Activation of Opioid μ-Receptors in the Commissural Subdivision of the Nucleus Tractus Solitarius Abolishes the Ventilatory Response to Hypoxia in Anesthetized Rats

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ABSTRACT

Background: The commissural subnucleus of the nucleus tractus solitarius (comNTS) is a key region in the brainstem responsible for the hypoxic ventilatory response (HVR) because it contains the input terminals of the carotid chemoreceptor. Because opioids inhibit the HVR *via* activating central μ-receptors that are expressed abundantly in the comNTS, the authors of the current study asked whether activating local μ-receptors attenuated the carotid body-mediated HVR.

Methods: To primarily stimulate the carotid body, brief hypoxia (100% N_2) and hypercapnia (15% CO_2) for 10 s and/or intracarotid injection of NaCN (10 μg/100 μl) were performed in anesthetized and spontaneously breathing rats. These stimulations were repeated after: (1) microinjecting three doses of μ-receptor agonist [d-Ala2, N-Me-Phe4, Gly-ol]-Enkephalin (DAMGO) (approximately 3.5 nl) into the comNTS; (2) carotid body denervation; and (3) systemic administration of DAMGO (300 μg/kg) without and with previous intracomNTS injection of d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH2, a μ-receptor antagonist.

Results: Study results showed that DAMGO at 0.25 and 2.5, but not 0.025 mM, caused a similar decrease in baseline ventilation (approximately 12%). DAMGO at 0.25 mM largely reduced (64%) the HVR, whereas DAMGO at 2.5 mM abolished the HVR (and the V_E response to NaCN) and moderately attenuated (31%) the hypercapnic ventilatory response. Interestingly, similar HVR abolition and depression of the hypercapnic ventilatory response were observed after carotid body denervation. Blocking comNTS μ-receptors by d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH2 significantly attenuated the HVR depression by systemic DAMGO with little change in the DAMGO modulatory effects on baseline ventilation and the hypercapnic ventilatory response.

Conclusion: The data suggest that opioids within the comNTS, *via* acting on μ-receptors, are able to abolish the HVR by affecting the afferent pathway of the carotid chemoreceptor.

What We Already Know about This Topic

- Although opioids profoundly depress the hypoxic ventilatory response (HVR), their site of action is not clear

What This Article Tells Us That Is New

- In rats, injection of a selective μ-opioid receptor agonist into the commissural subnucleus of the nucleus tractus solitarious could abolish HVR, suggesting action at this site is critical for opioid-induced respiratory depression

THE hypoxic ventilatory response (HVR), a crucial chemoreflex in mammals, is achieved predominantly by stimulating carotid chemoreceptors.[1] There are several lines of evidence demonstrating that the commissural subnucleus of the nucleus tractus solitarius (comNTS) receives most synaptic inputs from the carotid chemoreceptor and is essential for the carotid body-mediated HVR.[2–5] First, anatomic studies showed that after local injection of a retrograde tracer into the carotid body or anterograde tracer into the nodose-petrosal complex, the major and densest labeling of the terminals from the carotid sinus nerve (CSN) was observed in the comNTS in rats and cats.[6–9] Second, the HVR was abolished by microinjecting glutamate receptor antagonists into the comNTS, but the HVR was increased by injecting glutamate into this area.[10] Third, chemical lesions made in the nucleus tractus solitarius, including the comNTS, almost eliminated the HVR in both anesthetized and nonanesthetized rats.[2,3] Fourth, electrophysiologic stud-

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ies demonstrated that considerable comNTS neurons characterized by tonic firing were activated by stimulating chemoreceptors but not baroreceptors.[1,5]

It is well documented that opioids are able to profoundly depress and even eliminate the HVR mainly *via* activating central μ-opioid receptors in both animals and humans,[11–14] but the central site(s) responsible for this inhibition remains unknown. μ-Opioid receptors are widespread throughout the brainstem[15,16]; however, they are extraordinarily more prevalent in the nucleus tractus solitarius, especially the comNTS.[15–17] Interestingly enough, morphologic[15,18–20] and electrophysiologic[21–24] studies have demonstrated that μ-receptors exist on both fibers' terminals and neurons in the comNTS, and activating these receptors can inhibit the presynaptic glutamate release and hyperpolarize comNTS neurons. Therefore, we hypothesized that microinjection of the selective μ-receptors agonist DAMGO ([d-Ala2, N-Me-Phe4, Gly-ol]-Enkephalin) into the comNTS would attenuate or abolish the carotid body-mediated HVR. We also hypothesized that pretreating the comNTS with CTAP (d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH2), a μ-receptor antagonist, would largely diminish the systemic administration of DAMGO-induced HVR depression.

Materials and Methods

Sixty-three pathogen-free male Sprague-Dawley rats (350–450 g) were purchased from Charles River Laboratories, Inc. (Wilmington, MA), housed in the animal facility at Lovelace Respiratory Research Institute in filter-top cages, and provided with water and food *ad libitum*. The room was constantly ventilated and the temperature was kept at 23°C. The animals were quarantined for 2 weeks before experiments. The experimental protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by Lovelace Respiratory Research Institute's Institutional Animal Care and Use Committee (Albuquerque, NM), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, USA.

General Animal Preparation

The rats were anesthetized with urethane (1200 mg/kg, intraperitoneally). As needed, supplemental urethane (300 mg/kg, intraperitoneally) was administered to completely eliminate eye-blink and limb-withdrawal reflex throughout the experiment. The general animal preparation was the same as previously reported in rats.[25] In several rats, a cannula was inserted into the right common carotid artery for the intracarotid injection of sodium cyanide (NaCN). The CSN was bilaterally isolated and loosely looped by a thread for later section in some cases. Animals were placed into a rigid metal frame with the head fixed and centered in a stereotactic apparatus (Model 1404, Kopf, Tujunga, CA), and the calamus scriptorius (obex)[26,27] was exposed for microinjection. Also, 50% O_2 in nitrogen was served as a baseline throughout the experiment. The animal's core temperature was monitored with a rectal probe and maintained at 36.5–37.5°C by a water heating pad and radiant heat lamp.

Microinjection into the ComNTS

Loading DAMGO or CTAP. Unfilamented glass capillaries (1B100–3, 1/0.58 mm OD/ID, WPI, Sarasota, FL) were pulled in a horizontal pipette puller (DMZ-Universal Puller, Zeitz Instruments, München, Germany) and the long-shank micropipette was broken back to a desired tip size (approximately 15 μm, OD). The tip part of the micropipette was filled with DAMGO (averaged 3.5 nl, from 3.3–3.7 nl; Sigma–Aldrich, St. Louis, MO), CTAP (averaged 3.9 nl, from 3.4–4.3 nl; Sigma–Aldrich) or vehicle by quickly dipping the tip into the solution. The solution in the tip of the micropipette formed the shape of a truncated cone and the solution volume (V) was calculated by the equation: $V = 1/3 \times \pi\ (3.14) \times h \times (R_1^2 + R_1 \times R_2 + R_2^2)$, where h equals the length of the solution column and R_1 and R_2 equal the radius of each end of the solution in the micropipette tip. The micropipette was connected to a 5-ml syringe *via* a polyethylene tube. Both the syringe and the polyethylene tube were filled with distilled water. The preloaded solution in the micropipette was separated from the distilled water by an air column (approximately 10 cm long). The syringe was driven by a computerized infusion pump (Model 55–1111, Harvard Apparatus, South Nitick, MA) during microinjection.

Microinjection. The micropipette, as seen under an operating microscope (Photo-Zusatz, Carl Zeiss, Jena, Germany), was advanced by a micromanipulator into the calamus scriptorius or a site 1 mm lateral to the calamus scriptorius 0.3–0.5 mm deep from the dorsal surface. The pump-driven microinjection was continued for 4–6 s until we saw through the microscope the preloaded solution level in the micropipette descending below the dorsal surface. DAMGO (0.025, 0.25, and 2.5 mM) or CTAP (10 mM) was made in a solution of 0.9% saline containing red fluorescent microbeads (dilutions of 1:1, Lumafluor, Inc., Naples, FL).

Identification of Microinjection Site. At the end of the experiment the brain was fixed *in situ* by perfusing 0.1 M phosphate-buffered saline at a pH of 7.4 and then 4% paraformaldehyde in phosphate-buffered saline through the left ventricle of the heart. The brainstem was removed and subsequently sectioned at a 50-μm thickness by a slicing machine (Leica, CM 1850, Microsystems GMbH, Nussioch, Germany). The area marked by fluorescent beads was identified under a fluorescence microscope.

Chemoreceptor Stimulation

Three approaches were used to primarily stimulate the carotid chemoreceptor. First, the rats were exposed to a brief hypoxia (100% N_2 for 10 s), similar to other reports.[28,29] Second, NaCN (0.1 ml 0.1 mg/1 ml in saline) was intracarotid-injected within 2 s as reported previously.[30] Third, brief hypercapnia (15% CO_2 + 50% O_2 + 35% N_2) for 10 s

was administered to evoke the hypercapnic ventilatory response (HCVR).

Experiment Protocol

Study Series I was designed to test whether microinjecting DAMGO into the comNTS alters baseline cardiorespiratory variables and the HVR. The HVR to a brief hypoxia (100% N_2 for 10 s) was tested before and 5 min after microinjecting a given concentration of DAMGO into the comNTS. Three different concentrations of DAMGO, *i.e.*, 0.025, 0.25, and 2.5 mM, were administered in three groups of rats (n = 8 in each group). We selected the relatively small sample sizes mainly due to the consistency of our findings and a small variance in our pilot study. To further confirm the effect of DAMGO on the carotid body-mediated HVR, an intracarotid injection of NaCN (10 μg/100 μl) was given before and 5 min after 2.5 mM DAMGO was microinjected into the comNTS in four other rats. This concentration was chosen here and in the following experiments because of its ability to abolish the HVR in our pilot experiment.

Study Series II was carried out to serve as a control. To clarify whether DAMGO-induced cardiorespiratory changes were site-dependent, the same brief hypoxia was performed before and after 2.5 mM DAMGO was microinjected into the bilateral regions 1 mm lateral to the comNTS (n = 6, 3 for the right and 3 for the left side). Five additional rats that served as sham-operation control subjects had vehicle instead of DAMGO microinjected into the comNTS.

Study Series III was designed to test whether microinjecting 2.5 mM DAMGO into the comNTS affects the HCVR (n = 5). Rats were exposed to 15% CO_2 for 10 s before and 5 min after 2.5 mM DAMGO was microinjected into the comNTS.

Study Series IV was conducted to estimate to what extent the effect of DAMGO on the HVR and HCVR was similar to the effect of transecting the CSN. Ten rats were exposed to brief hypoxia and hypercapnia randomly before and 5 min after bilateral section of the CSN. A 5-min interval was allowed between the two stimulations. In five of them, subsequently, the same chemical stimulations were repeated 5 min after 2.5 mM DAMGO was microinjected into the comNTS to test to what extent the modulatory effects of microinjected DAMGO were dependent on the CSN input.

Study Series V was planned to evaluate the role comNTS μ-receptors played in the systemic DAMGO-induced HVR depression in six rats. HVR and HCVR were tested before and 5 min after intravenous administration of DAMGO (300 μg/kg). This dose was chosen to sufficiently depress HVR, by which the influence of blocking comNTS μ-receptors on this depressed HVR could be obvious. Two hours later, the same protocol was repeated 8–10 min after CTAP was microinjected into the comNTS. This 2-h interval was chosen because the inhibitory effect on eupneic ventilation and HCVR in our previous studies[25] and on HVR in our pilot study disappeared 1–2 h after systemic administration of DAMGO in anesthetized rats. In three other rats, intravenous DAMGO was repeated twice within a 2-h interval to test the reproducibility of the DAMGO effect on the HVR and HCVR over time.

Data Acquisition and Statistical Analysis

Raw data of the airflow, blood pressure, heart rate (HR), end-tidal pressure of carbon dioxide (P_{ETCO_2}), and rectal temperature were digitized, monitored, and recorded using a PowerLab/8sp (model ML 785; AD Instruments Inc., Colorado Springs, CO) connected to a computer using the PowerLab Chart 5 software. The airflow signals were integrated to generate tidal volume (V_T), respiratory frequency (f), and minute ventilatory volume (V_E). The cardiorespiratory baseline was determined by averaging the variables for 1 min immediately before and 5 min after administering DAMGO. The cardiorespiratory responses to the brief hypoxia (NaCN) or hypercapnia were determined by measuring the variables at the last 2-s period of the exposure and expressed by percentage change from the baseline (Δ%). All data are presented as means ± SE. Repeated analysis of two-way ANOVA was used to compare the differences of the cardiorespiratory variables (the baseline and their responses to the brief hypoxia or hypercapnia) induced by: (1) different DAMGO concentrations microinjected into the comNTS (outside of the comNTS); (2) systemic DAMGO alone and coupled with CTAP pretreating the comNTS; and (3) DAMGO microinjection in the CSN intact and denervated rats. If an overall test was significant, Tukey *post hoc* test was used for specific comparisons between individual groups. The software Statistica 6.0 (StatSoft, Inc., Tulsa, OK) was used for statistical analysis. The difference was considered significant at a $P < 0.05$.

Results

DAMGO Alters Baseline Cardiorespiratory Activity and its Responses to Hypoxia

We tested the effects of microinjecting three concentrations of DAMGO (0.025, 0.25, and 2.5 mM) into the comNTS on baseline cardiorespiratory activity. As shown in table 1, baseline cardiorespiratory variables were not altered by 0.025 mM DAMGO. However, 0.25 and 2.5 mM DAMGO significantly inhibited baseline V_E (by 10% and 12%, respectively) *via* lowering V_T and increased P_{ETCO_2}, with no difference between the two doses. Compared with the control values, these induced changes in baseline V_E and P_{ETCO_2} disappeared approximately 30 min (26 ± 6 min) later. In addition, blood pressure was similarly and significantly increased by 20% and 28% from 0.25 and 2.5 mM DAMGO, respectively. The increased blood pressure lasted for 33 ± 4 min with no change in HR.

Pure nitrogen exposure for 10 s markedly increased V_E, f, and V_T by 82%, 40%, and 28%, respectively. The evoked V_E response was not affected by 0.025 mM DAMGO microin-

Table 1. Baseline Cardiorespiratory Variables before and after Microinjecting DAMGO into the comNTS

Variables	Time Period	DAMGO (mM)		
		0.025	0.25	2.5
V_E (ml/min)	Before	195 ± 22	208 ± 28	199 ± 27
—	5 min after	197 ± 23	184 ± 26*	170 ± 22*
—	30 min after	—	202 ± 29	194 ± 25
f (breaths/min)	Before	106 ± 5	99 ± 4	97 ± 7
—	5 min after	108 ± 6	101 ± 5	98 ± 8
—	30 min after	—	103 ± 7	101 ± 8
V_T (ml)	Before	1.8 ± 0.2	2.1 ± 0.7	2.1 ± 0.2
—	5 min after	1.8 ± 0.3	1.8 ± 0.6*	1.7 ± 0.4*
—	30 min after	—	2.0 ± 0.7	1.9 ± 0.5
P_{ETCO_2} (mmHg)	Before	35 ± 3	34 ± 2	36 ± 2
—	5 min after	34 ± 4	39 ± 3*	41 ± 2*
—	30 min after	—	35 ± 4	36 ± 4
MBP (mmHg)	Before	101 ± 6	108 ± 8	105 ± 7
—	5 min after	105 ± 6	131 ± 9*	137 ± 5*
—	30 min after	—	113 ± 10	116 ± 9
HR (beats/min)	Before	381 ± 5	382 ± 25	371 ± 24
—	5 min after	390 ± 7	385 ± 26	375 ± 25
—	30 min after	—	389 ± 26	380 ± 28

n = 8 for each DAMGO concentration. Data are presented as mean ± SE.

* $P < 0.01$ compared variables between before and 30 min after administration of DAMGO.

comNTS = the commissural subnucleus of the nucleus tractus solitarius; DAMGO = [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; f = breath frequency; HR = heart rate; MBP = mean blood pressure; P_{ETCO_2} = end-tidal pressure of carbon dioxide; V_E = minute ventilation; V_T = tidal volume.

jected into the comNTS, but reduced by 64% and completely eliminated after 0.25 and 2.5 mM DAMGO were microinjected, respectively, *via* affecting both V_T and f responses (fig. 1 and 2). The responses of V_E, f, and V_T (79 ± 15%, 42 ± 9%, and 26 ± 7%) to nitrogen 1–2 h after the microinjection of DAMGO were not significantly different from the control values (82 ± 13%, 44 ± 8, and 27 ± 6%, $P > 0.05$). In addition, brief hypoxia depressed the mean blood pressure by 22% and increased the heart rate by 8%. However, DAMGO failed to alter the hypoxia-induced hypotension and tachycardia (fig. 3). As a control, microinjecting vehicle into the comNTS did not change the V_E (83 ± 9% *vs.*. 84 ± 10%, $P = 0.99$), f (44 ± 6% *vs.*. 45 ± 6%, $P = 0.98$), and V_T (27 ± 4% *vs.*. 26 ± 5%, $P = 0.99$) responses to the pure nitrogen or the baseline variables (table 2).

We also tested the ventilatory response to the intracarotid injection of NaCN (10 μg in 0.1 ml) before and after microinjecting 2.5 mM DAMGO into the comNTS. As a result,

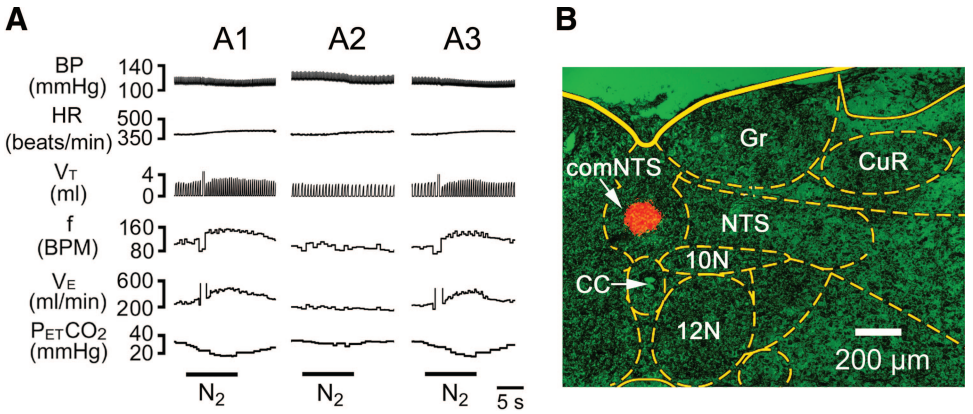

Fig. 1. (*A*) Experimental recordings of the cardiorespiratory responses to a brief hypoxia (100% N_2 for 10 s) before (*A1*), 5 min (*A2*), and 120 min (*A3*) after DAMGO (2.5 mM) was microinjected into the comNTS. Traces in sequence are arterial blood pressure (blood pressure), heart rate (HR), tidal volume (V_T), respiratory frequency (f, BPM = breaths/min), minute ventilation (V_E), and end-tidal pressure of carbon dioxide (P_{ETCO_2}), respectively. Sighs in V_T and V_E traces are truncated to focus on their changes. The bars on the bottom reflect the duration of a brief hypoxia. (*B*) A representative slice containing the comNTS, in which the injection location is stained by fluorescent microbeads (*red*). CC = central canal; comNTS = commissural part of the nucleus of the solitary tract (NTS); CuR = cuneate nucleus, rotundus part; DAMGO = [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; Gr = gracile nucleus; 10N = dorsal vagal motor nucleus; 12N = hypoglossal nucleus.

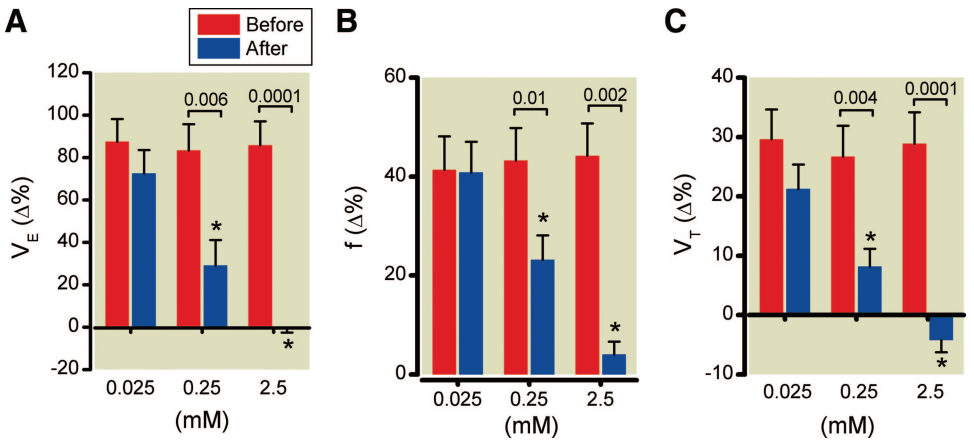

Fig. 2. The ventilatory (V_E, *A*), frequency (f, *B*), and tidal volume (V_T, *C*) responses to N_2 of 10 s before and after microinjecting different [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) doses into the commissural subnucleus of the nucleus tractus solitarius. Note: All the respiratory variables (V_E, f, and V_T) during the brief period of hypoxia were significantly higher than baseline values except those after 2.5 mM DAMGO microinjection. n = 8 in each group; data are presented as means ± SE; *P* values less than 0.05 are presented exactly with the exception that * = $P < 0.05$ compared with other DAMGO concentrations, whereas those more than 0.78 are not presented.

this dose of DAMGO eliminated the ventilatory response to NaCN in all four rats (fig. 4), similar to the abolition of the HVR to brief hypoxia.

Microinjection of DAMGO-induced Changes is Site-Dependent

To confirm the unique effect of microinjecting DAMGO into the comNTS on abolishing the HVR, we microinjected 2.5 mM DAMGO into the regions 1 mm left or right from the calamus scriptorius in six rats. Microinjections made in the right or left sides had a similar effect on the cardiorespiratory variables' responses to the brief hypoxia, so we grouped these data together. Collectively, these microinjections depressed the HVR by 26% without changing the blood pressure and HR responses to hypoxia (fig. 5) or the baseline cardiorespiratory variables (table 2).

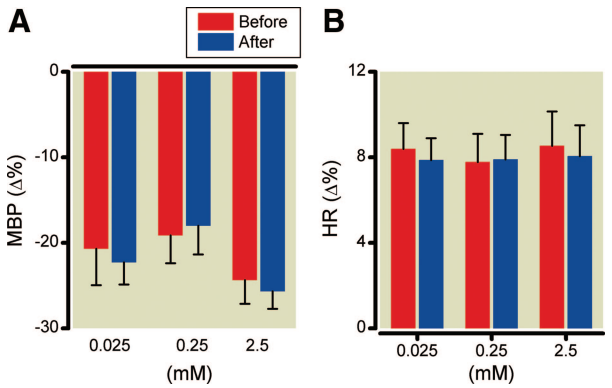

Fig. 3. Comparison of the cardiovascular responses to nitrogen for 10 s before and after [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO). The brief hypoxia significantly decreased mean blood pressure (MBP, *A*) and increased heart rate (HR, *B*) and these responses were not significantly altered by DAMGO. n = 8 in each group; data are presented as means ± SE; all *P* values are at least greater than 0.93.

DAMGO Depresses the Cardiorespiratory Responses to the Brief Hypercapnia

As shown in figure 6A, 15% CO_2 exposure for 10 s markedly increased V_E, f, and V_T by 92%, 18%, and 67%, respectively. 2.5 mM DAMGO microinjected into the comNTS significantly depressed the HCVR by 31% due to inhibiting the V_T response. The hypercapnic exposure significantly increased blood pressure and decreased HR. Interestingly, this evoked hypertension was depressed and bradycardia tended to be aggregated by microinjecting 2.5 mM DAMGO into the comNTS (fig. 6B). As a control, microinjecting vehicle into the comNTS did not change

Table 2. Effects on the Baseline Cardiorespiratory Variables after Microinjecting 2.5 mM DAMGO into the Outside of the comNTS and Vehicle into the comNTS

	DAMGO Outside the comNTS (n = 6)		Vehicle in the comNTS (n = 5)	
Variables	Before	After	Before	After
V_E (ml/min)	216 ± 14	213 ± 12	198 ± 25	201 ± 27
f (breaths/min)	108 ± 5	101 ± 4	108 ± 11	111 ± 12
V_T (ml)	2.0 ± 0.3	2.1 ± 0.4	1.8 ± 0.5	1.8 ± 0.6
P_{ETCO_2} (mmHg)	33 ± 4	33 ± 5	34 ± 4	33 ± 6
MBP (mmHg)	94 ± 5	95 ± 6	108 ± 6	107 ± 7
HR (beats/min)	401 ± 24	404 ± 25	394 ± 25	398 ± 26

Data are presented as mean ± SE.

comNTS = the commissural subnucleus of the nucleus tractus solitarius; DAMGO = [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; f = breath frequency; HR = heart rate; MBP = mean blood pressure; P_{ETCO_2} = end-tidal pressure of carbon dioxide; V_E = minute ventilation; V_T = tidal volume.

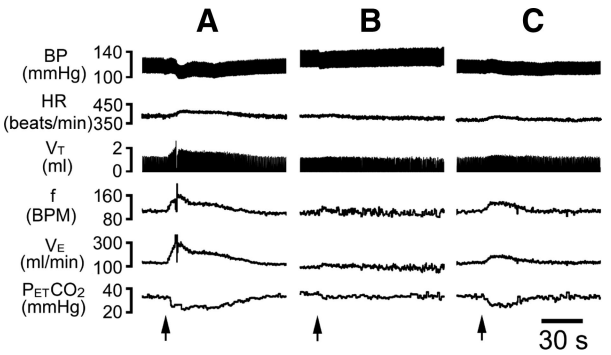

Fig. 4. Experimental recordings of the cardiorespiratory responses to intracarotid injections of NaCN (*arrows*) before (*A*), 5 min (*B*), and 60 min (*C*) after microinjecting [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) (2.5 mM) into the comNTS. Traces in sequence are arterial blood pressure (blood pressure), heart rate (HR), tidal volume (V_T), respiratory frequency (f, BPM = breaths/min), minute ventilation (V_E), and end-tidal pressure of carbon dioxide (P_{ETCO_2}), respectively. Note: 5 min after DAMGO injection, intracarotid injection of NaCN failed to evoke a remarkable respiratory response. Sighs in V_T, f, and V_E traces are truncated to focus on the responses. comNTS = the commissural subnucleus of the nucleus tractus solitarius.

the HCVR (101 ± 4% *vs.* 103 ± 12% for V_E, $P = 0.99$; 18 ± 3% *vs.* 17 ± 4% for f, $P = 0.97$; and 70 ± 6% *vs.* 73 ± 7% for V_T, $P = 0.98$;) or the cardiovascular response to carbon dioxide (13 ± 2% *vs.* 12 ± 2% for blood pressure, $P = 0.99$, and −2 ± 0.3% *vs.* −2 ± 0.4% for HR, $P = 0.96$).

Carotid Body Denervation Eliminates the HVR and Depresses the HCVR

Bilaterally transecting the CSN significantly depressed baseline V_E by 14% (196 ± 11 *vs.* 167 ± 19 ml/min, $P = 0.03$) due to inhibiting f (101 ± 7 *vs.*. 85 ± 9 breaths/min, $P = 0.01$) but not V_T (1.98 ± 0.17 *vs.*. 2.02 ± 0.25 ml, $P = 0.91$). Moreover, the transection failed to significantly change blood pressure (95 ± 10 *vs.* 103 ± 13 mmHg, $P = 0.87$) and HR (412 ± 20 *vs.* 403 ± 28 beats/min, $P = 0.93$). Similar to the high dose of DAMGO, bilaterally transecting the CSN abolished the HVR and depressed the HCVR (by 20%), although the amplitude of the latter was less than that induced by intracomNTS microinjection of 2.5 mM DAMGO (−31 ± 4% *vs.* −20 ± 3%, $P = 0.04$). CSN transection did not remarkably influence the cardiovascular responses to nitrogen and carbon dioxide with the exception that the blood pressure response to carbon dioxide was depressed. The typical recordings and the group data exhibiting the effect of bilateral CSN transection on the cardiorespiratory responses to brief hypoxia and hypercapnia are shown in figures 7 and 8, respectively.

In carotid body-denervated rats, intracomNTS injection of 2.5 mM DAMGO still caused (1) a depression of baseline V_E from 163 ± 13 to 147 ± 17 ml/min (10%, $P = 0.03$) mainly *via* lowering V_T from 2.06 ± 0.11 to 1.77 ± 0.20 ml ($P = 0.02$) without effect on f (79 ± 7 *vs.* 82 ± 11 breaths/min, $P = 0.86$); (2) an increase in baseline blood pressure (105 ± 12 *vs.* 129 ± 14 mmHg, $P = 0.0001$) but not HR (399 ± 32 *vs.* 404 ± 26 beats/min, $P = 0.97$); (3) a depression of HCVR by 16% (65 ± 9% *vs.* 54 ± 8%, $P = 0.03$) by inhibiting V_T response (48 ± 6% *vs.* 32 ± 5%, $P = 0.03$) with no effect on f response (12 ± 5% *vs.* 15 ± 7%, $P = 0.92$); and (4) no change in the abolished HVR to nitrogen (0.9 ± 2.2% *vs.* 0.4 ± 2.8% for V_E, $P = 0.93$; 8 ± 3% *vs.* 7 ± 4% for f, $P = 0.97$; and −7 ± 4% *vs.* −6 ± 5% for V_T, $P = 0.95$).

Blocking comNTS μ-Receptors Attenuates the HVR Depression Induced by Systemic DAMGO

Systemic administration of DAMGO significantly depressed the baseline V_E by 15% (208 ± 26 *vs.* 177 ± 30 ml/min, $P =$

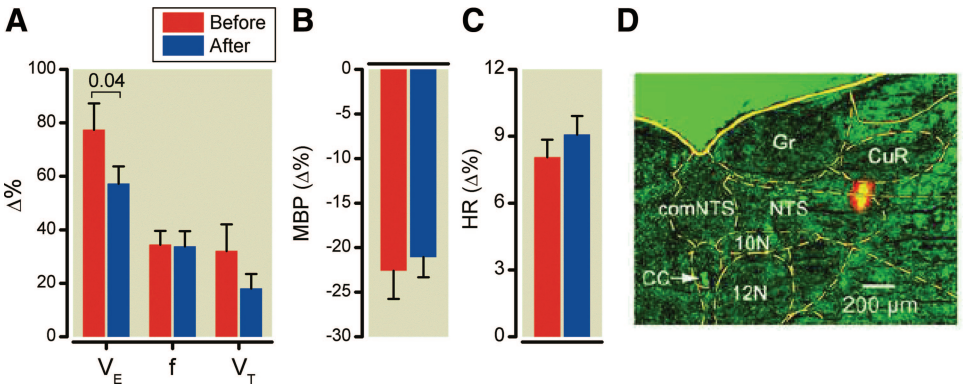

Fig. 5. The respiratory (*A*), mean blood pressure (*B*), and heart rate (*C*) responses to the brief hypoxia before and after 2.5 mM DAMGO was injected into the sites 1 mm lateral to the calamus scriptorius (n = 6). Data are presented as means ± SE. $P < 0.05$ are presented exactly and those more than 0.88 are not presented. f = breath frequency; HR = heart rate; MBP = mean blood pressure; V_E = minute ventilation; V_T = tidal volume. (*D*) A representative slice showing the location of injection 1 mm right to the comNTS stained by fluorescent microbeads (*red*). CC = central canal; comNTS = commissural part of the nucleus of the solitary tract (NTS); CuR = cuneate nucleus, rotundus part; DAMGO = [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; Gr = gracile nucleus; 10N = dorsal vagal motor nucleus; 12N = hypoglossal nucleus.

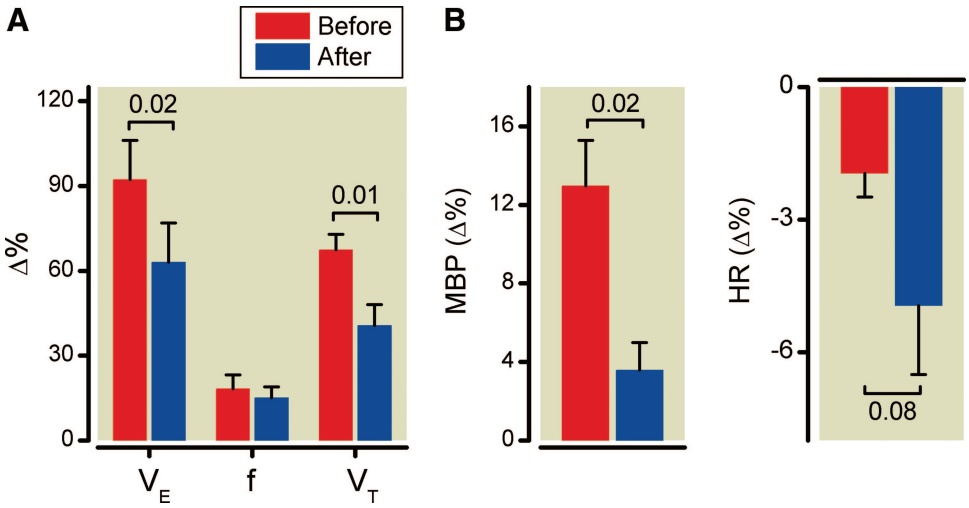

Fig. 6. The respiratory (*A*) and cardiovascular (*B*) responses to 15% CO_2 for 10 s before and after 2.5 mM DAMGO was injected into the commissural part of the nucleus of the solitary tract (comNTS). All of the cardiorespiratory variables significantly changed during hypercapnia exposure compared with the baseline level. n = 5; data are presented as means ± SE; *P* values ≤ 0.08 are presented exactly and those more than 0.75 are not presented. DAMGO = [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; f = breath frequency; HR = heart rate; MBP = mean blood pressure; V_E = minute ventilation; V_T = tidal volume.

0.01) due to the inhibition of f (110 ± 11 *vs.* 98 ± 11 breaths/min, $P = 0.03$) with little effect on V_T (1.83 ± 0.18 *vs.* 1.76 ± 0.23 ml, $P = 0.64$). This baseline V_E depression was not significantly changed by pretreating the comNTS with CTAP (−15 ± 3% *vs.* −13 ± 4%, $P = 0.86$). As illustrated in figure 9A, the brief hypoxia evoked an 85% increase in V_E, and this response was reduced to 30% after intravenous administration of DAMGO (65% depression of the HVR). Interestingly, this DAMGO-induced HVR depression (65%) became much smaller (13%) after pretreating the comNTS with CTAP. In other words, 80% of the HVR depression by intravenous administration of DAMGO was prevented by blocking comNTS μ-receptors. In contrast with the HVR, the same intravenous administration of DAMGO only decreased HCVR by 17% that was not significantly altered by blocking comNTS μ-receptors (fig. 9B). Because there was a 2-h interval between the first and second systemic injection of DAMGO, we tested the reproducibility of the effect of DAMGO over 2 h in three other rats. We found no remarkable differences between the first and second systemic DAMGO-induced depression in V_E during control (−18 ± 3% *vs.* −17 ± 3%), hypoxia (−67 ± 9% *vs.* −65 ± 8%), and hypercapnia (−16 ± 3% *vs.* −18 ± 3%).

Discussion

The major finding in this study is that, similar to bilaterally sectioning the CSN, microinjecting DAMGO into the comNTS totally abolishes the HVR but it mildly depresses the HCVR. Furthermore, blocking comNTS μ-receptors significantly attenuates the HVR depression induced by systemic administration of DAMGO.

Opioids reportedly are able to greatly depress the HVR in humans and animals[11,13,31–33] and even eliminate the HVR in some subjects.[34] Although an early report[35] showed that intracarotid injection of opioids had an inhibitory effect on CSN activity in anesthetized cats, recent studies point to a central inhibitory effect of opioids on the HVR.[14,36,37] To date, the key central sites responsible for this inhibition remain unknown. In the current study we found that the HVR was largely depressed (64%) by microinjecting 0.25 mM DAMGO into the comNTS, and eliminated by microinjecting 2.5 mM DAMGO. We also found that systemic DAMGO significantly depressed the HVR by 65% that was eliminated by blocking comNTS μ-receptors, indicating a key role that comNTS μ-receptors play in depressing the carotid body-mediated HVR induced by systemic administration of DAMGO. This finding is consistent with the highly expressed μ-opioid receptors in this area[15–17] and a key role this area plays in generating the HVR.[2,3,10]

An interesting issue is why such a small volume of DAMGO (approximately 3.5 nl) has the ability to totally abolish the HVR. It is well documented that the input from the carotid body terminates in the comNTS,[6–9] more accurately in the area tentatively called the "chemoreceptor projection site." The latter is identified within the region 0.2 mm rostral to 0.5 mm caudal, 0–0.5 mm lateral, and 0.3–0.5 mm deep to the calamus scriptorius.[5,10,28] Although the actual spread area of DAMGO is unknown in our study, it should be larger than the spread area of the fluorescent microbeads (approximately 200 μm), especially when a relative high concentration (2.5 mM) was used and the HVR measured 5 min after microinjection according to a previous report.[38] In agreement with our result, Vardhan *et al.*[10] reported that the maximum ventilatory excitatory responses were obtained when glutamate was microinjected into a subregion of the comNTS 200–300 μm around the midline.

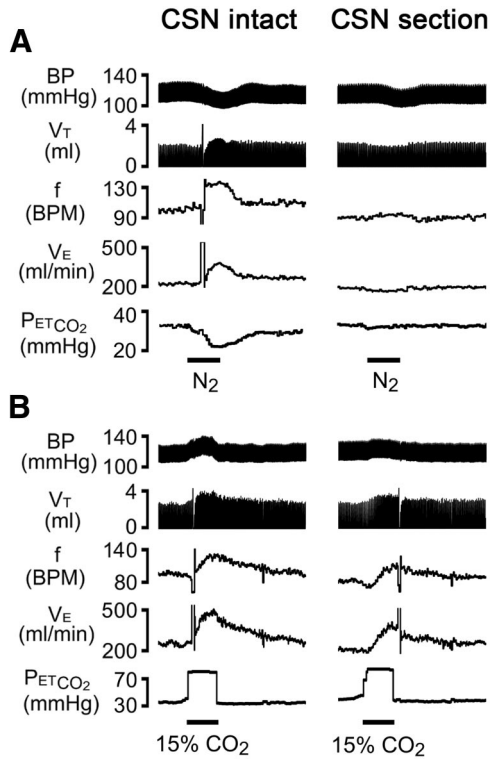

Fig. 7. Representative recordings showing the effects of bilateral section of carotid sinus nerves (CSN) on the cardiorespiratory responses to the brief hypoxia (*A*) and hypercapnia (*B*) in an anesthetized rat. Traces in sequence are arterial blood pressure (blood pressure), heart rate (HR), tidal volume (V_T), respiratory frequency (f, BPM = breaths/min), minute ventilation (V_E), and end-tidal pressure of carbon dioxide (P_{ETCO_2}), respectively.

Several investigators pointed out that a large lesion of the nucleus tractus solitarius, including the comNTS, caused from microinjection of approximately100 nl kainic or domoic acid, did not abolish but markedly reduced (70%) the HVR in rats.[2,3] One may question why HVR abolition is not induced by such a big chemical lesion but rather is produced by the limited volume of DAMGO microinjected into the comNTS. This paradox could be due to the fact that both kainic and domoic acid have a very high affinity for kainate receptors, intermediated affinity for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (also known as AMPA receptor), and low affinity for the *N*-methyl-D-aspartate receptor (also known as NMDA receptors).[39] Therefore, these acids might be insufficient to kill all key comNTS neurons, especially those expressing the *N*-methyl-D-aspartate receptors that are crucial in generating the HVR.[4]

The most likely mechanism by which DAMGO reduces and even abolishes the HVR, based on several lines of evidence, is that the activating comNTS μ-receptors with DAMGO blocks the afferent inputs from the carotid body. First, it has been established that the afferents from the carotid chemoreceptor make their initial central synapses predominantly in the comNTS,[6–9] which heavily expresses μ-receptors.[15–17] Second, the changes in the carotid body-mediated cardiorespiratory responses to hypoxia and hypercapnia induced by microinjecting DAMGO into the comNTS are highly similar to that by transecting the CSN in our study and others.[40–42] Third, we found that intra-comNTS microinjection of DAMGO no longer affected V_E during brief hypoxia in the CSN sectioned rats. Fourth, blocking glutamate receptors in the comNTS abolished the carotid body-mediated ventilatory responses.[10] More importantly, μ-receptor agonists can inhibit and even abolish the glutamate-mediated excitatory synaptic transmission in the nucleus tractus solitarius in a dose-dependent manner[21,43] and hyperpolarize most neurons tested in the nucleus tractus solitarius.[21] On the other hand, the comNTS contains respiratory-modulated neurons[44,45]; thus, we cannot rule out the possibility that the inhibitory effect of DAMGO on these neurons contributes to the depressed HVR. This inhibitory effect may account for some differences between the respiratory responses evoked by microinjecting DAMGO into the comNTS and bilaterally transecting the CSN. For example, microinjecting DAMGO abolishes the HVR *via* elimination of both the V_T and f responses, whereas transecting the CSN abolishes the HVR *via* elimination of the V_T and great reduction of the f response. Moreover, microinjection of DAMGO into the comNTS still depressed the baseline V_E after bilateral CSN section. Nevertheless, it is unlikely that the HVR abolition is the result of opioids' inhibitory effect on local respiratory-modulated neurons alone because of the widespread distribution of these neurons in the medulla and pons.[46–48] Because the HVR is mediated by carotid body afferents releasing glutamate in the comNTS,[1,10] further studies are needed to explore the role of local μ-receptors in control of the neurotransmission and carotid body second-order neuronal activity.

The brief hypercapnia was designed mainly to stimulate the carotid body in this study. Unlike the ventilatory response to brief hypoxia, the ventilatory response to brief hypercapnia (15% CO_2 for 10 s) was depressed (by 20%) but not abolished by bilateral transection of the CSN. This result supports a much less important role for the carotid body in controlling the HCVR than the HVR, as reported by other studies.[49,50] On the other hand, hyperoxia capable of attenuating the carotid body sensitivity to hypercapnia[51] was used as a baseline control in our study. Thus, it is also possible that the less important role of the carotid body observed here is partially due to the hyperoxia. Our data showed that after microinjecting DAMGO into the comNTS the brief hypercapnia-induced HCVR was reduced by 31%, which is slightly, but significantly, greater than the reduction (20%) produced by CSN transection. This greater inhibition likely results from the inhibitory effect DAMGO has on comNTS carbon dioxide -chemosensitive neurons, which is again supported by our observation that intracomNTS injection of DAMGO still depressed the HCVR by 17% in CSN-sectioned rats. Although hypercapnic exposure is transient (for

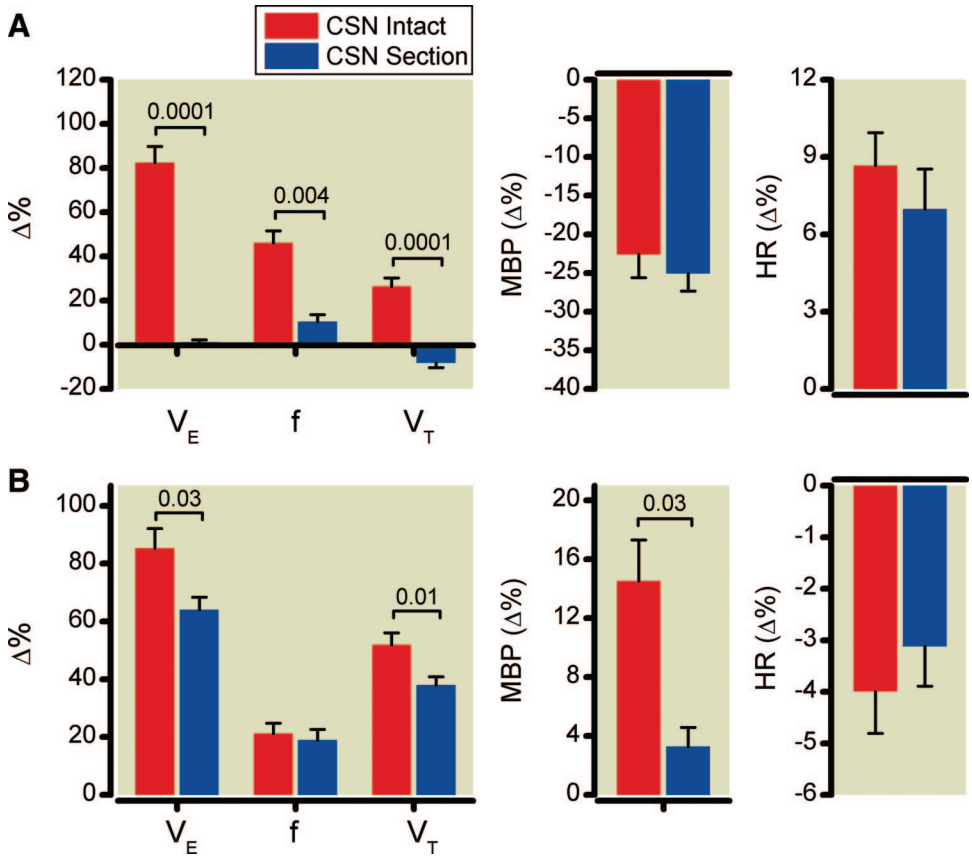

Fig. 8. Group data showing the effects of bilateral section of carotid sinus nerves (CSN) on the cardiorespiratory responses to the brief hypoxia (*A*) or hypercapnia (*B*). Note: All the cardiorespiratory variables during hypoxia or hypercapnia were significantly changed with the exception that V_E and V_T during hypoxia exposure were not significantly different from baseline after sectioning the CSN. n = 10; data are presented as means ± SE; *P* values less than 0.05 are presented exactly and those more than 0.82 are not presented. f = breath frequency; HR = heart rate; MBP = mean blood pressure; V_E = minute ventilation; V_T = tidal volume.

10 s) in our study, due to the high permeability of carbon dioxide to the blood-brain barrier,[52] the brief hypercapnia might also stimulate central carbon dioxide-chemosensitive neurons in addition to stimulating the carotid body. In fact, the comNTS contains carbon dioxide-chemosensitive neurons,[5,53] and the HCVR is depressed by activating μ-receptors in other carbon dioxide-chemosensitive areas such as the raphe.[25]

In this study, microinjecting a high dose of DAMGO into the comNTS increased blood pressure without changing HR. This pressor response clearly cannot be interpreted from the blockade of the CSN's input, as CSN denervation failed to significantly change cardiovascular variables and intra-comNTS injection of DAMGO still increased blood pressure after the CSN transection. In fact, a much larger dose and volume of DAMGO (3 mM, 100 nl) injected into the comNTS increased blood pressure and HR by approximately 70% and 14%, respectively, in the rats.[54] DAMGO induced the pressor response presumably *via* sympathetic pathways rather than parasympathetic pathways because all cardiovascular responses elicited by DAMGO were eliminated by complete C1 spinal transection but not by vagotomy.[54] Similar to the HVR abolition, the pressor response to DAMGO appears to be site specific because the same microinjections made outside of the comNTS failed to produce this response.

There are three major concerns in this study. First, we cannot rule out the interaction of the anesthetic and DAMGO. However, abolishing the HVR clearly is not the direct result of anesthesia. Second, intracomNTS DAMGO injection depressed the cardiovascular response to hypercapnia but not hypoxia. This difference may be due to the fact that hypotension and tachycardia in response to hypoxia is less dependent on the CNS. Extensive study has shown that systemic hypoxia causes vasodilatation mainly through the peripheral release of adenosine from the endothelium.[55] In contrast, both bilateral transection of the CSN and intra-comNTS DAMGO injection significantly decreased the hypercapnia-induced hypertension in our study. This finding points to the comNTS's involvement in this pressor response, consistent with the role of the brainstem, especially the nucleus tractus solitarius, in cardiovascular regulation,[54] including the hypercapnia-induced pressor response.[56] Third, the doses of μ receptor agonists that could sufficiently depress respiration are different in rodents and humans[57,58];

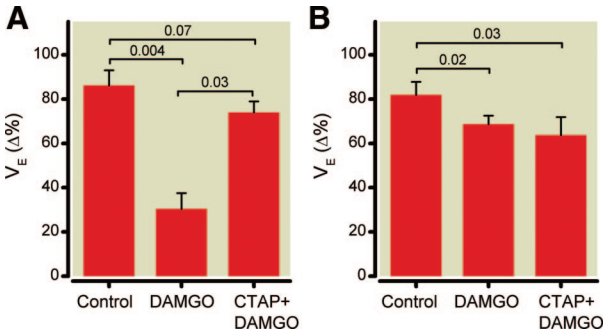

Fig. 9. Group data showing the effects of CTAP blocking comNTS μ-receptors on the depressed HVR (*A*) and HCVR (*B*) induced by systemic DAMGO. Control, averaged controls for DAMGO and CTAP+DAMGO (no difference between them); DAMGO, intravenous administration of DAMGO; CTAP+DAMGO, microinjection of CTAP into the comNTS before systemic DAMGO. n = 6; data are mean ± SE; *P* values ≤0.07 are presented exactly and those more than 0.94 are not presented. comNTS = the commissural subnucleus of the nucleus tractus solitarius; CTAP = d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH2; DAMGO = [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; HCVR = hypercapnic ventilatory response; HVR = hypoxic ventilatory response; V_E = minute ventilation.

therefore, the clinical relevance of the mechanism underlying the DAMGO-induced HVR depression in rats awaits further clarification.

In summary, our results show that microinjecting a limited volume (less than 4 nl) of high doses of DAMGO into the comNTS of anesthetized rats is capable of abolishing the HVR and mildly depressing the HCVR, which is similar to bilaterally sectioning the CSNs. Moreover, blocking comNTS μ-receptors significantly attenuates the HVR depression induced by the systemic administration of DAMGO. These results suggest a key role comNTS μ-receptors play in controlling the HVR.

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