTS also abolish the inhibitory effect of somatosensory input on the baroreflex, although lesions do not affect the cardiovascular response to arterial chemoreceptor stimulation by cyanide (52). This agrees with our observation that NK1R lesions in the caudal NTS have no effect on the ventilatory response to arterial chemoreceptor stimulation in normoxic rats.

In contrast, NK1R lesions cause hyperventilation after acclimatization to hypoxia (Fig. 3), indicating a change in the effect of NK1R cells in the caudal NTS on ventilatory reflexes. While there is evidence for plasticity in GABA receptors during chronic hypoxia, it is not easy to explain the changes we observed with chronic hypoxia in terms of GABAergic neurotransmission. GABA$_A$ receptors in the pons are up-regulated during chronic hypoxia (23) but dissociated NTS neurons from chronically hypoxic rats exhibit decreased sensitivity to GABA$_A$ receptor blockers (58). Also, inhibiting GABA neurotransmission in the caudal NTS decreases ventilatory drive in chronically hypoxic rats breathing normoxic gas (10). This is opposite the increase in ventilatory drive predicted by our results if the NK1R lesions destroyed the inhibitory cells with GABA receptors too. However, not all GABA cells in the NTS contain the NK1R and further experiments are necessary to determine if the same population of interneurons with NK1R and GABA$_A$ receptors contribute to the effects observed in all of these studies.
Changes to other neurotransmitter systems on the NK1R positive cells could also explain the effects of the lesion in chronic hypoxia. There is evidence in the dorsal horn of the spinal cord during chronic pain that persistent stimulation causes an up-regulation of the NK1R and increased excitability of the NK1R positive neurons (7, 24, 26). The number of cell bodies in the NTS positive for the NK1R was unchanged during chronic hypoxia, but that does not preclude a change in receptor number or function during chronic hypoxia, a condition that similarly to chronic pain involves chronic SP release (31).

Because at least a subset of the NK1R cells in the NTS regulate cardiovascular reflexes, they might also play an important role in cardiovascular control during chronic hypoxia. Activation of the peripheral chemoreflex also activates sympathetic neural activity, which can cause vasoconstriction and increased blood pressure (28). Perhaps this population of NK1R cells is important in preventing sympathetic over-activation during chronic hypoxia. Blood pressure was not measured in our animals so the effect of the lesion on blood pressure during chronic hypoxia is unknown, although lesion of these cells during normoxia does not change resting blood pressure (2).

4.6 Perspectives and Significance

We were surprised that NK1R lesion in the caudal NTS produced no effect on the hypercapnic ventilatory response in normoxic control animals, in contrast to similar lesions in the RTN or raphe (20, 45, 46). Like other areas of central CO₂-sensitivity, the NTS can clearly contribute to a central CO₂-sensitive ventilatory response (38) but unlike other areas, it is not necessary for a normal ventilatory response to CO₂ in intact animals. This supports the idea that multiple sites of central chemosensitivity play different roles in different conditions and may be interdependent, even with peripheral chemoreceptors (19, 38, 39).

The significant increase in ventilation and decrease in PaCO₂ with NK1R lesions in the caudal NTS after chronic hypoxia, but not before, demonstrates plasticity in the CNS
mechanisms controlling breathing and regulating arterial P_{CO_2}. The direction of this effect is opposite from what we predicted, with lesions increasing instead of decreasing ventilation. The result is not easily explained by the loss of NK1R-positive neurons that function as CO_2-sensitive central chemoreceptors or interneurons responding to SP release from carotid body chemoreceptor afferents. Further experiments are necessary to distinguish between plasticity in central CO_2-sensitivity, the processing of afferent input from arterial chemoreceptors, or other integrative mechanisms. Also, the exact phenotype of the cells killed by the SP-SAP lesions and their role in ventilatory control circuits remains to be determined.
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**Figure Legends**

Figure 1: **A:** Quantification of NK1R positive cell number in the NTS in brain sections from 4 levels; the first 3 encompass the caudal NTS under the Area Postrema and the fourth section is in the rostral NTS. Only animals with at least a 70% decrease in NK1R positive cells with SP-SAP injected (dashed line) were considered lesioned. * denotes p < 0.05 from controls (solid line). **B:** Location of caudal NTS as shown in D - E, approximately -13.8 mm from Bregma. **C:** Location of rostral NTS as shown in F and F1, approximately -13.3 mm from Bregma. **D:** Control animal injected with Blank-SAP. **D1:** Higher magnification of caudal NTS of D. NK1R positive cell bodies (arrows) and processes present. **E:** Animal injected with SP-SAP. **E1:** Higher magnification of caudal NTS of E. No NK1R cell bodies present, some processes present. **F:** NTS Rostral to SP-SAP injection site. **F1:** Higher magnification of F. NK1R positive cell bodies (arrows) and processes present. For **D - F**, NTS is located inside white dashed line. Red staining denotes NK1R immunoreactivity and Blue the cell nucleus (DAPI). CC = Central Canal; NTS = Nucleus Tractus Solitarii; DMNV = Dorsal Motor Nucleus of the Vagus; AP = Area Postrema; 4V = Fourth Ventricle.

Figure 2: Effect of the NK1R cell lesion on the hypercapnic ventilatory response (HCVR) in normoxic (N) and chronically hypoxic (CH) rats as a function of inspired and arterial CO₂. SP-Saporin lesions (dashed line) in the caudal NTS had no effect on any component of the HCVR in N or CH.
Figure 3: Effect of the NK1R cell lesion (dashed lines) on the hypoxic ventilatory response (HVR) in normoxic (N) and chronically hypoxic (CH) rats. Lesions in the caudal NTS increased ventilation and frequency only in chronic hypoxia.
<table>
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<th>$\text{PaO}_2$</th>
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<td>$F_{\text{IO}_2} = 0.10$</td>
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<td>7.27 ± 0.01*</td>
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<td>$F_{\text{IO}<em>2} = 0.21 F</em>{\text{ICO}_2} = 0.07$</td>
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Values are means ± SE. N = normoxic control; CH = chronically hypoxic; SP = Stable Substance P conjugated saporin; Blank = blank conjugated saporin. *denotes p < 0.05 from normoxic value. # denotes p < 0.05 from Blank value.