Neurokinin-1 receptor-expressing neurons in the ventral medulla are essential for normal central and peripheral chemoreception in the conscious rat

Eugene Nattie and Aihua Li

Department of Physiology, Dartmouth Medical School, Lebanon, New Hampshire

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Nattie, Eugene, and Aihua Li. Neurokinin-1 receptor-expressing neurons in the ventral medulla are essential for normal central and peripheral chemoreception in the conscious rat. J Appl Physiol 101: 1596–1606, 2006. First published August 10, 2006; doi:10.1152/japplphysiol.00347.2006.—Neurokinin-1 receptor immunoreactive (NK1R-ir) neurons and processes are widespread within the medulla, prominently at central chemoreceptor sites. Focal lesions of NK1R-ir neurons in the medullary raphe or retrotrapezoid nucleus partially reduced the CO₂ response in conscious rats. We ask if NK1R-ir cells and processes over a wide region of the ventral medulla are essential for central and peripheral chemoreception by cisterna magna injection of SSP-SAP, a high-affinity version of substance P-saporin. After 22 days, NK1R-ir cell loss was −79% in the retrotrapezoid nucleus and −65% in the A5 region, which lie close to the ventral surface, and −38% in the medullary raphe and −49% in the pre-Bötzhinger complex/rostral ventral respiratory group, which lie deeper. Dorsal chemoreceptor sites, the caudal nucleus tractus solitarius and the A6 region, were unaffected. At 8 and 22 days, these lesions produced 1) hyperventilation during air breathing in wakefulness (−8%) and in non-rapid eye movement (NREM) (−9%) and rapid eye movement (−14%) sleep, as measured over a 4-h period; 2) a substantially reduced ventilatory response to 7% CO₂ by 61% in wakefulness and 46–57% in NREM sleep; and 3) a decreased ventilatory response to 12% O₂ by 40% in wakefulness and 35% in NREM sleep at 8 days, with partial recovery by 22 days. NK1R-ir neurons in the ventral medulla are essential for normal central chemoreception, provide a drive to breathe, and modulate the peripheral chemoreceptor responses. These effects are not state dependent.

substance P; control of breathing; retrotrapezoid nucleus; medullary raphe; A5

NEUROKININ-1 (NK1) RECEPTOR immunoreactive (NK1R-ir) neurons and processes are widespread within the brain stem (5, 18, 30, 37, 54). Locations that are rich in NK1R-ir include all known brain stem chemoreceptor sites and the pre-Bötzhinger complex (PBC) (14, 17), a site that is important in the generation of a normal respiratory-like rhythm in reduced preparations (see Ref. 12) and in normal awake (14) and sleeping rats (27) and goats (55).

The natural ligand for the NK1R, substance P, is a peptide neuromodulator with known involvement in the control of breathing. In reduced preparations, substance P has strong stimulatory effects on respiratory system output (44, 45) and on neurons within the PBC (15, 40, 59). In anesthetized rats, focal injection of substance P into the nucleus tractus solitarius (NTS) or the ventral medulla stimulates breathing, while focal application of a substance P antagonist in the ventral medulla inhibits breathing (6, 7).

Central chemoreceptor sites that detect increases in CO₂ or H⁺ and stimulate breathing are widely distributed in the brain stem (8, 12, 23, 31, 32, 34, 35, 39). Ventral sites include the medullary raphe (MR) with involvement of serotonergic neurons (47, 48), the retrotrapezoid nucleus (RTN) with involvement of glutamatergic neurons (19, 29), the A5 region with possible involvement of catecholaminergic neurons (22), and PBC/rostral ventral respiratory group (rVRG) (34). Dorsal sites include the locus ceruleus (A6) (22, 43), the caudal NTS (cNTS) (36), and the fastigial nucleus of the cerebellum (57, 58). NK1R-ir is strikingly present at all of these central chemoreceptor locations within the brain stem (30, 37). It is not known whether it is present within the fastigial nucleus of the cerebellum. Focal application of a substance P antagonist in the ventral medulla (6, 7) and focal lesions of NK1R-ir neurons in the PBC (14), the RTN (37), and the MR (39) each reduced the ventilatory response to increased CO₂.

Substance P and NK1Rs are also involved in the ventilatory response to hypoxia, which is mediated through the carotid body via the NTS with modulation by the RTN (51). Genetically modified mice, which do not express NK1Rs, have a reduced ventilatory response to hypoxia as adults (46), as do rats after focal application of a substance P antagonist in the ventral medulla (6, 7), suggesting that NK1R-expressing neurons within the ventral medulla participate in hypoxic chemoreception.

We hypothesize that NK1R-ir neurons and processes within the ventral medulla are essential for central and peripheral chemoreception and contribute to the drive to breathe under normal air-breathing conditions. To test this hypothesis, we produced a widespread lesion of NK1R-expressing neurons and processes within the ventral medulla by injection into the cisterna magna of SSP-SAP (high-affinity version of substance P-saporin) (56), a specific and potent toxin for NK1R-expressing neurons. We produced large lesions of NK1R-ir neurons in the ventral medulla of conscious rats, which reduced ventilation during air breathing and the ventilatory response to 7% CO₂ at 8 and 22 days after the injection and the ventilatory response to 12% oxygen at 8 days with partial recovery at 22 days. The lesions did not affect oxygen consumption, body temperature, the frequency of spontaneous deep breaths, or sleep cycling.

METHODS

General. The methods are essentially the same as in a recently published study from our laboratory (22), except that the injections of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
were of SSP-SAP into the cisterna magna. All procedures followed the guidelines of the National Institutes of Health for animal use and care and were approved by the Dartmouth College Institutional Animal Use and Care Committee. A total of 20 male Sprague-Dawley rats (250–350 g) were used, of which 13 completed the entire 4-wk protocol. There were two groups: SSP-SAP injection lesion (n = 7) and IgG-SAP injection control (n = 6). The rats were anesthetized with ketamine (100 mg/kg im) and xylazine (15 mg/kg ip). To monitor the depth of anesthesia, we tested hindlimb withdrawal and corneal reflexes, and responses resulted in administration of supplemental anesthesia in the form of one-fourth of the initial dose. The skull and a portion of the abdomen were shaved, and the skin cleansed with betadine and alcohol. The head was placed into a Kopf stereotaxic holder, and two EEG electrodes were screwed into the right side of the skull. One was over the frontal cortex (2 mm anterior to bregma and 2 mm lateral to midline), another was over the parietal cortex (3.5 mm posterior to bregma, 2 mm lateral), and an earth lead was placed between the two 3 mm lateral to midline. For the electromyogram (EMG), a pair of wire electrodes was threaded through the nuchal muscles. Through a separate incision, a sterile tetralogy temperature probe (TA-F20, Data Sciences, St. Paul, MN) was placed in the abdominal cavity. After at least 7 days of recovery and baseline data collection, each rat was reanesthetized with the same doses of ketamine and xylazine and received a microinjection of either SSP-SAP or IgG-SAP into the cisterna magna. A dorsal incision was made over the neck muscles, which were then retracted to expose the atlanto-occipital membrane. The microinjection was made using a 10-μl Hamilton syringe with a 28-gauge needle inserted into the cistern down and around the medulla to reach the ventral medullary region. The needle remained in position for another 5 min before removal. After each procedure, the wound was sutured.

Protocol. The rats were housed in a room in the Animal Research Facility with a light rest period from midnight to noon and a dark active period from noon to midnight. Food and water were available ad libitum. All of the experiments were performed between 8 AM and 3 PM. Each rat was studied over a period of 4–6 h. After at least 7–days recovery from the EEG/EMG placement, baseline ventilation (VE), tidal volume (VT), frequency (f), and oxygen consumption were measured in each rat while it breathed room air, 7% CO2 in air, and 12% O2 in nitrogen during sleep and wakefulness. Each rat was placed into the plethysmograph and allowed 30–40 min for acclimatization. Then breathing was recorded for 140–60 min of room air exposure followed by exposure to 7% CO2 for 40 min. The plethysmograph was then opened for a 60-min recovery period. The plethysmograph was again sealed, and 30-min room air exposure is followed by 30- to 40-min exposure to 12% O2. The lengths of exposure to the three gases allowed time for both awake and non-rapid eye movement (NREM) sleep periods. [These rats also cycle through rapid eye movement (REM) sleep, but in the rat these are very brief and quite variable during our relatively brief protocol; hence we will not report REM data except for the 4- and 24-h recording periods.] On a separate day, each rat was recorded over a period of 4 consecutive hours while breathing room air, with analysis of VE and of periodic deep breaths or sighs.

After obtaining these baseline data, each rat then received one injection (250–280 ng in 2.5–2.8 μl) of one of the following agents: mouse IgG-SAP or SSP-SAP (Advanced Targeting Systems, San Diego, CA). Ventilatory measurements were again made on days 8 and 22 after the injection. Sleep cycling was measured in a subset of rats during 24 h of air breathing during the preinjection baseline period and at 10 and 22 days after the injection. All rats also had a second 4-h period of room air breathing analyzed for VE and for periodic deep breaths or sighs. These were defined as a VT that was at least two times larger than any of the five previous breaths and were counted over a 4-h period of room air breathing in both NREM sleep and wakefulness. These 4- and 24-h periods occurred on separate days from the CO2 and hypoxic tests.

Data analysis. We determined the times of sleep and wakefulness using EEG and EMG electrode signals, the fast Fourier transform of the EEG signal analyzed in 3.6-s epochs at delta (0.5–3 Hz), theta (6–9 Hz), and sigma (10–17 Hz) frequency bands, and behavioral observations. We used criteria as previously described (37, 39). In our case, we measured awake breathing only during quiet wakefulness, as in active wakefulness the activity of the rat in the plethysmograph interferes with reliable measurement of breathing. For 24-h sleep cycling data and for analysis of sleep states in the 40– to 60-min and the 4-h periods of air breathing, we used Sleep Sign sleep analysis software (Kissei Comtec America) to determine the amount of time the rat spent in wakefulness and NREM and REM sleep. Ventilation was measured using a flow-through whole body plethysmography, as previously described (37, 39). The volume of the plethysmograph was 7.6 l, with a 3.5-liter top to protect the head pedestal. The plethysmograph was connected by a high-resistance leak to a similarly sized reference chamber. The inflow gas for the plethysmograph chamber was humidified and controlled by a flowmeter at a minimum of 1.4 l/min to prevent rebreathing of exhaled gas. This flow also helped to keep plethysmograph temperature constant. The outflow was matched to the inflow via a flowmeter connected to a vacuum system. Approximately 100 ml/min of outflow gas were sampled for O2 and CO2 analyzers (Applied Electrochemistry). We measured chamber pressure by transducer and calibrated the plethysmograph with multiple 0.3-ml injections. We measured chamber temperature by thermometer and rat body temperature by telemetry continuously.

For calculation of ventilatory data, we used the DataPac 2000 system to determine the pressure deflections and the respiratory cycle time for each of 400 or more breaths at defined sleep and wake periods breathing air, 7% CO2, and 12% O2. Sighs, sniffing, and recording artifacts were edited from analysis. These data were exported to SigmaPlot (Jandel Scientific software) with f, VT, and VE per 100 g body wt calculated for each breath using plethysmograph and body temperatures for that time period. We averaged data from two to four measurement periods for NREM sleep and wakefulness as a baseline before 7% CO2 and 12% O2 exposure and one or two periods during the 30–40 min of CO2 and 12% O2 exposure. In our two experimental groups, we compared VE, Vt, and f breathing air, 7% CO2, and 12% O2 in NREM sleep or wakefulness on baseline and on days 8 and 22. We defined the “CO2 response” as a change (Δ) in VE, which is VE breathing 7% CO2 minus that in air, and the “hypoxic response” as a ΔVE, which is VE breathing 12% O2 minus that in air. Oxygen consumption was calculated by the Fick principle using the difference in O2 content between inspired and expired gas and the flow rate through the plethysmograph and normalized to milliliters per grams body weight per hour. We monitored CO2 content of the outflow gas continuously to ensure that no buildup of CO2 took place within the chamber. In the 4-h air-breathing experiment, we summed VE values obtained in each state, wakefulness and NREM and REM sleep, over the 4-h period. Oxygen consumption was averaged from nine measurements made over the 4-h period. The box washout time was long enough to preclude assignment of oxygen consumption values to arousal states.

Anatomy. Our goal was to substantially reduce the numbers of NK1R-ir neurons and processes within a widespread area of the ventral medulla, an anatomical site that would require multiple tissue injections in vivo. We used a conjugate of the ribosomal toxin SSAP with SSP, a substance P-like peptide that has a higher affinity for the NK1R than endogenous substance P (56). The SSP binds to endogenous NK1Rs, the conjugate is internalized, the ribosomal toxin is released, and the cell is killed (56). We made our injections into the cisterna magna as the cerebrospinal fluid flows laterally from the cistern down and around the medulla to reach the ventral medullary surface. Our injections could also reach the cervical spinal cord. Given the knowledge regarding the presence of central chemoreception at the ventral medulla, we focused our attention at that site.
Twenty-three days after injection and following all physiological experiments, the rats were anesthetized with ketamine and an overdose of phenobarbital, and then they were transcardially perfused with 200 ml saline followed by 300–500 ml of chilled 4% paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4). The brain was removed and postfixed overnight in 4% paraformaldehyde at 4°C, and then it was cryoprotected for 48 h in 30% sucrose. The brains were sectioned at 30-μm thickness on a Leica cryostat for immunohistochemical staining of NK1R-ir and tyrosine hydroxylase (TH)-ir. All immunohistochemical procedures were performed by using free-floating sections at room temperature. We used 0.1 M phosphate buffer for NK1 and TH quenched by 3% H2O2 and then blocked with 5% normal goat serum. The sections were incubated with either a rabbit polyclonal antibody against NK1R (1:10,000; ATS CA; Chemicon), or a mouse monoclonal antibody against TH (1:10,000, Sigma) for 48 h at 4°C, followed by a biotinylated goat anti-rabbit IgG for NK1 or anti-mouse IgG for TH overnight at 4°C (1:500, Vector Laboratories, Burlingame, CT). An avidin-biotin-horseradish peroxidase procedure with diaminobenzidine was used to visualize NK1 and TH staining. These sections were used for all cell counts. All of the sections were mounted and dehydrated with graded alcohol (25–100% EtOH), cleared with xylene, and coverslipped.

Cell counting methods. Brain stem sections were cut at 30-μm thickness in a cryostat with one of every three successive sections stained for NK1R-ir or TH-ir. To evaluate the effects of our SSP-SAP injections into the cisterna magna on brain stem NK1R-ir neurons, we counted them in putative central chemoreceptor regions as follows. For all cell counts, we identified the caudal aspect of the facial nucleus and labeled that as −11.6 mm from bregma, an approach used by Guyenet and colleagues (18, 54). For the RTN, we counted all NK1R-ir cell bodies from −300 μm rostral to the rostral pole of the facial nucleus to 200 μm caudal to the caudal pole of the facial nucleus from just lateral to the pyramids to the lateral aspect of the facial nucleus (9, 37). We counted one section out of every three in six control and four lesioned rats.

For the MR, we counted one out of every three sections from −2,000 μm rostral to −2,000 μm caudal to the caudal end of the facial nucleus (39). We counted all NK1R-ir cell bodies within the midline and parapyramidal regions in six control and four lesioned rats (39).

For the cNTS, we counted NK1R-ir neurons, beginning at the rostral aspect of the area postrema and caudally for 720 μm (36). We counted one of every three sections in four control and five lesioned rats.

For the PBC/tVRG, we counted NK1R-ir neurons, as described by Wang et al. (54) and Guyenet et al. (18). They used a small rectangle to limit the area counted; we used a similarly sized circle (the area visualized by the ×20 objective). We counted every sixth section from −11.7 to −13.3 relative to bregma in four control and five lesioned rats.

For A6 and A5, the NK1R-ir pattern consisted largely of small, dense fibers with few cell bodies. Given that the catecholaminergic neurons in these sites express NK1Rs, we estimated our lesion effect by counting TH-ir neurons as TH marks neurons that synthesize catecholamines, which here express the NK1R. For A5, we again (22) used the approach of Byrum and Guyenet (4), counting cells in one out of every three sections within a small rectangle on the ventrolateral pons and medulla over −2 mm in four control and four lesioned rats. For A6, we counted cells in one out of every three sections over the entire length of the locus ceruleus, −1.5 mm, along the lateral margin of the fourth ventricle (22). We expressed all counts per 180 μm of rostral-to-caudal axis. While we cannot be certain that each catecholamine (CA) neuron in A5 and A6 contains NK1R-ir, it was more reliable to count the TH-ir-stained cells.

Statistics. Mean counts of somatic cell profiles of NK1R-ir neurons in each region of interest in each rat were compared between IgG-SAP and SSP-SAP groups by t-test. Within each region of interest, cell counts were compared between IgG-SAP and SSP-SAP-treated groups by a repeated-measures (RM) ANOVA, with treatment and distance along the rostral-caudal axis as the categorical variables.

We measure in each rat the VE (VT and f) as a RM variable over the experimental time course, baseline, and 8 and 22 days. First, we examine the effects of treatment (lesion vs. control), state (awake vs. NREM sleep), and gas (room air-CO2 or room air-hypoxia) as categorical variables. Since there was no state effect in any of the within-subjects analyses, we then examined ventilation (VT and f) as RM separately in the awake and NREM states, with treatment and gas (room air-CO2 or room air-hypoxia) as categorical variables. Significant interactions in the within-subjects analysis between ventilation and treatment, gas, or treatment × gas were followed by a post hoc analysis of VE values in room air or hypercapnia (hypoxia) at the different times comparing treatment to control using a Bonferroni correction for multiple comparisons. We also analyzed the effects of treatment and state on the CO2 and hypoxic responses measured as the ΔVE (CO2 or hypoxia-room air). In the absence of any significant interaction between ΔVE and state, we examined ΔVE as a RM separately in the awake and NREM state, with treatment as the categorical variable. Significant interaction in the within-subjects analysis between ΔVE and treatment allowed post hoc comparison between the ΔVE values at different times, with a Bonferroni correction for multiple comparisons. For the 4-h air-breathing experiments, ventilation or oxygen consumption were the RM variables, with treatment and state as categorical variables.

RESULTS

Oxygen consumption and body temperature. Body temperature and oxygen consumption did not differ significantly between the IgG-SAP and SSP-SAP groups on any day, nor did it differ with time in the 40- to 60-min or in the 4-h air-breathing experiment. For example, in the 40- to 60-min air-breathing experiment, baseline oxygen consumption in the SSP-SAP group was 1.11 ± 0.03 ml·100 g−1·h−1, and in the IgG-SAP group it was 1.12 ± 0.03 ml·100 g−1·h−1, and baseline body temperature was 37.7 ± 0.1°C in the control group and 37.6 ± 0.1°C in the SSP-SAP group. In the 4-h air-breathing experiment, oxygen consumption at baseline and 8 and 22 days in the IgG-SAP controls was 1.09 ± 0.04, 1.10 ± 0.08, and 1.05 ± 0.06 ml·100 g−1·h−1, while in the SSP-SAP lesion group it was 1.12 ± 0.03, 1.19 ± 0.03, and 1.06 ± 0.06 ml·100 g−1·h−1, respectively. Average body temperature was 38.0 to 38.3°C in the control group and 37.9 to 38.0°C in the SSP-SAP group at baseline and 8 and 22 days. During the exposure to 7% CO2, there was no significant difference in oxygen consumption between IgG-SAP controls and SSP-SAP-lesioned rats at baseline and 8 or 22 days, nor was there a difference in body temperature between the two groups. Oxygen consumption remained unchanged during the CO2 test.

During the exposure to 12% O2, there was no significant difference in oxygen consumption between the two groups, nor was there a difference during the exposure period. Body temperature decreased in both groups during the exposure period by 0.3–0.6°C, but there was no difference between the IgG-SAP and SSP-SAP groups. Oxygen consumption did not change during the 12% O2 exposure.

Cell counts. The lesion effects of our SSP-SAP injections into the cisterna magna were greatest in areas exposed to the cerebrospinal fluid overlying the ventral medulla. Figure 1 shows schematically the locations of our regions of interest with respect to NK1R-ir neurons and central chemoreception.
and the overall percent decrease in cell counts produced in each region by the SSP-SAP injections. In the RTN, the number of NK1R-ir neurons counted decreased 79% from 45 ± 12 (n = 6) in controls to 10 ± 4 (n = 4) (P < 0.001; t-test). In the MR, they decreased 38% from 167 ± 63 (n = 6) to 93 ± 21 (n = 4) (P = 0.038, ranked sum test). In the PBC/rVRG, they decreased 49% from 99 ± 7 (n = 4) to 50 ± 15 (n = 5) (P < 0.001; t-test). There was no significant effect on the number of NK1R-ir neurons in the cNTS. For the A5 and A6 regions, we estimated the lesion effect by counting TH-ir neurons, since these catecholaminergic neurons express NK1Rs, and there are few NK1R-ir cells but many processes. In the dorsal A6 region, there was no significant difference. In the ventral A5 region, the number of TH-ir neurons decreased 62% from 85 ± 10 (n = 4) to 33 ± 8 (n = 4) (P < 0.001; t-test).

The regions with greatest loss of NK1R-ir neurons, the A5 region and the RTN, lie closest to the ventral surface, the site of access to the brain stem of our injections into the cisterna magna. Figure 2 shows the details of the NK1R-ir neuron counts for the RTN (A) and the TH-ir neuron counts for the A5 region (B). For the RTN, the number of NK1R-ir neurons is significantly less in the SSP-SAP group compared with controls (P < 0.001, treatment effect; RM ANOVA). For the A5 region, the number of TH-ir neurons is significantly less in the SSP-SAP group compared with controls (P < 0.004; treatment effect, RM ANOVA). Of the regions with an intermediate NK1R-ir cell loss, the PBC/rVRG and MR, the PBC/rVRG lies deeper below the ventral medullary surface, while the MR has some neurons close to the ventral surface more rostrally and others that lie deeper more caudally.

Figure 3 shows the details of the NK1R-ir neuron counts for the MR (A) and the PBC/rVRG region (B). For the PBC/rVRG, the number of NK1R-ir neurons is significantly less in the SSP-SAP group compared with controls (P < 0.002; treatment effect, RM ANOVA). For the MR, the number of NK1R-ir neurons is significantly less in the SSP-SAP group compared with controls (P < 0.001; treatment effect, RM ANOVA). By inspection of Fig. 3A, it appears that the loss of NK1R-ir neurons is most pronounced at more rostral aspects of our cell count distribution in a region in which serotonergic neurons have greater proximity to the ventral surface. Since there was no significant interactive effect in the ANOVA, we cannot show this to be statistically significant. Our dorsal regions of interest, the cNTS and the A6 region, had no significant cell loss; the SSP-SAP did not appear to reach these neurons.

Breathing in air. Analysis of 4 h of continuous breathing in air with the rats spontaneously cycling through wakefulness and NREM and REM sleep allowed us to detect a significant decrease in V\textsubscript{E} in lesioned compared with control rats in all three states (Fig. 4). An ANOVA with V\textsubscript{E} as a RM and treatment and state as categorical variables showed an overall effect of state (P < 0.001), a significant RM effect on V\textsubscript{E} (P < 0.001), and a significant interaction of V\textsubscript{E} and treatment (P < 0.001). Oxygen consumption was not significantly different in
with wakefulness, but the effects of treatment or gas did not vary by state.

When only the awake data are examined, as shown in Fig. 5, absolute $V_e$ breathing 7% CO$_2$ was significantly lower in SSP-SAP-treated rats at both 8 and 22 days ($P < 0.01$; post hoc with Bonferroni adjustment; $P < 0.001$ interaction between treatment and gas breathed) as was $V_t$ and $f$ (data not shown).

Breathing in CO$_2$. We examined the results of the hypercapnia and hypoxia experiments separately. We studied $V_e$ as a RM over time with state (awake, NREM sleep), treatment (IgG-SAP, SSP-SAP), and gas (air, 7% CO$_2$) as categorical variables. In the CO$_2$ experiments, there was a significant effect of state as a categorical variable ($P < 0.001$), but there was no significant interaction between state and $V_e$ as a RM. In these experiments, $V_e$ was lower in NREM sleep compared...
The \( \dot{V_e} \) while breathing air was also significantly lower at 8 days in the SSP-SAP group \((P < 0.05; 76.3 \text{ vs. } 89.4 \text{ breaths/min})\).

When only the NREM data are examined, as shown in Fig. 5, absolute \( \dot{V_e} \) breathing 7% CO\(_2\) was significantly lower in SSP-SAP-treated rats at both 8 and 22 days \((P < 0.01; \text{ post hoc with Bonferroni adjustment}; P < 0.001 \text{ interaction with treatment})\) and \( \dot{V_T} \) \((P < 0.05)\) and \( f \) \((P < 0.01)\) (data not shown). The \( \dot{V_e} \) while breathing air was significantly lower at 8 days in the SSP-SAP group \((P < 0.05)\).

To emphasize the effects of SSP-SAP lesions on the CO\(_2\) response and to control for any independent variation in room air and CO\(_2\) stimulated values, we analyzed the change (\( \Delta \)) in ventilation, \( \dot{V_T} \), and \( f \), comparing these values in the CO\(_2\)-stimulated condition to the corresponding air-breathing condition. We examined \( \Delta \dot{V_e} \), \( \Delta \dot{V_T} \), and \( \Delta f \) separately as a RM over time, with state (awake, NREM sleep) and treatment (IgG-SAP, SSP-SAP) as categorical variables. These results are shown in Figs. 6 and 8.

State had a significant effect as a categorical variable on \( \Delta \dot{V_e} \) without any significant interactions on the variation of \( \Delta \dot{V_e} \) over the time course of the experiment. The \( \Delta \dot{V_e} \) responses to 7% CO\(_2\) are, in general, lower in NREM sleep. In wakefulness, as shown in Fig. 6, \( \Delta \dot{V_e} \), \( \Delta \dot{V_T} \), and \( \Delta f \) breathing 7% CO\(_2\) were significantly lower in SSP-SAP-treated rats \((P < 0.01; \text{ post hoc with Bonferroni adjustment}; P < 0.001 \text{ interaction with treatment})\) at both 8 and 22 days. These were large effects, a 61% decrease in \( \Delta \dot{V_e} \) at 8 and 22 days in wakefulness for example. The results in NREM sleep were similar, a 46% decrease at 8 days and a 57% decrease at 22 days (data not shown).

Breathing in hypoxia. As in the hypercapnia experiments, in the hypoxia experiments we studied absolute \( \dot{V_e} \) as a RM over time with state (awake, NREM sleep), treatment (IgG-SAP, SSP-SAP), and gas (air, 12% O\(_2\)) as categorical variables. There was a significant effect of state as a categorical variable \((P < 0.001)\), but there was no significant interaction between state and \( \dot{V_e} \) as a RM. In these experiments, \( \dot{V_e} \) was lower in NREM sleep compared with wakefulness, but the effects of treatment or gas did not vary by state. There was a significant interaction between \( \dot{V_e} \) and treatment \((P < 0.001)\), gas \((P <\)
0.01), and treatment × gas (P < 0.001). For VT and f, there was a RM effect and a significant interaction between them and gas, but no significant interaction between them and gas × treatment. This precluded any post hoc analysis of VT and f.

When only the awake data are examined, as shown in Fig. 7, absolute ventilation breathing 12% O2 was significantly lower in SSP-SAP-treated rats at both 8 days (P < 0.01; post hoc with Bonferroni adjustment; P < 0.001 interaction with treatment) and 22 days (P < 0.05; post hoc with Bonferroni adjustment; P < 0.001 interaction with treatment), although the decrease was −35% at 8 days and −18% at 22 days. There was a trend toward a lesser response at 22 days. The ΔVT was significantly lower in SSP-SAP-treated rats (P < 0.01) only at 8 days. The pattern of results was the same in NREM sleep. The Δf did not differ significantly between IgG-SAP and SSP-SAP groups in wakefulness or in NREM sleep.

Sighs. There was no effect of our lesion on the frequency or duration of periodic deep breaths during 4 h of room-air breathing. The control and treated groups had between 19 and

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Fig. 7. SSP-SAP lesions decrease VT in 12% O2 in both wakefulness and NREM sleep without an effect on air breathing. The effect of IgG-SAP (solid symbols, n = 6, means ± SE) or SSP-SAP (open symbols, n = 7, means ± SE) injections on VT, while breathing air or 12% O2 in wakefulness (top) or NREM sleep (bottom) is shown at 0, 8, and 22 days after injection. *P < 0.005 for the lesion group compared with the same day for the control group (see text for details).

To emphasize the effects of SSP-SAP lesions on the hypoxic response and to control for any independent variation in room air and hypoxic stimulated values, we analyzed ΔVE, ΔVT, and Δf separately as a RM over time, with state (awake, NREM sleep) and treatment (IgG-SAP, SSP-SAP) as categorical variables, as shown in Fig. 8.

State had a significant effect as a categorical variable on ΔVE without any significant interactions on the variation of ΔVE over the time course of the experiment. The ΔVE responses to 12% O2 are, in general, lower in NREM sleep. In wakefulness, as shown in Fig. 8, ΔVE breathing 12% O2 was significantly lower (−40%) in SSP-SAP-treated rats (P < 0.01 post hoc with Bonferroni adjustment; P < 0.001 interaction with treatment) but only at 8 days. In NREM sleep, ΔVE was significantly lower at both 8 and 22 days (P < 0.01, post hoc with Bonferroni adjustment; P < 0.001 interaction with treatment), although the decrease was −35% at 8 days and −18% at 22 days. There was a trend toward a lesser response at 22 days. The ΔVT was significantly lower in SSP-SAP-treated rats (P < 0.01) only at 8 days. The pattern of results was the same in NREM sleep. The Δf did not differ significantly between IgG-SAP and SSP-SAP groups in wakefulness or in NREM sleep.

Sighs. There was no effect of our lesion on the frequency or duration of periodic deep breaths during 4 h of room-air breathing. The control and treated groups had between 19 and

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<th>Days after injection</th>
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Fig. 8. SSP-SAP lesions transiently decrease the hypoxic response in both wakefulness and NREM sleep. The “hypoxic response” refers to the absolute value of each variable in 12% O2 minus that in air. The effect of IgG-SAP (●, n = 6, means ± SE) or SSP-SAP (○, n = 7, means ± SE) injections on the “hypoxic response” of ΔVE (top), ΔVT (middle), and Δf (bottom) in wakefulness is shown at 0, 8, and 22 days after injection. *P < 0.005 for the lesion group compared on the same day with the control group (see text for details). The hypoxic responses in NREM sleep are similar (data not shown, see text).
Sleep. We measured sleep cycling over 24 h in two IgG-SAP controls and three SSP-SAP-lesioned animals at 10 and 22 days after the injections. All rats spent more time awake in the dark phase than in the light phase, e.g., 54 vs. 33% for IgG-SAP controls and 54 vs. 38% for SSP-SAP lesioned at 10 days, and, conversely, less time in NREM sleep, e.g., 40 vs. 58% for IgG-SAP controls and 39 vs. 54% for SSP-SAP lesioned at 10 days. REM time was the same in both dark and light conditions. There was no effect of our SSP-SAP injection-produced lesions on sleep cycling or on the diurnal rhythm. In the 4-h air exposures, there was no treatment effect, with the time spent awake being 36–40%, in NREM sleep 55–59%, and in REM sleep 2–5%. In the 40- to 60-min air-breathing period of the CO2 and hypoxic tests, there was no treatment effect, with the time spent awake being 47–55% and that spent in NREM sleep 45–53%.

DISCUSSION

Protocol. To obtain breathing data during 7% CO2 and 12% O2 tests in both wakefulness and NREM sleep required a 30- to 40-min exposure to each gas. The data were obtained throughout this exposure period and averaged within each state. Any time-related effects that might occur within this exposure period have been ignored to obtain state-specific results. The use of a parallel control group allows us to make treatment-specific conclusions on these averaged data.

To evaluate the level of VE during air breathing, we obtained data over a 4-h period during a separate experimental day. This longer period breathing only air allowed us to detect hypoventilation.

Lesion size and pattern. Our results showed the greatest cell loss of NK1R-ir neurons at the RTN (−79%) and A5 region (−65%), sites close to the ventral medullary surface, with lesser cell loss at the PBC/rVRG (−49%) and MR (−38%), sites located slightly deeper. There was no effect at dorsal sites, i.e., the cNTS and the A6 region. In the MR, we observed greater cell loss more rostrally at the level of the facial nucleus. As one examines sections caudal to the facial nucleus, more serotonergic neurons appear to be dorsally located, suggesting that, even in the MR, our SSP-SAP injections were predominantly affecting neurons or processes near to or at the ventral medullary surface.

Resting ventilation. Focal-specific lesions of NK1R-ir neurons and processes in the PBC that produced >80% cell loss in rats resulted in hypoventilation and disordered breathing rhythm in wakefulness and sleep (14). By day 4 after such PBC injections, there is a REM sleep-specific disordered breathing (27). Here our lesion size in the PBC/rVRG region was ~49%, a value much below the 80% threshold. We did not observe disordered breathing in wakefulness or in NREM or REM sleep. We speculate that even the REM sleep-specific disordered breathing requires a PBC lesion size of >49%. In contrast to these data in the rat, Wenninger et al. (55) observed abnormal breathing rhythms in goats with a 29% decrease in PBC NK1R-ir neurons. The goat appears to be more sensitive to PBC lesions than does the rat. We conclude that the hypoventilation in our study cannot be directly attributed to the moderately sized lesion in the PBC.

Focal lesion of NK1R-ir neurons in the MR alone with 31% cell loss did not affect resting VE (39); thus we cannot attribute the hypoventilation directly to the loss of MR NK1R-ir neurons. In our prior study with focal SP-SAP lesions of NK1R-ir processes and neurons in the RTN only (37), we observed a lesion-induced hypoventilation. With unilateral RTN lesions producing a 44% loss of NK1R-ir processes and soma, there was a 10–12% decrease in VE in wakefulness and NREM sleep, respectively, although this did not reach statistical significance. With bilateral RTN lesions producing a 44–47% reduction in NK1R-ir processes and soma, arterial blood-gas analysis showed an 11% decrease in alveolar ventilation, and measurements of VE showed an ~20% decrease. Here with substantial lesions of the RTN (−79%), we observed a significant hypoventilation, as detected over a 4-h measurement period, which was ~8% in wakefulness, ~9% in NREM sleep, and ~14% in REM sleep. Based on the comparison to prior single-site lesions, we attribute this hypoventilation to the loss of RTN NK1R-ir neurons and processes. The chemical drive to breathe that arises from the RTN is present in wakefulness and in NREM and REM sleep. Given the substantial effect on the CO2 response of the lesions in this study, we might have expected a more marked degree of hypoventilation. Certainly the presence of intact and partially functioning carotid bodies and central chemoreception at deeper medullary locations that were unaffected by these lesions would act to counter the loss of the drive to breathe that arises from the more ventral medullary sites that were more severely lesioned. It seems clear that, in unanesthetized animals, a wide variety of lesions or types of site-specific inhibition within the sphere of central chemoreceptive regions can alter the CO2 response, with little or lesser effect on VE during air breathing (see below). This is analogous to the situation following carotid body denervation in which the CO2 response is reduced by ~40% but the degree of hypoventilation is less (50). The control system does not tolerate hypoventilation, but seems to function well with quite varying CO2 sensitivities.

The reduced CO2 response. Our large and widespread lesions of ventral medullary NK1R-ir neurons and processes resulted in a very large decrease in the ventilatory response to CO2, a 61% decrease in an awake, conscious rat that was sustained for 8–22 days, and a 46–57% decrease during NREM sleep. We know of no other manipulation of central chemoreception that has produced an inhibitory effect of equivalent magnitude in a conscious animal. We contend that central chemoreception, as observed in the conscious animal, includes the participation of several chemoreceptor sites within the hindbrain (8, 12, 20, 23, 29, 31, 32, 34–36, 48). Sites that induce an increase in VE when focially acidified by focal delivery of CO2 include the following: the RTN, rVRG, cNTS, A6, fastigial nucleus, and the MR. We will focus our discussion on the four chemoreceptor regions that had substantial loss of NK1R-ir neurons in this study: the MR, RTN, A5, and PBC/rVRG.

There is evidence to support the involvement of serotonergic neurons and the MR as one central chemoreceptor site. Serotonergic neurons of the MR are CO2 sensitive in culture and in slices (47, 48); focal CO2 stimulation within the MR in vivo increases VE in conscious rats (35) and goats (20); focal inhibition of serotonergic neurons in vivo by reverse microdialysis of the 5-HT1A receptor agonist 8-hydroxy-2-(di-n-pro-
pletion of the CO2 response (caudal to the facial nucleus do not decrease the CO2 response with and without blockade of downstream synaptic connec-

During NREM sleep (41). Also, reverse microdialysis of mus-

lar neuronal processes, but few neurons (30, 37). In this study, the widespread lesions of NK1R-ir neurons in the MR (the region that had loss of NK1R-ir neurons in this study) actually increases the ventilatory response to CO2 (53).

MR serotonergic neurons do not express NK1Rs (21, 39), but many of them do contain substance P (2, 25, 42). Cell-specific lesions in the MR producing loss of either 28% of serotonergic or 31% of NK1R-ir neurons decreased the ventilatory response to CO2 in vivo by about the same amount (15–20%), while simultaneous lesions of both serotonergic and NK1R-ir neurons did not show any additional effect (39). Our interpretation was that serotonergic neurons within the MR that contain substance P detect changes in CO2, release substance P, and stimulate abundant adjacent neural processes that express NK1Rs. In this study, the widespread lesions of NK1R-ir neurons include these processes within the MR, and the effect of our lesions includes then the inhibition of the MR serotonergic neuron contribution to central chemoreception. How-

ever, the loss of MR NK1R-ir neurons/processes alone cannot explain the large effect we observed on the CO2 response. A 28% cell loss of tryptophan hydroxylase-ir neurons (neurons that express tryptophan hydroxylase, a key enzyme in serotonin synthesis) limited to the MR by injection of anti-SERT-SAP (a conjugate of an antibody to the serotonin transport protein and the cell toxin SAP) reduced the CO2 response by 15–18% (39). Here we had only a slightly larger loss of NK1R-ir cells within the MR (~38%) but a substantially larger decrease in the CO2 response (~62%).

There is evidence to support the involvement of RTN neu-

rons in central chemoreception. RTN neurons identified as glutamatergic by their expression of mRNA for the vesicular GLUT2 transporter are CO2 sensitive in slices and in vivo, with and without blockade of downstream synaptic connections in the “central pattern generator” (19, 29); focal CO2 stimulation of the RTN by reverse microdialysis increases ventilation in rats (23); focal inhibition of RTN neurons by muscimol decreases the ventilatory response to CO2 in adult rats and in newborn piglets (10); and lesions of RTN neurons by an excitatory amino acid toxin (ibotenic acid) in adult rats decreases the ventilatory response to CO2 (1), as do focal RTN lesions of NK1R-ir neurons and processes (37).

We do not know if RTN glutamatergic neurons express NK1Rs. However, substance P is present in the RTN (25), and RTN neurons that express c-fos when activated by CO2 contain preprotachykinin mRNA, indicating that they contain sub-

stance P or neurokinin A (42). Given the evidence that it is the glutamatergic neurons in the RTN that respond to CO2, it seems reasonable to presume that they also contain substance P. The pattern of NK1R-ir in the RTN shows a rich network of neuronal processes, but few neurons (30, 37). In this study, the widespread lesions of NK1R-ir neurons include these processes, as well as the NK1R-ir neurons within the RTN, and the overall effect of our lesions includes the attenuation of the RTN contribution to central chemoreception. Focal unilateral injection of SSP-SAP into the RTN produced a 44% loss of NK1R-ir and a 30% reduction in the CO2 response in wakefulness (36). Following cisterna magna injections of SSP-SAP, there was a 79% loss of NK1R-ir neurons bilaterally and a 61% reduction in the CO2 response. This comparison of lesion size and physiological effects suggests that the RTN lesions of NK1R-ir neurons/processes could have made a major contribution to the overall 61% reduction in the CO2 response in this study. We suggest that, in the RTN, as in the MR, the CO2 responsive glutamatergic neurons stimulate adjacent NK1R-ir processes by release of substance P.

The role of the A5 region in central chemoreception is poorly understood. CA neurons do express NK1Rs (5) and are also putative chemodetectors (22, 43), but we do not believe that the effect of our lesions on A5 neurons can wholly explain the substantially decreased ventilatory response to CO2 in this study. In recent experiments, we produced widespread lesions of CA neurons by injection into the fourth ventricle of anti- 

dopamine β-hydroxylase-SAP (an antibody to dopamine β-hydroxylase conjugated to SAP to kill CA neurons) (22). These injections reduced the number of A5 CA neurons by 78%. Other CA sites were also substantially affected, yet the CO2 response was reduced by only 28%.

The effects of our SSP-SAP injections on NK1R-ir neuron numbers in the PBC and the rVRG could have influenced the CO2 response. In rats, Gray et al. (14) produced extensive SP-SAP lesions of NK1R-ir neurons within the PBC. Both an abnormal breathing rhythm and a reduced CO2 response were observed with >80% loss of NK1R-ir neurons. Our PBC lesions were well below this value. We conclude that our 49% loss of PBC NK1R-ir neurons is not the sole cause of the 61% decrease in the CO2 response. CA neurons could themselves be chemosensitive and are likely the destination of chemoreceptor-derived information via the suggested substance P neurotransmission from MR and RTN. The smaller PBC lesion could contribute to the decreased CO2 response. With respect to the rVRG, focal reverse microdialysis of a hypercapnic solution does increase Ve, indicating the presence of chemoreception at that site (34). As in the PBC, neurons in the rVRG could themselves detect CO2, or they could be influenced by substance P inputs from MR and RTN. In either case, the loss of NK1R-ir neurons there could contribute to the observed 61% decrease in the CO2 response.

The remaining response to CO2 after the cisterna magna SSP-SAP lesions can be explained by the participation of other chemoreceptors at the carotid body, the A6 region, the cNTS, and the fastigial nucleus of the cerebellum. Recent work has helped to describe the relative importance of central and peripheral chemoreceptors in the CO2 response. Studies in conscious dogs with denervation of the carotid body on one side and control of the milieu of the carotid body on the other side by perfusion (50) maintain the tonic input from the carotid bodies to the medullary respiratory neurons, something missing in denervation experiments. This work shows that the peripheral chemoreceptor contributes ~40% of the overall response. In studies of conscious rats after acute carotid de-

nervation, the ventilatory response to CO2 is reduced by
30–40% (12a, 26, 49). Thus much of the remaining response present after our SSP-SAP-induced NK1R-ir cell loss could be attributable to input from the carotid bodies. However, our SSP-SAP-lesioned rats also demonstrate a reduced ventilatory response to hypoxia that is present at 8 days after the injection. This indicates that the processing of the CO2-related afferent excitatory input from the carotid body may also be affected by the NK1R-ir neuronal loss. Insofar as the RTN (38, 51) and the MR (53) may modulate the effects of afferent input from the carotid bodies, we conclude that the remaining CO2 response in our SSP-SAP-lesioned rats is likely due mainly to stimulation at other central chemoreceptor sites and to some amount of carotid body input.

The hypoxic response. We observed a decrease in the hypoxic response at 8 days following the SSP-SAP, agreeing with observations in the adult NK1R knock-out mouse (46), which showed a reduced hypoxic response. In that our substantial lesions of NK1R-ir neurons are limited to the ventral medulla, NK1R-ir neurons in these lesion sites must participate in the reduced hypoxic ventilatory response. NTS neurons, which contain preprotachykinin mRNA for substance P/neurokinin A (42), could project to ventral medullary sites, or, alternatively, ventral medullary neurons with NK1Rs could project to the NTS and modulate afferent inputs at that site. The observation that injection of substance P antagonists into the ventral medulla in anesthetized rats decreases the hypoxic response supports a modulatory role for NK1R-ir neurons in the ventral medulla on the hypoxic response. RTN chemosensitive glutamatergic neurons receive afferent input from NTS neurons that are excited by hypoxic stimulation (51). These neurons could modulate the hypoxic response, and the loss of NK1R-ir processes in the RTN could diminish this function. In a similar fashion, some MR serotonergic neurons participate in the hypoxic response (11), and muscimol inhibition of the MR produces complex temperature-dependent effects on the hypoxic response in conscious adult rats (53).

The recovery of the hypoxic response by 22 days was surprising in that the CO2 response showed no such recovery. Rats do show rapid recovery of the hypoxic response following carotid body denervation (49). In our case, the carotid bodies were intact, and the loss of the hypoxic response was due to loss of modulation by, or participation from, ventral medullary neurons and/or processes lesioned by the SSP-SAP. The partial recovery of the hypoxic response likely reflects plasticity within the medulla involving the ventral medulla and/or the NTS. In this study, this plasticity is greater for the hypoxic than for the hypercapnic response.

Significance. We propose that substance P is released from glutamatergic chemosensitive neurons of the RTN and from serotonergic chemosensitive neurons of the MR in response to CO2 stimulation. The MR serotonergic and RTN glutamatergic neurons could be stimulated by CO2, either directly or indirectly, e.g., by ATP release from nearby surface cells (glia?) (13) or via carotid body afferents through the NTS (51). In either case, the subsequent release of substance P stimulates NK1Rs, which are part of, or are connected to, the respiratory rhythm and pattern generators. We also propose that neurons in the MR and RTN (possibly the same CO2-sensitive neurons) are activated in hypoxia and release substance P to modulate the hypoxic response (11, 38, 51). This substance P system contributes to the chemical drive to breathe present under resting conditions, as shown by the hypoventilation following the lesions.

Of interest is the human disease multiple system atrophy in which there is severe loss of NK1R-ir neurons in the ventral medulla (3). These patients have disordered breathing and an abnormal ventilatory response to hypoxia (3). Their response to hypercapnia has not been reported.

GRANTS

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REFERENCES


