ANESTHESIOLOGY

Dose-dependent Respiratory Depression by Remifentanil in the Rabbit Parabrachial Nucleus/Kölliker–Fuse Complex and Pre-Bötzinger Complex

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Opioid-sensitive inputs to respiratory rate and rhythm originate in the pre-Bötzinger complex, the parabrachial nucleus, and the Kölliker–Fuse nucleus

What This Article Tells Us That Is New

- The hypothesis that opioid-induced respiratory depression is due to combined depression of parabrachial nucleus/Kölliker–Fuse complex activity and pre-Bötzinger complex activity was tested in a decerebrate, hyperoxic, and moderately hypercapnic rabbit preparation at steady-state intravenous remifentanil infusions that depressed the respiratory rate by 50% and after a remifentanil bolus that produced apnea

- Sequential naloxone microinjection into the bilateral Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex did not completely reverse respiratory depression produced by the steady-state remifentanil concentrations and reversed respiratory depression from apneic remifentanil doses even less effectively

- This suggests that opioids depress respiratory drive to the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex and that depression of drive reduced the activity of these areas, especially at high opioid concentrations

ABSTRACT

Background: Recent studies showed partial reversal of opioid-induced respiratory depression in the pre-Bötzinger complex and the parabrachial nucleus/Kölliker–Fuse complex. The hypothesis for this study was that opioid antagonism in the parabrachial nucleus/Kölliker–Fuse complex *plus* pre-Bötzinger complex completely reverses respiratory depression from clinically relevant opioid concentrations.

Methods: Experiments were performed in 48 adult, artificially ventilated, decerebrate rabbits. The authors decreased baseline respiratory rate ~50% with intravenous, "analgesic" remifentanil infusion or produced apnea with remifentanil boluses and investigated the reversal with naloxone microinjections (1 mM, 700 nl) into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex. In another group of animals, naloxone was injected only into the pre-Bötzinger complex to determine whether prior parabrachial nucleus/Kölliker–Fuse complex injection impacted the naloxone effect. Last, the μ-opioid receptor agonist [D-Ala,^{2}N-MePhe,^{4}Gly-ol]-enkephalin (100 μM, 700 nl) was injected into the parabrachial nucleus/Kölliker–Fuse complex. The data are presented as medians (25 to 75%).

Results: Remifentanil infusion reduced the respiratory rate from 36 (31 to 40) to 16 (15 to 21) breaths/min. Naloxone microinjections into the bilateral Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex increased the rate to 17 (16 to 22, $n = 19$, $P = 0.005$), 23 (19 to 29, $n = 19$, $P < 0.001$), and 25 (22 to 28) breaths/min ($n = 11$, $P < 0.001$), respectively. Naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex prevented apnea in 12 of 17 animals, increasing the respiratory rate to 10 (0 to 12) breaths/min ($P < 0.001$); subsequent pre-Bötzinger complex injection prevented apnea in all animals (13 [10 to 19] breaths/min, n = 12, $P = 0.002$). Naloxone injection into the pre-Bötzinger complex alone increased the respiratory rate to 21 (15 to 26) breaths/min during analgesic concentrations (n = 10, $P = 0.008$) but not during apnea (0 [0 to 0] breaths/min, n = 9, $P = 0.500$). [D-Ala,^{2}N-MePhe,^{4}Gly-ol]-enkephalin injection into the parabrachial nucleus/Kölliker–Fuse complex decreased respiratory rate to 3 (2 to 6) breaths/min.

Conclusions: Opioid reversal in the parabrachial nucleus/Kölliker–Fuse complex *plus* pre-Bötzinger complex only partially reversed respiratory depression from analgesic and even less from "apneic" opioid doses. The lack of recovery pointed to opioid-induced depression of respiratory drive that determines the activity of these areas.

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R ecent studies of opioid-induced respiratory depression have expanded the focus from the respiratory rhythm generator in the pre-Bötzinger complex to areas that provide inputs to those pre–Bötzinger complex neurons that mediate inspiratory on- and off-switch and thus

determine inspiratory and expiratory phase duration.[1] We have previously shown in acute *in vivo* rabbit studies that naloxone injection into the parabrachial nucleus partially reversed respiratory rate depression from "analgesic" remifentanil concentrations,[2] whereas injections into the pre-Bötzinger complex did not.[3] These results were supported by similar studies in dogs.[4,5] Our last study supported the idea of an additional opioid-sensitive source of inputs to the respiratory rhythm generator outside of the parabrachial nucleus and pre-Bötzinger complex.[2] Since then, the importance of the Kölliker–Fuse nucleus for the control of respiratory phase duration,[6–8] as well as for opioid-induced respiratory depression,[9–11] has been highlighted by multiple investigators. In particular, a novel mouse model showed that respiratory depression including from high morphine doses was substantially attenuated when μ-opioid receptors were knocked out selectively in Kölliker–Fuse nucleus neurons.[9–11]

We recently showed in our *in vivo* rabbit model that glutamatergic disfacilitation of the parabrachial nucleus *and* Kölliker–Fuse nucleus was necessary to achieve maximal respiratory rate depression.[8] We thus hypothesized that opioid-induced respiratory depression was due to combined depression of parabrachial nucleus *and* Kölliker–Fuse nucleus activity. After initial experiments showed that the opioid effect could not be fully reversed in these areas, we extended our hypothesis to include the pre-Bötzinger complex. In contrast to our previous studies that used *non-vagotomized* animals,[2,3] the current experiments were performed in *vagotomized* animals to prevent the possibility that small changes in respiratory parameters were confounded by ventilator-induced respiratory rate entrainment.

We used local microinjections of the opioid antagonist naloxone into the bilateral Kölliker–Fuse nucleus, parabrachial nucleus, *and* pre-Bötzinger complex to antagonize the respiratory depression from intravenous remifentanil. To determine whether the magnitude of opioid reversal depended on the opioid dose,[11] we used both analgesic concentrations (*i.e.*, steady-state remifentanil infusions that depressed respiratory rate by ~50%),[2,3,11–14] as well as "apneic" concentrations (*i.e.*, a remifentanil bolus that just resulted in apnea under control conditions).[10] To clarify whether the observed pre-Bötzinger complex effect depended on prior opioid reversal in the parabrachial nucleus/Kölliker–Fuse complex, we added experiments where we injected naloxone solely into the pre-Bötzinger complex. Once we had determined that naloxone reversal of the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex prevented apnea from the apneic remifentanil bolus, we further investigated whether naloxone injection also prevented apnea from very high remifentanil concentrations (*i.e.*, up to 10 times the threshold apneic bolus).

Last, to gauge the degree to which parabrachial nucleus/Kölliker–Fuse complex neurons could be depressed by μ-opioid receptor agonists, we injected the μ-opioid receptor agonist [D-Ala,²N-MePhe,⁴Gly-ol]-enkephalin (DAMGO) at high, supraclinical concentrations into the bilateral parabrachial nucleus/Kölliker–Fuse complex.

Materials and Methods

Surgical Preparation

The research was approved by the Subcommittee on Animal Studies of the Zablocki Veterans Affairs Medical Center, Milwaukee, Wisconsin, and the Institutional Animal Care and Use Committee, Medical College of Wisconsin, Milwaukee, Wisconsin, in accordance with provisions of the Animal Welfare Act, the Public Health Service Guide for the Care and Use of Laboratory Animals, and Veterans Affairs policy. Experiments were carried out on adult (3 to 4 kg), pathogen-free, New Zealand White rabbits of either sex. Anesthesia was induced with 5 vol% sevoflurane *via* facemask and ventilated *via* tracheotomy with an anesthesia machine (Ohmeda CD, GE, Datex Ohmeda, USA). Anesthesia was maintained with 1.5 to 3% isoflurane. Inspiratory oxygen fraction, expiratory carbon dioxide concentration and expiratory isoflurane concentration were continuously displayed with an infrared analyzer (POET II, Criticare Systems, USA). Skin was infiltrated with lidocaine 1% before each skin incision. Femoral arterial and venous lines were used for blood pressure monitoring, infusion of solutions, and bolus drug administration, respectively. Care was taken to increase anesthetic depth for any signs of "light anesthesia" (*e.g.*, an increase in blood pressure or lacrimation). Lactated Ringer's solution with 3 μg/ml epinephrine was continuously infused at 1 ml/h. At this rate, the infusion did not result in appreciable changes in heart rate and blood pressure from baseline. Infusion rate was increased as needed to counteract or prevent hypotension in response to drug injections and/or from blood loss but maintained as constant as possible during the recording phase. The animal was maintained at 37.0 ± 0.5°C with a warming blanket. The animal was placed in a stereotaxic frame (David Kopf Instruments, USA), and blunt precollicular decerebration with complete removal of the forebrain was performed through a parietal craniotomy. After decerebration, isoflurane was either discontinued or continued at subanesthetic levels (0.3 to 0.4 vol%) for blood pressure control. This sedative concentration equals ~0.2 minimum alveolar concentration,[15] which is associated with a decrease of 10% or less in respiratory rate and peak phrenic activity.[16,17] Volatile anesthetics add to but do not amplify the remifentanil effect[16] (*i.e.*, slight variation in isoflurane concentration between experiments would affect the baseline respiratory rate and peak phrenic activity, but not the dose dependency of the remifentanil effect). Isoflurane concentration was not changed during the experimental protocol. Decerebration eliminates the need for further general anesthesia, but it may reduce forebrain and midbrain inputs to the respiratory center and thus cause a minor increase in apneic

threshold.[18,19] The brainstem was exposed *via* occipital craniotomy and partial removal of the cerebellum. The animals were paralyzed with rocuronium (15 mg/kg subcutaneous bolus), followed by pancuronium 2 mg/h infusion to avoid motion artifacts during neural/neuronal recording. Bilateral vagotomy was performed to achieve peripheral deafferentation to avoid interference of the mechanical ventilation with the underlying central respiratory rhythm and respiratory neuronal activity. Respiratory rates after vagotomy were comparable to our previous studies in nonvagotomized animals.[2,3] The phrenic nerve and in some experiments the vagus nerve were recorded with fine bipolar electrodes through a posterior neck incision. The complete surgical preparation required 6 to 7 h.

Throughout the experiment, the animals were ventilated with a hyperoxic gas mixture (F_{IO_2} 0.6) to achieve functional denervation of the peripheral chemoreceptors and thus rule out that the observed drug effects were due to effects on the carotid bodies. Mild hypercapnia (expiratory carbon dioxide, 45 to 55 mmHg) was used to emulate the hypercarbia encountered clinically during opioid use in patients. Mild hypercapnia may also have compensated for the loss of respiratory drive with decerebration because respiratory rates were similar to rabbit preparations using normocapnia in anesthetized, nondecerebrate preparations.[12,20] Blood pressure was maintained stable throughout the experiment by adjusting the intravenous infusion rate. At the end of the experiment, the animals were euthanized with intravenous potassium chloride.

Neuronal Recording, Microinjection Procedures, and Measured Variables

All neuronal recording and microinjection techniques have been well established by our research group and have been previously described in detail.[21,22] In short, extracellular neuronal recordings were obtained using multibarrel micropipettes (20- to 40-μm tip diameter) consisting of three drug barrels and a recording barrel containing a 7-μm-thick carbon filament. The barrels were filled with the glutamatergic agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 50 μM, 70 nl/injection) and the opioid receptor antagonist naloxone (1 mM, 700 nl/injection), which were dissolved in artificial cerebrospinal fluid. The microinjected volume was determined *via* height changes in the meniscus in the respective pipette barrel with a 100× monocular microscope and calibrated reticule (resolution ~3.5 nl). Respiratory neuronal discharge was recorded extracellularly from neuronal aggregates and individual neurons and classified by the temporal relationships relative to the phrenic neurogram. The neuronal and neural activity and pressure microejection marker signals were recorded using a digital acquisition system. These variables were also continuously displayed and recorded along with the phrenic neurogram, vagal neurogram, discharge rate meter, respiratory rate, arterial blood pressure, and airway carbon dioxide concentration on a computerized chart recorder (Powerlab/16SP; ADInstruments, Australia). Before and after drug injection, steady-state conditions were obtained for respiratory parameters. Postexperiment LabChart data were exported to SigmaPlot 11 (Systat Software, USA) for data reduction, data plotting, and statistical analysis. Between 10 and 50 consecutive respiratory cycles were averaged over 1 to 2 min with the number of cycles dependent on the respiratory rate. Using the phrenic neurogram, we determined the respiratory rate, inspiratory and expiratory duration, and peak phrenic activity. Using the vagal neurogram, we determined peak vagus activity. In rabbits, inspiratory phase timing of the vagal neurogram closely matches the phrenic neurogram without the postinspiratory activity typically observed in rats[10] but with minor activity during midexpiration. Peak vagus activity was calculated as the amplitude between minimal vagus nerve activity before the start of inspiration and peak vagus nerve activity during inspiratory phase. Because changes in peak phrenic activity closely reflect changes in respiratory tidal volume but the absolute value does not correspond with the absolute tidal volume,[23] peak phrenic activity and peak vagus activity were normalized to the respective control values for all calculations.

Identification of the Parabrachial Nucleus, Kölliker–Fuse Nucleus, and Pre-Bötzinger Complex

We previously characterized the locations of the Kölliker–Fuse nucleus, parabrachial nucleus,[8] and pre-Bötzinger complex[22] in our model through stereotaxic coordinates, neuronal recordings, and typical respiratory rate response to AMPA injection. For protocols investigating only the parabrachial nucleus and Kölliker–Fuse nucleus, we inserted the micropipette in a grid-wise fashion starting at the caudal end of the inferior collicle at 1.5 mm lateral from midline and moved lateral and caudal with 0.5-mm steps (0.47 mm rostro-caudal, corrected for the 20° angle of the stereotaxic frame). In areas where neuronal activity was encountered, we microinjected AMPA (50 μM, 70 nl) starting at the ventral limit of the tonic neuronal activity and then in 1-mm steps more dorsally. The area of maximal AMPA-induced tachypnea was labeled as "parabrachial nucleus," and the area of maximal bradypnea was labeled as "Kölliker–Fuse nucleus" (fig. 1). The parabrachial nucleus was located 1.0 ± 0.9 mm caudal from the inferior collicle, 2.6 ± 0.7 mm lateral to midline, and 7.7 ± 1.9 mm ventral to the dorsal surface, and the Kölliker–Fuse nucleus was located 1.1 ± 0.2 mm caudal, 0.7 ± 0.3 mm lateral, and 1.8 ± 0.7 mm ventral to the parabrachial nucleus (n = 12). Because the parabrachial nucleus to Kölliker–Fuse nucleus distance was consistent and matched our previous study,[8] in later experiments we functionally identified the parabrachial nucleus and used the location 1 mm caudal, 0.5 mm lateral and, 2 mm ventral to the parabrachial nucleus for Kölliker–Fuse nucleus injections.
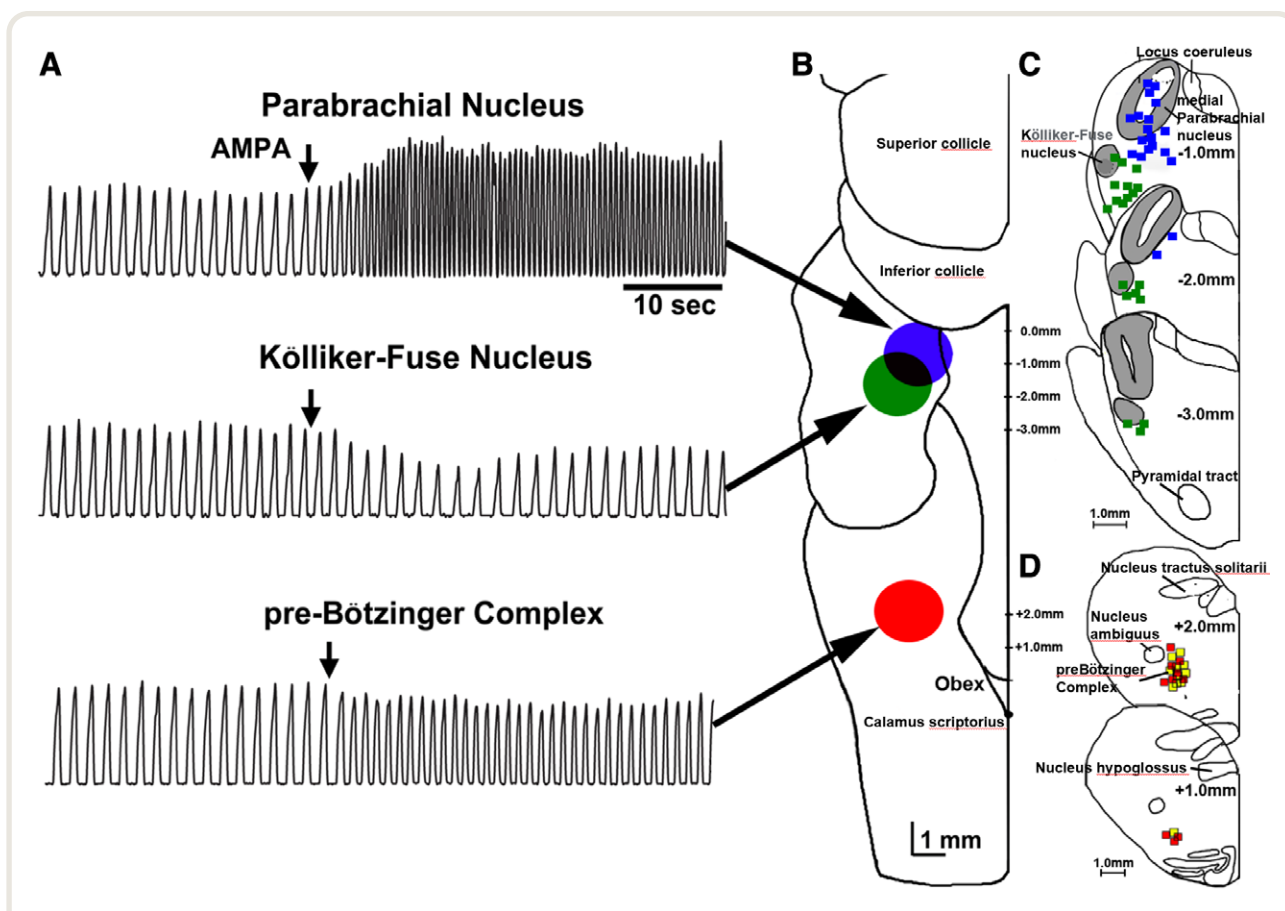
Fig. 1. Brainstem locations of naloxone microinjection. (*A*) Phrenic neurogram tracings illustrate the functional identification of the parabrachial nucleus, Kölliker–Fuse nucleus, and pre-Bötzinger complex through typical responses to injection of the glutamate receptor agonist α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; *vertical arrows*). (*B*) Dorsal view of the brainstem. Superimposed are the approximate distribution areas of the naloxone injections into the parabrachial nucleus (*blue*), Kölliker–Fuse nucleus (*green*), and pre-Bötzinger complex (*red*). We estimate an effective spherical diffusion radius for our injection volume of 1 to 1.2 mm.[22] There is little overlap between the parabrachial nucleus and Kölliker–Fuse nucleus injections in the brainstem, because the Kölliker–Fuse nucleus is located 2 mm ventral to the parabrachial nucleus. (*C*) Stereotaxic coordinates of the naloxone injection sites for parabrachial nucleus injections (*blue squares*) and Kölliker–Fuse nucleus injections (*green squares*), projected over coronal slices of the rostral pons. Coordinates for the bilateral injections were averaged for each animal. For clarity, injection sites less than 1.5 mm caudal to the inferior collicle are summarized in the slice labeled "-1.0mm"; coordinates between 1.5 and 2.5 mm caudal to the inferior collicle are summarized in the slice labeled "-2.0mm"; and injection sites 2.5 mm or more caudal to the inferior collicle are summarized in the slice labeled "-3.0mm." To account for residual cerebellar tissue covering the dorsal brainstem in our preparation, we subtracted 5 mm from the measured stereotaxic depth coordinate in all animals (*i.e.*, the depicted depth of injection is an approximation). (*D*) Stereotaxic coordinates of the naloxone injections into the pre-Bötzinger complex after injection into the parabrachial nucleus and Kölliker–Fuse nucleus (*yellow squares*, cohort A) or solely into the pre-Bötzinger complex (*red squares*, cohort B), projected over coronal slices of the caudal medulla oblongata. Coordinates for the bilateral injections were averaged for each animal. Injection sites less than 1.5 mm rostral to obex are summarized in the slice labeled "+1.0mm." Injection sites 1.5 mm or more rostral to obex are summarized in the slice labeled "+2.0mm." Please see "Identification of the Parabrachial Nucleus, Kölliker–Fuse Nucleus, and Pre-Bötzinger Complex" for the average stereotaxic coordinates. The outlines of the maps are redrawn from histologic sections obtained for our previous studies in adult rabbits.[8,22] The atlas of Meessen and Olszewski[46] was used for comparison. Please note the different scale for (*D*).

For protocols including the pre-Bötzinger complex, we identified the pre-Bötzinger complex as the area with inspiratory and expiratory neuronal activity where AMPA injection caused maximal tachypnea (fig. 1). The pre-Bötzinger complex was located on average 2.1 ± 0.7 mm rostral to obex, 2.7 ± 0.4 mm lateral from midline and 5.0 ± 0.5 mm ventral to the dorsal surface (*n* = 32). The parabrachial nucleus was located 9.6 ± 0.7 mm rostral to the pre-Bötzinger complex at the same distance lateral from midline and, dependent on the thickness of the residual cerebellar peduncle, 3.2 ± 1.2 mm ventral to the pre-Bötzinger complex (*n* = 23). Complete, bilateral functional identification of all areas including the

time required for respiratory rate to return to baseline after each AMPA injection required 4 to 5h.

Opioid Effect Sites at Analgesic IV Remifentanil Concentrations

Experimenters were not blinded to the experimental conditions, and we did not perform formal randomization to experimental protocols. Because the effect of naloxone microinjection persists more than 2h, only one complete protocol was performed per animal.

To determine how much opioid effects in the Kölliker–Fuse nucleus and parabrachial nucleus contributed to systemic opioid-induced respiratory depression at analgesic opioid doses, we infused remifentanil intravenously at $0.15 \pm 0.08\ \mu g \cdot kg^{-1} \cdot min^{-1}$ until the respiratory rate was depressed by approximately 50% (fig. 2). In rabbits, a ~50% respiratory rate depression was associated with loss of response to ear pinch and pedal withdrawal reflex.[14,24] Its fast onset and short half-life that is independent of the duration of infusion[12,25] make remifentanil the ideal drug to investigate opioid effects at steady-state concentrations. After reaching steady-state effect for 10 to 15 min, naloxone (1 mM,

700 nl) was microinjected bilaterally into the Kölliker–Fuse nucleus, and 5 min were allowed to obtain maximal effect. Subsequently, naloxone was injected into the bilateral parabrachial nucleus. After interim analysis revealed that naloxone injections into the Kölliker–Fuse nucleus and parabrachial nucleus did not lead to complete reversal of respiratory rate depression, for all subsequent protocols, we additionally injected naloxone into the bilateral pre-Bötzinger complex. Volume and timing for Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex microinjections had been established in previous studies.[8,22] Animals in which naloxone was injected into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex are included in cohort A.

To determine whether the effect of pre-Bötzinger complex injections depended on prior naloxone reversal in the parabrachial nucleus and Kölliker–Fuse nucleus, in a separate set of animals (cohort B), we infused remifentanil until steady-state and injected naloxone solely into the pre-Bötzinger complex. At the end of the experiments, naloxone (15 to 40 μg/kg) was injected intravenously to document that the respiratory rate returned to preremifentanil control. We have previously described that artificial cerebrospinal
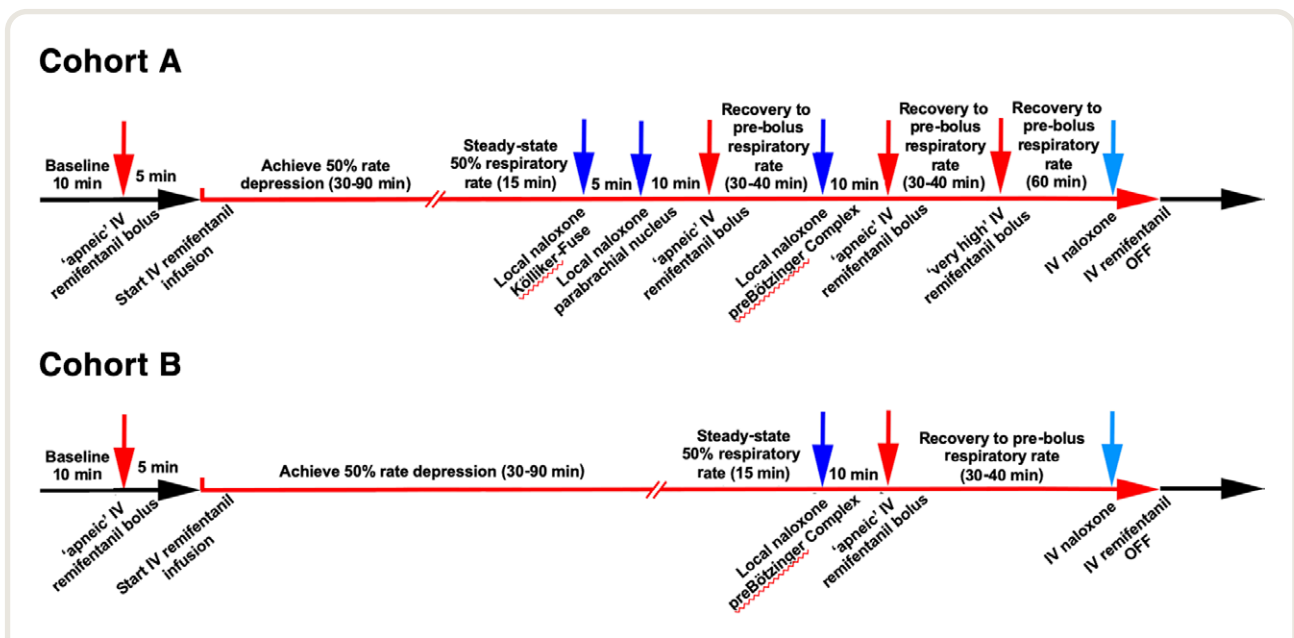
Fig. 2. Injection sequence for intravenous remifentanil and local naloxone microinjections. The initial intravenous remifentanil bolus (*red arrow*) was chosen to cause apnea for more than 30 s. Once the respiratory activity returned, the remifentanil infusion was started (*red line*). In many animals, one or more adjustments of the infusion rate were necessary to achieve the targeted respiratory rate depression of 50%. Steady-state respiratory rate depression was confirmed for 15 min before the start of the brainstem injections. The remifentanil dose rate was continued unchanged throughout the entire injection sequence. For cohort A, naloxone (*blue arrows*) was microinjected into the bilateral Kölliker–Fuse nucleus and parabrachial nucleus in 19 animals and subsequently into the pre-Bötzinger complex in 12 of these animals. Apneic IV remifentanil boluses (*red arrows*) were given after naloxone injections into the pons and after injection into the pre-Bötzinger complex. After each IV remifentanil bolus, we awaited recovery of the respiratory rate to the prebolus level before subsequent injections. In six animals, we also tested a very high IV remifentanil dose. For cohort B, naloxone was microinjected only into the bilateral pre-Bötzinger complex. Because the subsequent apneic IV remifentanil bolus continued to cause apnea in the majority of animals, no very high IV remifentanil bolus was given in this cohort.

fluid and naloxone injections into the parabrachial nucleus or pre-Bötzinger complex did not have any independent effects and did not repeat those control injections in the current study.[2,3,26]

Opioid Effect Sites at Apneic IV Remifentanil Concentrations

To determine whether the contributions of the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex to opioid-induced respiratory depression depended on the opioid concentration, we injected a remifentanil bolus that was sufficient to cause apnea for more than 30 s (fig. 2). Because the exact dose to achieve apnea varied between animals, we chose 10 μg (~3 μg/kg) as a standard dose. However, boluses were repeated with larger doses when the initial bolus did not result in apnea. Because repeat doses required return of the respiratory rate to baseline, this added 30 to 60 min to the experiment. In cohort A, the apneic bolus was repeated after naloxone injection into the parabrachial nucleus/Kölliker–Fuse nucleus and again after naloxone injection into the pre-Bötzinger complex. In cohort B, the apneic bolus was repeated after naloxone injection solely into the pre-Bötzinger complex.

To compare the effect sites of analgesic and apneic remifentanil concentrations *in the same animals*, the entire experimental sequence consisted of the apneic intravenous remifentanil bolus, after which we started the remifentanil infusion and waited until respiratory rate reached steady-state depression (~50% control) for 10 to 15 min. If respiratory rate was substantially higher or lower than 50%, we adjusted the remifentanil infusion rate and waited until a new steady-state was obtained for more than 10 min (*i.e.*, for an additional 20 to 30 min). Once a satisfactory infusion rate was established, the rate was not changed throughout the entire naloxone injection protocol. In cohort A, at steady-state respiratory depression, we performed bilateral naloxone microinjections into the Kölliker–Fuse nucleus and, after a 5-min wait, into the parabrachial nucleus. When respiratory parameters had reached steady state after the parabrachial nucleus injection (5 to 10 min), we repeated the apneic bolus. The concurrent remifentanil infusion meant that the plasma concentrations after the repeat apneic bolus were somewhat higher than after the initial bolus; however, the time requirement to discontinue the remifentanil infusion, inject and recover from the apneic bolus, restart the infusion, and achieve the same steady-state conditions as before would have been prohibitive. To obtain paired data for analgesic and apneic concentrations, we chose to continue the remifentanil infusion. After the apneic bolus we waited for respiratory parameters to recover to pre–bolus values and performed bilateral naloxone microinjections into the pre-Bötzinger complex. After the effects of the naloxone injection had reached steady state (5 min), we repeated the apneic remifentanil bolus. After recovery of the respiratory rate to the prebolus rate, we injected naloxone

intravenously to document that the control respiratory rate had not changed. Only then was the remifentanil infusion discontinued.

In cohort B, the initial remifentanil bolus and infusion rate were determined in the same fashion, but naloxone was injected only into the pre-Bötzinger complex. The entire experiment including surgical preparation, functional identification of the injection sites, and the remifentanil/naloxone protocol required 16 to 18 h. Depending on whether the experiments required functional identification of the parabrachial nucleus and Kölliker–Fuse nucleus in addition to the pre-Bötzinger complex, the first remifentanil injection occurred between 6 and 8 PM, and the experiments often lasted past midnight. Hemodynamics, end-tidal carbon dioxide, and nerve recordings were remarkably stable throughout the entire experiment.

Effects of Very High Remifentanil Concentrations on Areas outside the Parabrachial Nucleus/Kölliker–Fuse complex and Pre-Bötzinger Complex

Initial experiments showed that naloxone injection into the parabrachial nucleus/Kölliker–Fuse nucleus and pre-Bötzinger complex reliably prevented apnea from the apneic remifentanil bolus (10 μg). We sought to determine whether naloxone reversal of these areas would be able to prevent apnea including from very high remifentanil doses or whether very high remifentanil doses would also depress other respiratory-related areas (*e.g.*, respiratory drive to the parabrachial nucleus/Kölliker–Fuse nucleus and pre-Bötzinger complex or respiratory motor output). In a subgroup of cohort A, we completed the naloxone injection sequence into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex to test the reversal of analgesic and apneic remifentanil concentrations as described under "Opioid Effect Sites at Analgesic IV Remifentanil Concentrations" and "Opioid Effect Sites at Apneic IV Remifentanil Concentrations." After the final apneic remifentanil bolus, we allowed the respiratory rate to recover to prebolus values with the remifentanil infusion running. We then injected 10- to 50-μg bolus doses of remifentanil intravenously in short sequence until we observed apnea in the phrenic neurogram or to a maximal dose of 100 μg of remifentanil. For this analysis, we also determined peak vagus activity.

Effects of Supraclinical Opioid Concentrations, Compared to Glutamate Receptor Blockade in the Parabrachial Nucleus and Kölliker–Fuse Nucleus

We investigated whether maximal opioid receptor activation in the parabrachial nucleus and Kölliker–Fuse nucleus resulted in similar effects as disfacilitating neuronal activity with glutamate antagonist injection.[8] In a separate set of animals, we injected the μ–opioid receptor agonist DAMGO at supraclinical concentrations (100 μM, 700 nl) bilaterally

into the functionally identified parabrachial nucleus and Kölliker–Fuse nucleus. We statistically compared the opioid effects with results from our previous study in which parabrachial nucleus and Kölliker–Fuse nucleus activity was eliminated using microinjections of the non–*N*-methyl-D-aspartate (NMDA) receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 1 mM, 700 nl/injection) and NMDA receptor antagonist D(−)-2-amino-5-phosphonopentanoic acid (AP5; 5 mM, 700 nl/injection).[8]

Initial comparison of the two data sets showed a difference in baseline respiratory rate. Because both DAMGO and AP5/NBQX injections reduced the respiratory rate to the single digits, a difference in the change in respiratory parameters between groups could have been due to the difference in baseline rate. In response to peer review, we matched the baseline respiratory rates in both groups. We performed two additional experiments using DAMGO injections and excluded one animal with a respiratory rate of less than 20 breaths/min, and we excluded animals with respiratory rates of more than 37 breaths/min from the original AP5/NBQX data set.[6–8,27] Of note, the selection of data subsets may have biased the results toward the properties of these animals. In our adult outbred rabbit model, we observe a natural variation in baseline respiratory rate between ~20 and 40 breaths/min that remains remarkably constant over the course of many hours. Individual baseline rate does not clearly correlate with sex, age, or weight and varies even between animals of the same litter when experiments are performed in the same week. However, we cannot rule out that despite our efforts at consistency in surgical preparation and experimental conditions, respiratory control and rate were influenced by unrecognized factors that may have changed since our previous study.[6–8,27] It is also possible that pontine function is different in animals with very high or low baseline respiratory rates and that the interpretation of our data is limited to animals with baseline respiratory rates between 20 and 37 breaths/min.

Control Studies: Effects of Naloxone or Artificial Cerebrospinal Fluid Injections into the Parabrachial Nucleus, Kölliker–Fuse Nucleus, and Pre-Bötzinger Complex

To ensure that the effect of local naloxone microinjections we observed during remifentanil infusion represented a reversal of the extrinsic opioid effect rather than antagonism of endogenous opioid receptor activation or γ-aminobutyric acid type A (GABA$_A$) receptor antagonism,[28] we injected naloxone (1 mM, 700 nl) sequentially into the bilateral Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex. We also injected artificial cerebrospinal fluid (700 nl), which was used as solvent for all injected drugs, into the bilateral Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex to rule out an independent effect of the solvent.

Statistical Analysis

Statistical analysis was performed using SigmaPlot 11 (Systat Software, USA). We did not perform a formal power analysis, and no adjustments were made for interim analyses. Comparable studies have used 4 to 9 rats,[10,29] 4 to 11 mice,[11,13] 8 to 16 rabbits,[2,3] and 10 to 21 dogs[4,5] per protocol.

Because the experiments were technically very difficult and labor-intensive, we included data from a few animals where a single data point (injection of the apneic remifentanil dose into either the parabrachial nucleus/Kölliker–Fuse complex or pre-Bötzinger complex) was missing. The total number of animals is indicated for each comparison. To eliminate the problem of "missing values," we calculated the difference (Δ) for each variable between naloxone injection into the Kölliker–Fuse nucleus and IV remifentanil, between naloxone injection into the parabrachial nucleus and Kölliker–Fuse nucleus, between naloxone injection into the pre-Bötzinger complex and parabrachial nucleus, and between naloxone injection into the pre-Bötzinger complex and IV remifentanil. Testing revealed that not all Δ values were normally distributed (Shapiro–Wilk test). For paired data, we uniformly used the Wilcoxon Signed Rank test to test each Δ against no change (null hypothesis). Within the same protocol, we used the Mann–Whitney U test to compare the effects of naloxone injection into the pre-Bötzinger complex between animals in which naloxone had been previously injected into the parabrachial nucleus/Kölliker–Fuse complex and those in which naloxone was solely injected into the pre-Bötzinger complex (unpaired data). Hypothesis testing was two-tailed. The critical value for significant differences was adjusted according to the number of comparisons for each protocol according to Bonferroni (*i.e.*, $P < 0.01$ for analgesic remifentanil concentrations [five comparisons] and $P < 0.0125$ for apneic remifentanil concentrations [four comparisons]). Results for the different remifentanil concentrations were analyzed separately without additional correction for multiple comparisons. Inputs to inspiratory on- and off-switch were calculated from the values for inspiratory and expiratory duration as described in the appendix. For "Effects of Very High Remifentanil Concentrations on Areas outside the Parabrachial Nucleus/Kölliker–Fuse Complex and Pre-Bötzinger Complex," the values for inspiratory duration and peak phrenic activity were log transformed, and the adjusted correlation coefficients (R^2, squares of Pearson's correlation) were compared using bootstrap analysis (R 3.5.0, R Foundation for Statistical Computing, Austria). In accordance with the exploratory nature of the study, for additional comparisons (*e.g.*, between different remifentanil concentrations, between respiratory parameters, or between peak phrenic and peak vagus activity), we used Cohen's *d*. This is defined as the difference between two means $(\mu_1 - \mu_2)$, divided by the pooled SD (s): $d = (\mu_1 - \mu_2)/s$. The pooled SD is weighted for the number of samples (n) in each group: $s = (s_1^2 \star (n_1 - 1) + s_2^2 \star (n_2 - 1)/(n_1 + n_2 - 2))^{1/2}$.

A Cohen's *d* of 0.5 or more is considered a "medium" difference, 0.8 or more is considered a "large" difference, and 2 or more is considered a "huge" difference.[30] In addition, the groups were compared using the Mann–Whitney U test without corrections for multiple comparisons. The parameters are presented as medians (25 to 75% range).

Primary outcomes were the changes in respiratory parameters with local microinjection of naloxone at one remifentanil concentrations or microinjection of DAMGO, respectively. Secondary outcomes were comparisons between respiratory parameters (*e.g.*, inspiratory and expiratory duration) at the same remifentanil concentration, between the effects on different areas (*i.e.*, parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex) at the same remifentanil concentration, between different opioid concentrations, and between phrenic and vagal nerve activity.

Results

In total, 48 animals were used in our studies. Three animals died during the surgical preparation, and four animals developed significant respiratory slowing during AMPA mapping and were removed from further study. No animal died during the remifentanil/naloxone injection sequence.

Opioid Effect Sites at Analgesic IV Remifentanil Concentrations

To determine the effect of analgesic remifentanil concentrations on the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex, we microinjected naloxone into the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex (cohort A) or the pre-Bötzinger complex alone (cohort B) during remifentanil infusion, dosed to depress the baseline respiratory rate by 50%. Cohort A consisted of eight female (3.5 ± 0.9 kg) and 11 male (2.8 ± 0.4 kg) animals, and cohort B consisted of four female (3.7 ± 0.6 kg) and six male (3.3 ± 0.4 kg) animals. In cohort A, the continuous remifentanil infusion depressed the respiratory rate from 36 to 16 breaths/min (fig. 3B, left). Sequential, bilateral microinjections of naloxone into the bilateral Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex (n = 12) increased the respiratory rate to 17 (*P* = 0.005), 23 (*P* <0.001), and 25 breaths/min (*P* <0.001), respectively. For the 25 to 75% range, please see table 1. In Cohort B, remifentanil infusion decreased the respiratory rate from 33.5 to 17 breaths/min (fig. 3B, right). Naloxone microinjection solely into the pre-Bötzinger complex increased the respiratory rate to 20.5 breaths/min (*P* = 0.005). The effect of naloxone injection into the pre-Bötzinger complex was similar with or without prior naloxone injection into the parabrachial nucleus/Kölliker–Fuse nucleus (*P* = 0.242; fig. 3B, blue bracket).

In cohort A, remifentanil infusion decreased peak phrenic activity from 100 to 84% of control (fig. 3C, left).

Naloxone injections into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex increased peak phrenic activity to 87 (*P* = 0.022), 88 (*P* <0.001), and 91% (*P* = 0.898), respectively. In Cohort B, remifentanil infusion decreased peak phrenic activity from 100 to 89%, and naloxone injection into the pre-Bötzinger complex did not reverse the depression (96%, *P* = 0.039; fig. 3C, right). The effect of naloxone injection into the pre-Bötzinger complex was similar with or without prior naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex (*P* = 0.193; fig. 3C, blue bracket).

In cohort A, remifentanil infusion increased inspiratory duration from 0.78 to 1.5 s (fig. 3D, left). Naloxone injections into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex decreased inspiratory duration to 1.43 (*P* = 0.012), 1.2 (*P* <0.001), and 1.16 s (*P* = 0.007), respectively. In cohort B, remifentanil increased inspiratory duration from 0.84 to 1.32 s, which naloxone injection into the pre-Bötzinger complex did not change (1.35 s, *P* = 0.375; fig. 3D, right). The effect of naloxone injection into the pre-Bötzinger complex was similar with and without prior naloxone injection into the parabrachial nucleus/Kölliker–Fuse nucleus (*P* = 0.149; fig. 3D, blue bracket).

In cohort A, remifentanil infusion increased expiratory duration from 0.84 to 2.22 s (fig. 3E, left). Naloxone injections into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex decreased expiratory duration to 2.01 (*P* = 0.008), 1.50 (*P* <0.001), and 1.28 s (*P* <0.001), respectively. In cohort B, remifentanil infusion increased expiratory duration from 1.08 to 2.26 s, which was decreased by naloxone injection into the pre-Bötzinger complex to 1.68 s (*P* = 0.002; fig. 3E, right). The decrease in expiratory duration with naloxone injection into the pre-Bötzinger complex alone was greater than the decrease after prior naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex (*P* < 0.001; fig. 3E, blue bracket).

In cohort A, remifentanil infusion decreased inputs to inspiratory off-switch from 185 to 129% of apneic threshold (fig. 3F, left). Naloxone injections into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex increased these inputs to 131% (*P* = 0.002), 143% (*P* < 0.001), and 146% (*P* = 0.024), respectively. In cohort B, remifentanil decreased inputs to inspiratory off-switch from 161 to 136%, which naloxone injection into the pre-Bötzinger complex did not change (134%, *P* = 0.064; fig. 3F, right). The effect of naloxone injection into the pre-Bötzinger complex was similar with and without prior naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex (*P* = 0.460; fig. 3F, blue bracket).

In cohort A, remifentanil decreased inspiratory on-switch from 175 to 112% of apneic threshold (fig. 3G, left). Naloxone injections into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex increased these inputs to 116 (*P* = 0.007), 129 (*P* <0.001), and 138% of apneic threshold (*P* <0.001), respectively. In cohort B,
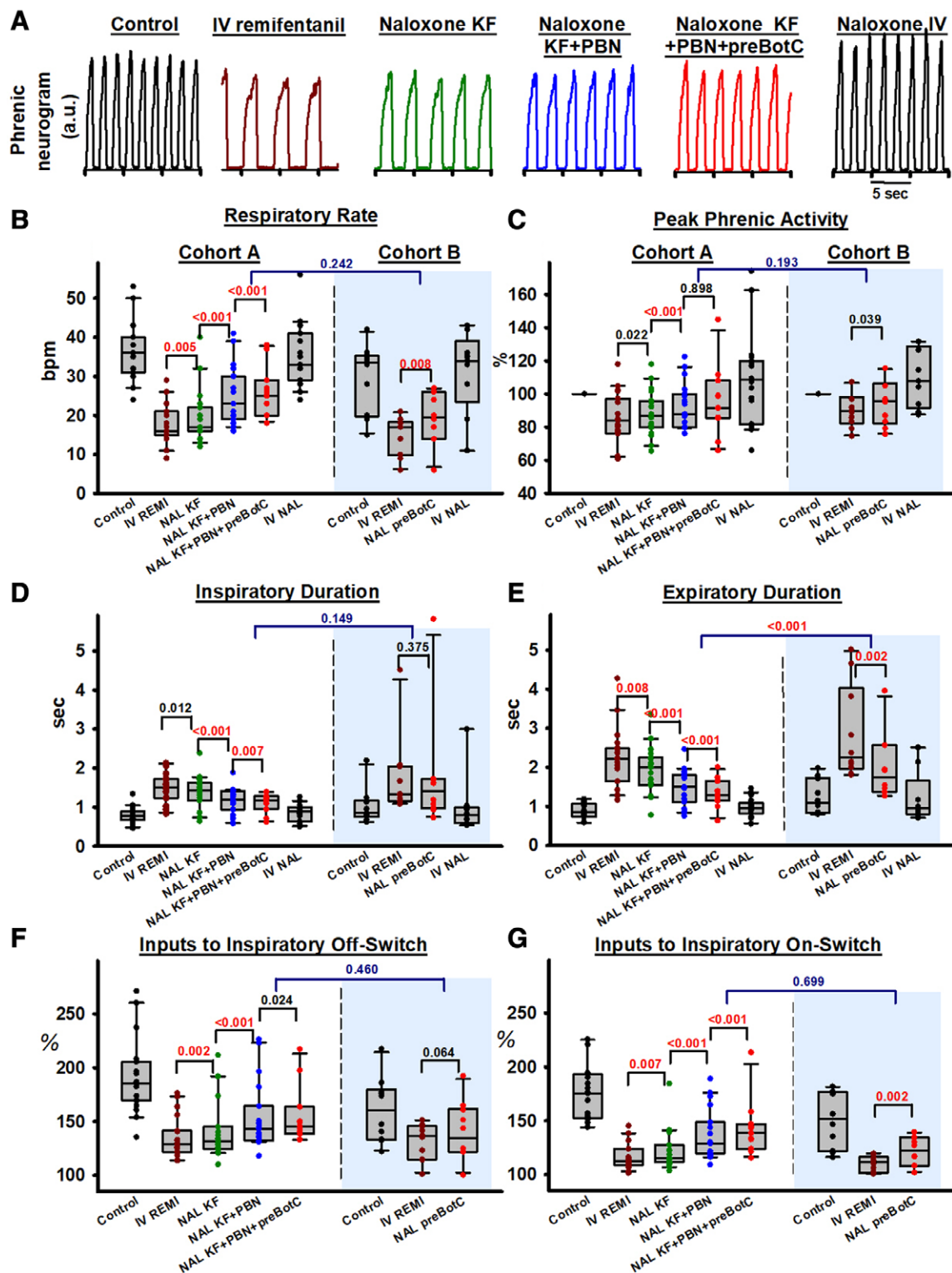
Fig. 3. Analgesic remifentanil (REMI) concentrations. Bilateral naloxone (NAL) injections into the Kölliker–Fuse (KF) nucleus, the parab-rachial nucleus (PBN), and the pre-Bötzinger complex (preBotC) significantly reversed the respiratory rate depression from intravenous remifentanil. The analgesic remifentanil dose rate was chosen to achieve ~50% respiratory rate depression. (*A*) Phrenic neurogram tracings during control conditions and sequential drug injections in an individual rabbit. (*B–E*) Pooled data for changes in respiratory rate and other

inputs to inspiratory on–switch decreased with remifentanil infusion from 152 to 112% of apneic threshold and increased with naloxone injection into the pre-Bötzinger complex to 122% ($P = 0.002$; fig. 3G, right). The effect of naloxone injection into the pre-Bötzinger complex was similar with and without prior naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex ($P = 0.699$; fig. 3G, blue bracket), suggesting that the observed difference in expiratory duration may have been due to the slower respiratory rate and longer expiratory duration before naloxone injection (see the appendix).

Additional analysis showed that naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex (fig. 3B, left) increased the respiratory rate more than injection into the pre-Bötzinger complex (data pooled for cohorts A+B; fig. 3B, left and right; Cohen's $d = 0.8$; $P = 0.033$). Naloxone microinjection into the parabrachial nucleus/Kölliker–Fuse nucleus decreased expiratory duration (fig. 3E, left) more than inspiratory duration (fig. 3D, left, Cohen's $d = 0.9$, $P = 0.008$), as did the subsequent injection into the pre-Bötzinger complex (fig. 3, D and E, left; Cohen's $d = 1.6$; $P = 0.004$). Naloxone injection into the pre-Bötzinger complex alone decreased expiratory duration (fig. 3E, right) more than inspiratory duration (fig. 3D, right; Cohen's $d = 1.6$; $P < 0.001$).

Opioid Effect Sites at Apneic IV Remifentanil Concentrations

To determine the effect of apneic remifentanil concentrations on the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex, we administered an intravenous remifentanil bolus that caused apnea under control conditions before and after microinjection of naloxone into the parabrachial nucleus/Kölliker–Fuse complex and after additional naloxone injection into the pre-Bötzinger

Fig. 3. (*Continued*) respiratory parameters. (*F, G*) Values for inputs to inspiratory on- and off-switch were derived from the values for inspiratory and expiratory duration and are presented as percentages of apneic threshold with the apneic threshold equal to 100% (appendix). The data for cohort A are presented on the *left side* of each panel. Sequential naloxone injections into the Kölliker–Fuse nucleus and the parabrachial nucleus were performed in 19 animals. In 12 of these animals, naloxone was subsequently injected into the pre-Bötzinger complex. The data for cohort B are presented on the *right side* of each panel (*shaded*). In a separate group of 10 animals, naloxone was injected only into the pre-Bötzinger complex. *Black brackets* indicate that the difference (Δ) between values from two sequential injections was tested against no change (Wilcoxon signed rank test). *Blue brackets* indicate comparison of the Δ values from pre-Bötzinger complex injection with and without prior naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex (Mann–Whitney rank sum test). The levels of significance below the critical $P = 0.01$ are highlighted in *red*. The phrenic neurogram traces and pooled data are color-coded for the same condition to facilitate reader orientation.

complex (cohort A, 17 animals), or after naloxone injection into the pre-Bötzinger complex alone (cohort B, nine animals). In cohort A, in 12 of 17 animals, naloxone microinjection into the parabrachial nucleus/Kölliker–Fuse nucleus prevented apnea from the intravenous remifentanil bolus that had caused apnea under control conditions. Respiratory rate increased from 0 to 10 breaths/min ($P < 0.001$; fig. 4B, left). For the 25 to 75% range, please see table 1. After additional naloxone microinjection into the pre-Bötzinger complex (n = 12), the repeat remifentanil bolus did not cause apnea in any animal, and the respiratory rate was increased to 13 breaths/min ($P = 0.002$). In cohort B, naloxone microinjection into the pre-Bötzinger complex alone prevented apnea from the IV remifentanil bolus only in 2 of 9 animals (fig. 4B, right), and the median respiratory rate was not increased (0 breaths/min, $P = 0.500$). Compared to the effect of naloxone microinjection into the pre-Bötzinger complex alone, pre-Bötzinger complex injection after naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex caused a larger increase in the respiratory rate ($P = 0.006$; fig. 4B, blue bracket).

Because inspiratory duration, expiratory duration, and peak phrenic activity cannot be measured during apnea, we extrapolated these variables from the first breath after apnea (see fig. 4A, control). If no apnea was observed, we averaged parameters for approximately six to eight breaths at the lowest respiratory rate after the bolus (fig. 4A, NAL KF+PBN and NAL KF+PBN+preBötC). In cohort A, the apneic remifentanil bolus reduced peak phrenic activity from 100 to 47% (fig. 4C, left). Naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex (56%, $P = 0.030$) and the pre-Bötzinger complex (63%, $P = 0.064$) did not significantly increase peak phrenic activity. In cohort B, the remifentanil bolus depressed peak phrenic activity from 100 to 61% (fig. 4C, right), and naloxone injection into the pre-Bötzinger complex did not change peak phrenic activity (69%, $P = 0.250$). The naloxone effect was similar with and without prior naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex ($P = 0.596$; fig. 4C, blue bracket).

The apneic remifentanil bolus increased inspiratory duration from 0.78 to 2.96 s (fig. 4D, left). Naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex reduced the increase to 1.87 s ($P = 0.009$), whereas naloxone injection into the pre-Bötzinger complex caused no further change (1.92 s, $P = 0.375$). In cohort B, the apneic remifentanil bolus increased inspiratory duration from 0.84 to 2.20 s (fig. 4D, right). Naloxone injection into the pre-Bötzinger complex further *increased* inspiratory duration to 4.65 s ($P = 0.008$). The naloxone effect on inspiratory duration was not significantly different with prior parabrachial nucleus/Kölliker–Fuse complex reversal ($P = 0.016$; fig. 4D, blue bracket).

In cohort A, the apneic remifentanil bolus increased expiratory duration from 0.85 to 17.48 s (fig. 4E, left).

Table 1. Summary Data for Analgesic and Apneic Remifentanil Concentrations in Cohorts A and B

Characteristics	Control	IV Remifentanil	Naloxone Kölliker–Fuse Nucleus	Naloxone Parabrachial Nucleus	Naloxone Kölliker–Fuse + Parabrachial Nucleus	Naloxone pre-Bötzinger Complex	IV Naloxone
Cohort A: analgesic remifentanil concentration, naloxone injection into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex							
Number of animals	19	19	19	19		12	19
Breaths/min	36 (31–40)	16 (15–21)	17 (16–22)	23 (19–29)		25 (22–28)	33 (28.5–40)
Inspiratory duration, s	0.78 (0.69–0.89)	1.5 (1.23–1.72)	1.43 (1.21–1.60)	1.20 (1.01–1.38)		1.16 (1.02–1.26)	0.88 (0.70–0.95)
Input to inspiratory off-switch, %	185 (169–201)	129 (122–141)	131 (125–143)	143 (134–157)		146 (140–157)	174 (164–199)
Expiratory duration, s	0.85 (0.74–1.06)	2.22 (1.80–2.48)	2.01 (1.61–2.24)	1.50 (1.15–1.79)		1.28 (1.15–1.52)	0.96 (0.82–1.11)
Input to inspiratory on-switch, %	175 (153–192)	112 (109–120)	116 (112–125)	129 (120–146)		138 (128–146)	163 (149–179)
Peak phrenic activity, %	100	84 (77–96)	87 (80–95)	88 (81–99)		91 (86–104)	109 (91–119)
Cohort B: analgesic remifentanil concentration, naloxone injection into the pre-Bötzinger complex							
Number of animals	10	10	—	—		10	10
Breaths/min	34 (22–35)	17 (11–18)	—	—		21 (15–26)	33 (28.5–40)
Inspiratory duration, s	0.85 (0.79–1.09)	1.32 (1.18–1.94)	—	—		1.35 (1.00–1.71)	0.80 (0.78–0.94)
Input to inspiratory off-switch, %	161 (135–177)	136 (117–144)	—	—		134 (122–158)	183 (164–188)
Expiratory duration, s	1.08 (0.84–1.61)	2.26 (2.02–3.57)	—	—		1.68 (1.39–2.40)	0.96 (0.85–1.37)
Input to inspiratory on-switch, %	152 (125–176)	112 (103–115)	—	—		122 (104–133)	162 (134–175)
Peak phrenic activity, %	100	89 (84–96)	—	—		96 (84–103)	108 (94–127)
Cohort A: apneic remifentanil concentration, naloxone injection into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex							
Number of animals	17	17			17	12	
Breaths/min	36 (31–40)	0 (0–0)			10 (0–12)	13 (10–19)	
Inspiratory duration, s	0.78 (0.69–0.89)	2.96 (1.96–4.24)			1.87 (1.35–2.19)	1.92 (1.41–2.57)	
Input to inspiratory off-switch, %	185 (169–201)	105 (102–117)			118 (113–135)	117 (109–133)	
Expiratory duration, s	0.85 (0.74–1.06)	17.5 (14.2–19.9)			3.73 (2.97–14.5)	2.73 (1.58–4.58)	
Input to inspiratory on-switch, %	175 (153–192)	100 (100–100)			102 (100–105)	107 (102–126)	
Peak phrenic activity, %	100	47 (42–65)			56 (52–77)	63 (56–78)	
Cohort B: apneic remifentanil concentration, naloxone injection into the pre-Bötzinger complex							
Number of animals	9	9			—	9	
Breaths/min	33 (20–35)	0 (0–0)			—	0 (0–0)	
Inspiratory duration, s	0.84 (0.77–1.13)	2.2 (1.7–2.9)			—	4.65 (2.89–7.88)	
Input to inspiratory off-switch, %	176 (147–186)	112 (106–122)			—	101 (100–106)	
Expiratory duration, s	1.14 (0.85–1.71)	21.2 (13.1–24.8)			—	20.6 (6.90–22.0)	
Input to inspiratory on-switch, %	147 (122–175)	100 (100–100)			—	100 (100–100)	
Peak phrenic activity, %	100	61 (32–71)			—	69 (65–72)	

The values are given as means (25 to 75%). Analgesic concentrations are given as intravenous (IV) infusion. Apneic concentrations are given as IV bolus. For analgesic concentrations, respiratory parameters were determined separately after naloxone injection into the Kölliker–Fuse nucleus and into the parabrachial nucleus. The apneic bolus was only performed after naloxone injection into the Kölliker–Fuse nucleus *and* into the parabrachial nucleus. Inputs are normalized to the apneic threshold. Peak phrenic activity is normalized to control.
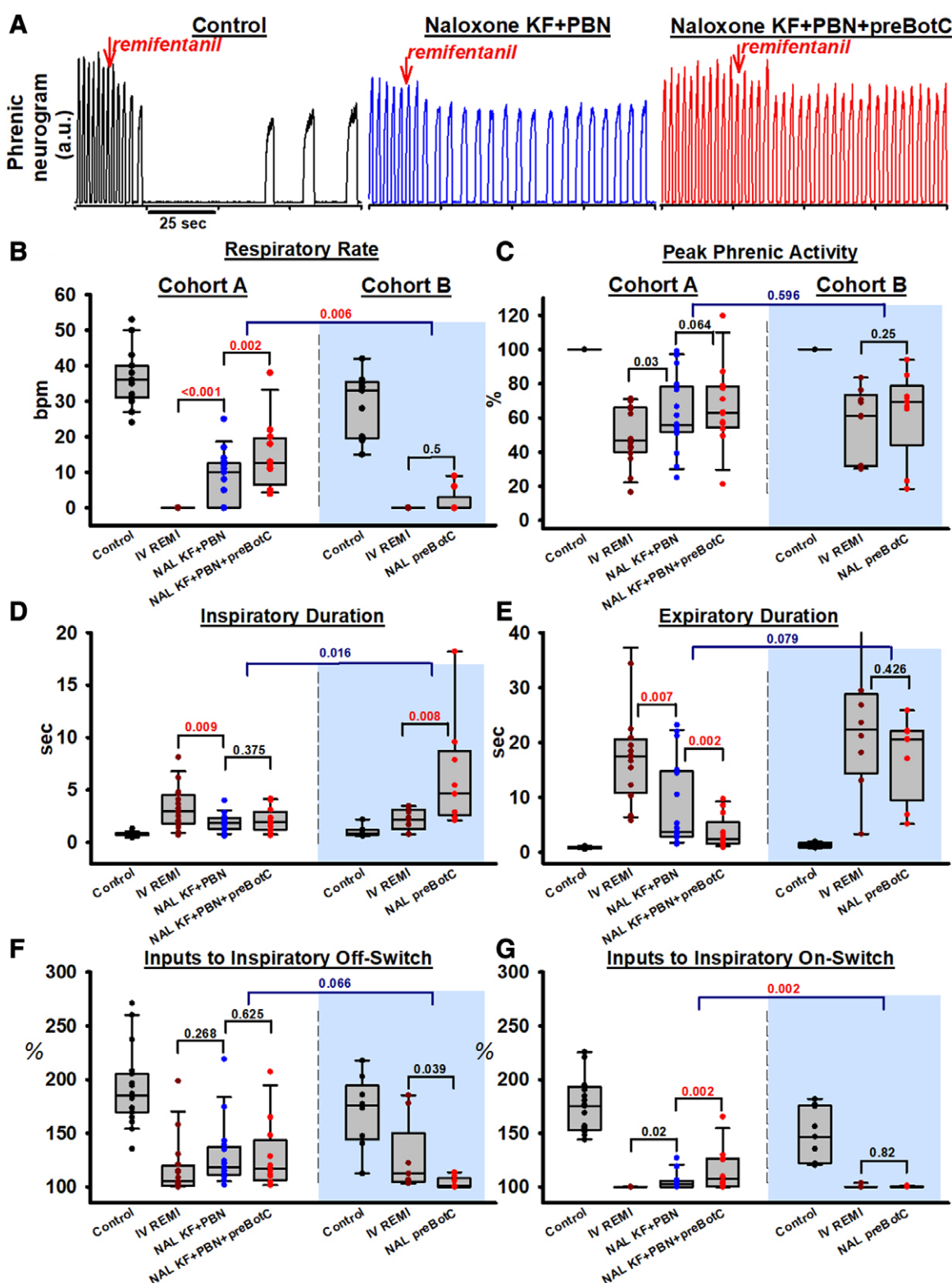
Fig. 4. Bilateral naloxone (NAL) injections into the Kölliker–Fuse nucleus (KF), parabrachial nucleus (PBN), and pre-Bötzinger complex (preBotC) prevented respiratory rate depression and apnea from an intravenous remifentanil (REMI) bolus (~10 µg). (*A*) Phrenic neurogram tracings from the same rabbit shown in figure 3 show that the same remifentanil bolus (*red arrow*) that elicited apnea during control conditions (*left*) only moderately depressed

Naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex reduced the increase to 3.73 s (*P* = 0.007), and injection into the pre-Bötzinger complex further reduced the increase to 2.73 s (*P* = 0.002). In cohort B, the apneic remifentanil bolus increased expiratory duration from 1.14 to 21.2 s (fig. 4E, right), and naloxone injection into the pre-Bötzinger complex did not change it (20.58 s, *P* = 0.426). The naloxone effect on expiratory duration was not different after prior parabrachial nucleus/Kölliker–Fuse complex reversal (*P* = 0.079; fig. 4E, blue bracket).

In cohort A, the apneic remifentanil bolus decreased inspiratory off-switch from 185 to 105% of apneic threshold (fig. 4F, left). Naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex (118%, *P* = 0.268) and pre-Bötzinger complex (117%, *P* = 0.625) did not significantly change these inputs. In cohort B, remifentanil decreased inputs to inspiratory off-switch from 176 to 112%, and naloxone injection into the pre-Bötzinger complex further decreased inputs to inspiratory off-switch to 101% (*P* = 0.039; fig. 4F, right). The naloxone effect on inputs to inspiratory off-switch was not significantly different with prior parabrachial nucleus/Kölliker–Fuse complex reversal (*P* = 0.066; fig. 4F, blue bracket).

In cohort A, the apneic remifentanil bolus decreased inputs to inspiratory on-switch from 175 to 100% of apneic threshold (fig. 4G, left). Naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex increased inputs to 102% (*P* = 0.020), and subsequent injection into the pre-Bötzinger complex increased inputs to 107% (*P* = 0.002). In cohort B, inputs to inspiratory on-switch decreased with the remifentanil bolus from 147 to 100% of apneic threshold (fig. 4G, right) and did not change with naloxone injection

(100%, *P* = 0.82). Inputs to inspiratory on-switch increased more after prior parabrachial nucleus/Kölliker–Fuse complex reversal (*P* = 0.002; fig. 4G, blue bracket).

Additional analysis showed that naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex decreased expiratory duration (fig. 4E, left) more than inspiratory duration (fig. 4D, left; Cohen's *d* = 0.9, *P* = 0.057), as did the subsequent injection into the pre-Bötzinger complex (fig. 4, D and E, left; Cohen's *d* = 1.6; *P* < 0.001). Naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex increased inputs to inspiratory on-switch more at analgesic (fig. 3G, left) than apneic remifentanil concentrations (fig. 4G, left; Cohen's *d* = 1.2; *P* <0.001), as did the subsequent injection into the pre-Bötzinger complex (figs. 3 and 4G, left; Cohen's *d* = 2.4; *P* < 0.001). Inputs to inspiratory off-switch also increased more with naloxone injection into the pre-Bötzinger complex at analgesic than at apneic concentrations (fig. 3 and 4F, left; Cohen's *d* = 1.3; *P* = 0.003). After naloxone injection solely into the pre-Bötzinger complex, there was a substantial difference between the lack of effect on expiratory duration (fig. 4E, right) and the increase in inspiratory duration (fig. 4D, right; Cohen's *d* = 1.1; *P* = 0.093) and also between the corresponding decrease in inputs to inspiratory off-switch (fig. 4F, right) and the lack of effect on inspiratory on-switch (fig. 4G, right; Cohen's *d* = 1.0; *P* = 0.005). The percentage of control inputs to inspiratory on- and off-switch above the apneic threshold that could be recovered with naloxone injections into the respective brainstem areas at analgesic and apneic concentrations are presented in table 2.

The Quotient of Peak Phrenic Activity/Inspiratory Duration and Respiratory Drive

To determine whether the quotient of peak phrenic activity and inspiratory duration (PPA/TI) is an adequate surrogate for "respiratory drive," we analyzed how intravenous remifentanil bolus injections affected peak phrenic activity and inspiratory duration. As illustrated for a single animal in fig. 5 (A through C), inspiratory duration and peak phrenic activity changed concomitantly after an apneic remifentanil bolus; however, the remifentanil-induced increase in inspiratory duration was substantially reduced with naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex and the pre-Bötzinger complex, whereas the maximal depression of peak phrenic activity did not change much. Correlation analysis using data from all animals, log-transformed to allow linear regression analysis and analyzed separately for opioid dose and naloxone injection (fig. 5, D through F), showed that the predictive properties of ln(inspiratory duration) for ln(peak phrenic activity/inspiratory duration) were consistently higher than ln(peak phrenic activity) for all but one data set. We conclude that systemic opioids affect excitatory inputs to phase switching mechanisms differently from peak phrenic activity. Consequently,

Fig. 4. (*Continued*). respiratory rhythm after naloxone injection into the Kölliker–Fuse nucleus and parabrachial nucleus (*middle*) and even less after subsequent naloxone injection into the pre-Bötzinger complex (*right*). (*B–E*) Pooled data for changes in respiratory rate and other respiratory parameters. Please note the different time scale for inspiratory (*D*) and expiratory (*E*) duration. (*F, G*) Values for inputs to inspiratory on- and off-switch were derived from the values for inspiratory and expiratory duration and are presented as percentages of apneic threshold with an apneic threshold of 100% (appendix). The data for cohort A are presented on the *left side* of each panel. The data were available for apneic bolus after naloxone injection into the Kölliker–Fuse nucleus and parabrachial nucleus in 17 animals and for 12 animals after additional injection into the pre-Bötzinger complex. The data for cohort B are presented on the *right side* of each panel (*shaded*). In a separate group of nine animals, naloxone was injected only into the pre-Bötzinger complex. *Black brackets* indicate that the difference (Δ) between values from two sequential injections was tested against no change (Wilcoxon signed rank test). *Blue brackets* indicate comparison of the Δ values from pre-Bötzinger complex injection with and without prior naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex (Mann–Whitney rank sum test). Levels of significance below the critical *P* = 0.0125 are highlighted in *red*.

Table 2. Percentage of Inputs to Respiratory Phase Duration Restored with Naloxone Injections into the Parabrachial Nucleus/Kölliker–Fuse Complex and the Pre-Bötzinger Complex at Analgesic and Apneic Remifentanil Concentrations

Remifentanil	Respiratory Phase	Respiratory Drive, %	Parabrachial Nucleus/Kölliker–Fuse Complex, %	Pre-Bötzinger Complex, %
Cohort A analgesic	Inspiratory duration	29 (9 to 49)	24 (13 to 30)	4 (2 to 11)
	Expiratory duration	41 (25 to 55)	25 (16 to 33)	22 (8 to 24)
Cohort A apneic	Inspiratory duration	77 (62 to 92)	14 (3 to 25)	−3 (−7 to 10)*
	Expiratory duration	90 (82 to 98)	0.002 (0 to 4.7)	7 (0.1 to 16)
Cohort B analgesic	Inspiratory duration	44 (26 to 52)		12 (6 to 17)
	Expiratory duration	54 (52 to 62)		26 (19 to 30)
Cohort B apneic	Inspiratory duration	98 (95 to 100)		−12 (−69 to −4)*
	Expiratory duration	100 (100 to 100)		0 (0 to 0)

The values are relative to the total inputs above the apneic threshold during control conditions. For example, analgesic remifentanil concentrations depressed inputs to expiratory duration by ~88% (equaling a decrease in inputs from 2 to 1.12). Altogether, 22% of the input was reversed with naloxone injection into the pre-Bötzinger complex and 25% with naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex, suggesting that 41% was due to depression of respiratory drive. The original data and statistics are provided in Table 1, in figs. 3 and 4, and under "Opioid Effect Sites at Analgesic IV Remifentanil Concentrations" and "Opioid Effect Sites at Apneic IV Remifentanil Concentrations." Cohort B did not receive naloxone reversal of the parabrachial nucleus/Kölliker–Fuse complex.

*Negative values indicate a decrease in inspiratory duration by apneic remifentanil concentrations in the pre-Bötzinger complex. Median (25 to 75% range).

the quotient of peak phrenic activity and inspiratory duration is not a reliable reflection of drive to all components of the respiratory rhythm and pattern generator. In the following analysis and discussion, we will use the term respiratory drive more broadly as inputs to the mechanisms of phase switch and motor activity and not limited to the concept of drive as the quotient of peak phrenic activity and inspiratory duration.

Effects of Very High Remifentanil Concentrations on Areas outside the Parabrachial Nucleus/Kölliker–Fuse Complex and Pre-Bötzinger Complex

To determine whether very high remifentanil concentrations affect respiratory rate and tidal volume outside the respiratory rhythm generator, we injected up to 100 μg of remifentanil IV after naloxone reversal of the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex. In six animals, the very high remifentanil bolus substantially decreased respiratory rate from 23.5 (20.5 to 28) breaths/min to 11 (7.2 to 12.5) breaths/min ($P = 0.031$). Peak phrenic activity was depressed to 5 (0 to 38)% of the prebolus amplitude ($P = 0.031$). In 3 of 6 animals, peak phrenic activity was completely abolished by the remifentanil bolus (phrenic apnea; fig. 6A). The very high remifentanil bolus depressed peak vagal activity to 50 (41 to 55)% of prebolus amplitude ($P = 0.031$), but rhythmic vagal respiratory activity continued during phrenic apnea (fig. 6B). In comparison, the initial apneic remifentanil bolus before naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex (see 2.5.) generated apnea in the phrenic and vagal neurogram (fig. 6C). At baseline, vagal inspiratory activity closely matched phrenic activity, which confirms that the central respiratory pattern

generator controls the phase timing of respiratory pump muscles as well as airway motor activity.[31] The concomitant phrenic and vagal apnea observed after the initial apneic remifentanil bolus indicated that remifentanil completely depressed the respiratory rhythm generator (*i.e.*, parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex function). The observation that after naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex, the very high remifentanil bolus still completely depressed phrenic activity (fig. 6A) but that the respiratory rhythm continued as reflected in the continued phasic vagal activity (fig. 6B) suggests (1) that naloxone successfully prevented complete depression of the respiratory rhythm generator and (2) that the very high remifentanil concentration directly depressed inspiratory premotor and/or phrenic motoneurons. Vagal premotor and motoneurons appeared to be less sensitive to direct opioid depression. Before naloxone microinjections into the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex, the initial apneic remifentanil bolus depressed peak phrenic activity more than peak vagus activity (both calculated from the first breath after apnea, relative to the respective peak activity before the bolus; Cohen's $d = 2.1$; $P = 0.015$; fig. 6C). This difference was also observed at the maximal remifentanil effect after the very high remifentanil bolus (Cohen's $d = 1.1$; $P = 0.065$; fig. 6D).

We also observed that although naloxone reversal in the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex prevented cessation of the respiratory rhythm, the very high remifentanil bolus still caused significant depression of the respiratory rate. Assuming that the naloxone injections were sufficient to locally antagonize all opioid effects, this suggests that remifentanil depressed the activity of the parabrachial nucleus/Kölliker–Fuse complex
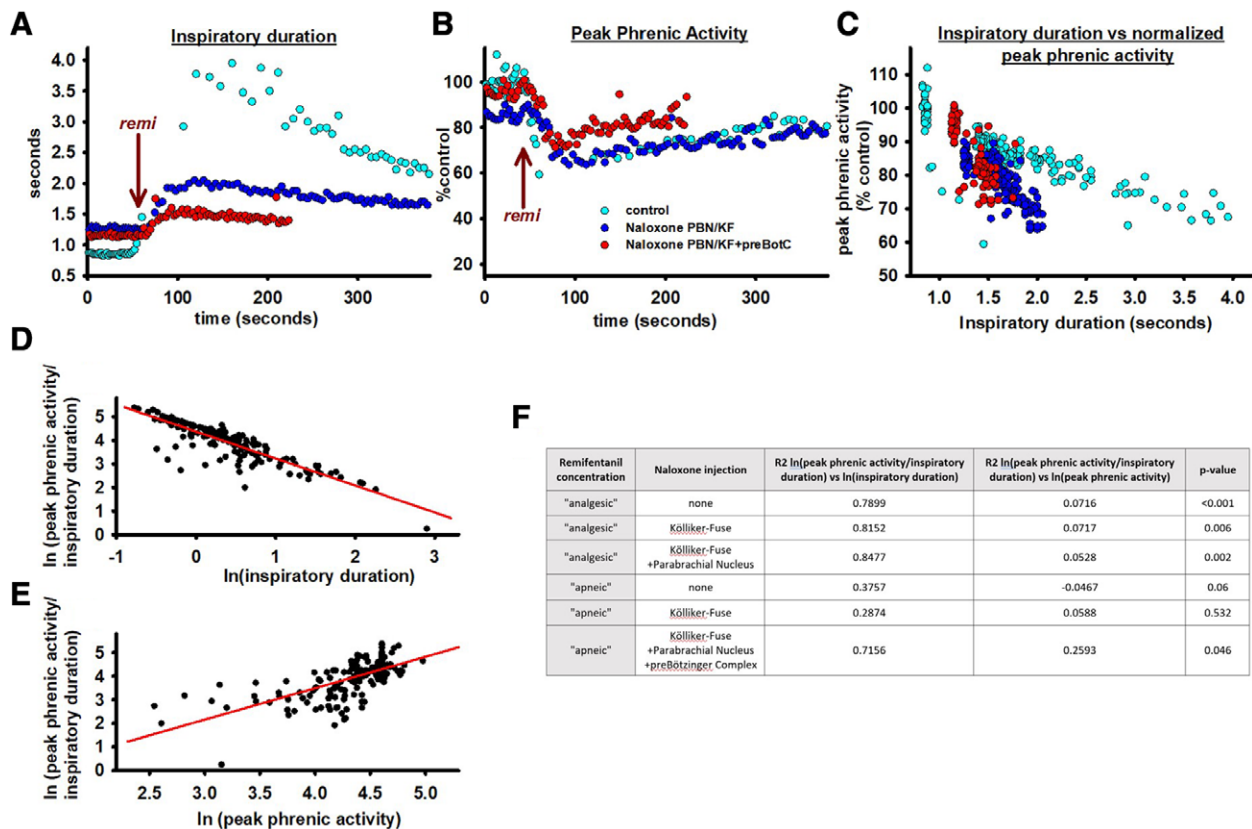
Fig. 5. Opioids depress inspiratory phase timing differently from peak phrenic activity. (*A–C*) Data from the rabbit shown in figs. 3A and 4A. (*A*) The increase in inspiratory duration from the apneic remifentanil bolus (*red arrow*), plotted for each breath *versus* time, was smaller after naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex (PBN/KF) (*blue*; lowest respiratory rate, 12 breaths/min) and pre-Bötzinger complex (preBotC; *red*; lowest respiratory rate, 19 breaths/min). (*B*) The decrease in peak phrenic activity, normalized to control, was attenuated less. Please note that inspiratory duration was increased, and peak phrenic activity was decreased before remifentanil injection after naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex and the pre-Bötzinger complex because of the continuous remifentanil infusion (see "Opioid Effect Sites at Apneic IV Remifentanil Concentrations"). (*C*) Naloxone reversal greatly decreased the prolongation of inspiratory duration from the apneic remifentanil bolus from 4 to ~1.5 s, whereas peak phrenic activity was always depressed 30 to 40%. (*D–F*) Pooled data from all remifentanil protocols (*n* = 171) illustrate the correlation between inspiratory duration, peak phrenic activity, and respiratory drive, here defined as the quotient of peak phrenic activity and inspiratory duration. The predictive properties of ln(inspiratory duration) for ln(peak phrenic activity/inspiratory duration) were higher than ln(peak phrenic activity) in all but one data set. (*F*) Correlation coefficients (*R*2, squares of Pearson's correlation) for each data set, and bootstrap analysis for adjusted correlation coefficients.

and pre-Bötzinger complex by decreasing the respiratory drive to these areas.
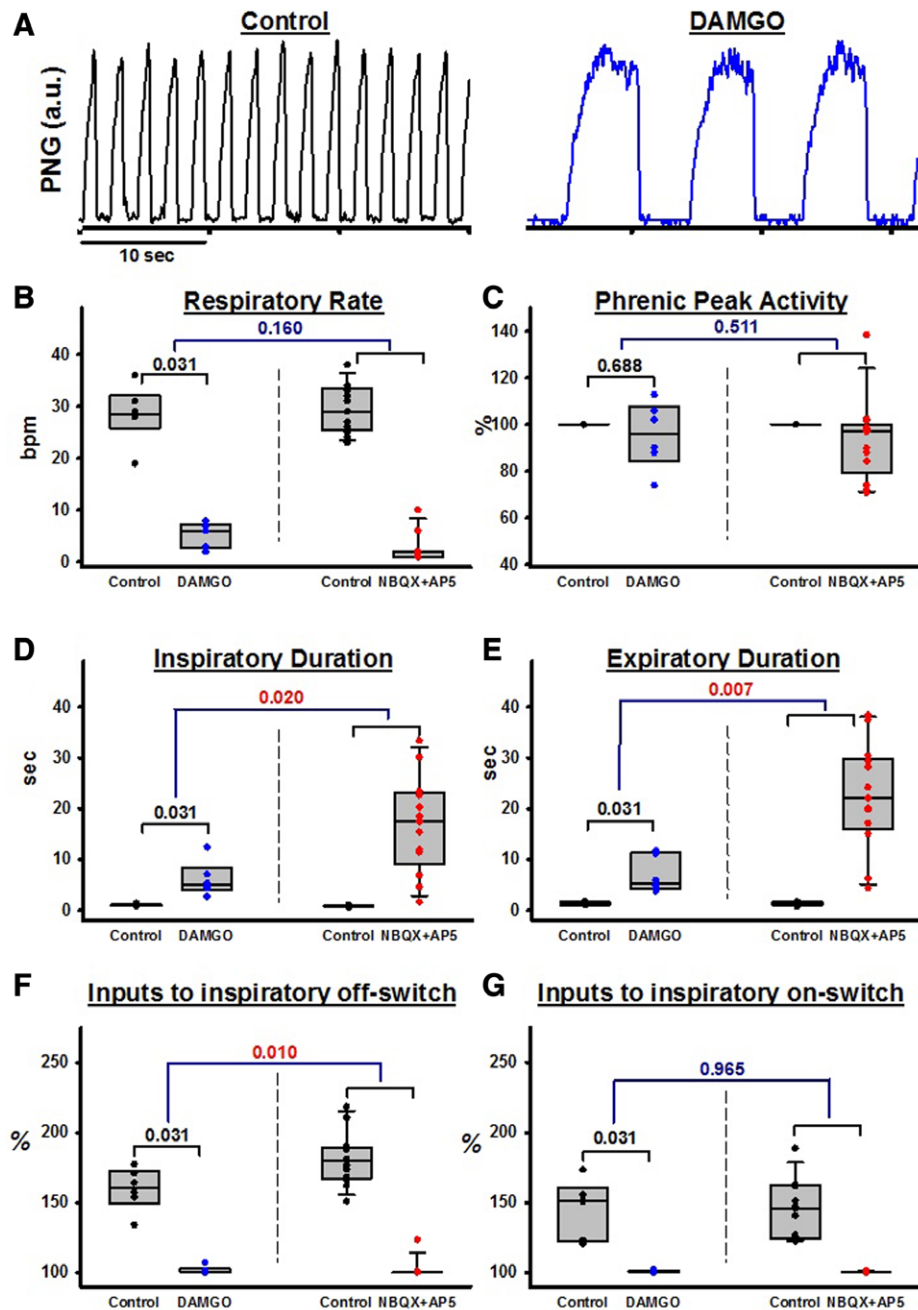
Effects of Supraclinical Opioid Concentrations, Compared to Glutamate Receptor Blockade in the Parabrachial Nucleus and Kölliker–Fuse Nucleus

To determine the effect of maximal opioid receptor activation, in six animals, the μ–opioid agonist DAMGO was injected into the bilateral parabrachial nucleus and Kölliker–Fuse nucleus (fig. 7). These data were compared to glutamate antagonist injections in a subset of 13 animals from our previous study,[8] which were selected to match the

average respiratory rate at control. The levels of significance for comparisons between the effects of DAMGO and glutamate antagonists in the two studies are indicated by blue brackets in fig. 7. DAMGO injection depressed respiratory rate from 29 (28 to 31) to 6 (4 to 7) breaths/min ($P = 0.031$; fig. 7B, left). This was similar to the effect of the glutamate receptor antagonists NBQX and AP5, which depressed the respiratory rate from 29 (26 to 33) to 2 (1 to 2) breaths/min ($P = 0.160$; fig. 7B), blue bracket). DAMGO injection did not depress peak phrenic activity (100% to 96 [81 to 105]%, $P = 0.688$; fig. 7C, left), which was similar to NBQX and AP5 (100% to 97 [84 to 99]%; $P = 0.511$; fig. 7C, blue bracket). DAMGO increased inspiratory duration from 0.98
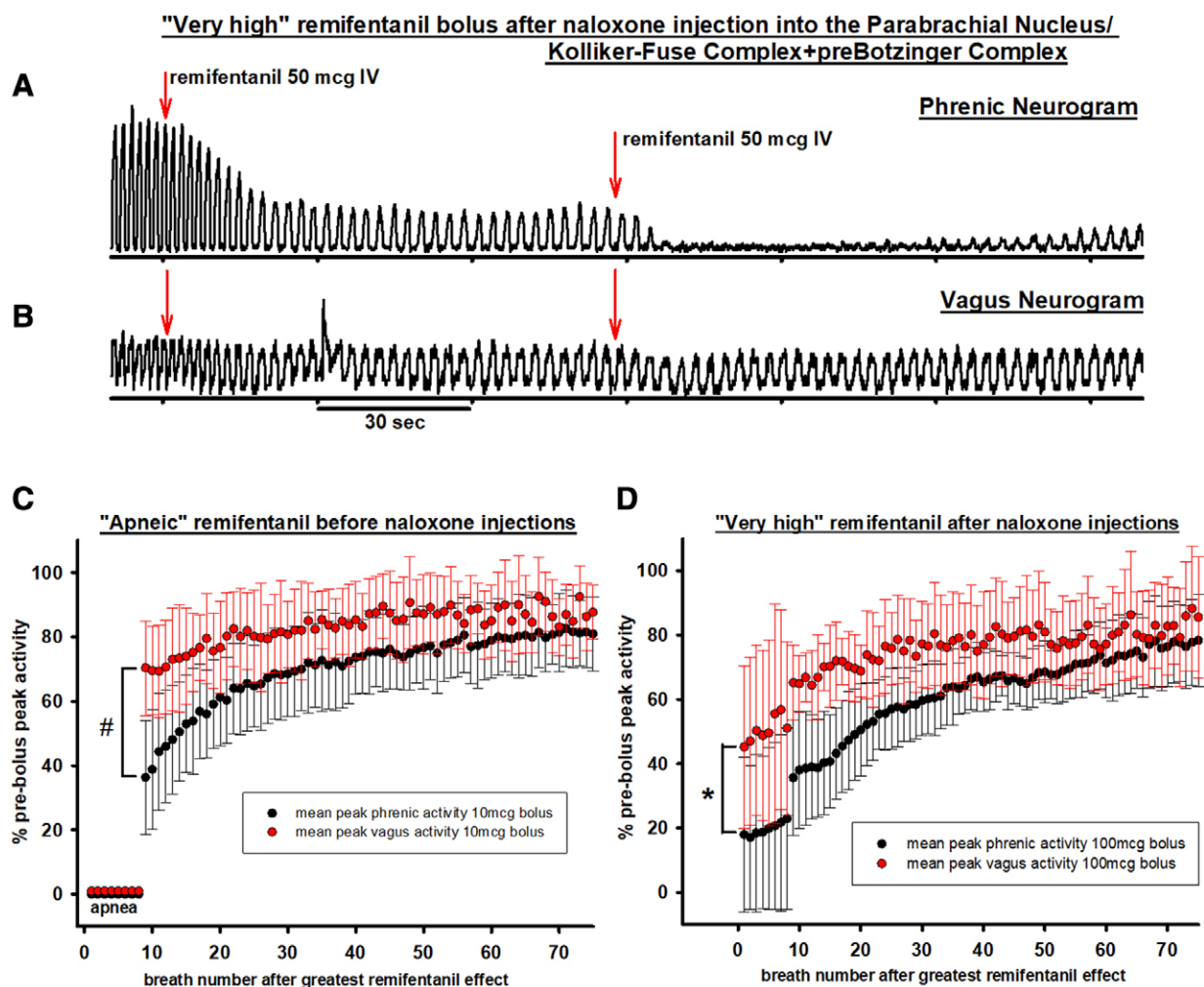
Fig. 6. Effect of very high remifentanil concentrations on areas outside the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex. (*A*) Phrenic neurogram tracings from an individual rabbit show that high bolus doses of intravenous remifentanil (total of 100 μg, *red arrows*) *after* naloxone microinjection into the bilateral parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex decreased peak phrenic activity to 0. (*B*) However, during phrenic apnea, the respiratory rhythm continued in the vagus neurogram. The continued rhythm confirmed that opioid antagonism in the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex successfully prevented inhibition of the respiratory rhythm generator even by very high remifentanil concentrations. The depression of peak phrenic activity (*A*) was likely due to direct inhibition of inspiratory premotor and/or motoneurons. In all animals, respiratory rate decreased by 15 (14 to 16) breaths/min (*n* = 6). The slowing of the respiratory rate after the initial remifentanil bolus suggests that respiratory drive to the respiratory rhythm generator was decreased by very high remifentanil concentrations. (*C, D*) We performed additional analysis to determine whether opioids depressed peak phrenic activity more than peak vagus activity. Peak phrenic and peak vagus activity was calculated relative to peak activity before the intravenous remifentanil bolus and pooled for six animals (means ± SD). (*C*) Peak phrenic and peak vagus activity for each breath after recovery from apnea from the 10-μg remifentanil bolus *before* naloxone injection into the brainstem showed that phrenic activity was more depressed by remifentanil. (*D*) Peak phrenic and peak vagus activity for each breath starting at maximal depression from the 100-μg remifentanil bolus *after* naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex again showed that phrenic activity was more depressed. Complete loss of phrenic motor output (apnea) was observed in 3 of 6 animals. The statistical difference between pooled peak phrenic and peak vagus activity for the first breath after apnea at maximal depression: #Cohen's *d* = 2.1, *P* = 0.015; *Cohen's *d* = 1.1, *P* = 0.065. Mann–Whitney U test.

Fig. 7. Microinjection of supraclinical concentrations of the μ-opioid agonist [D-Ala,²N-MePhe,⁴Gly-ol]-enkephalin (DAMGO; 100 μM, 700 nl) into the bilateral parabrachial nucleus/Kölliker–Fuse complex severely depressed the respiratory rate. We compared the effect size with data from 13 animals from our previous study using microinjections of the non–N-methyl-D-aspartate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 1 mM, 700 nl) and N-methyl-D-aspartate receptor antagonist D(–)-2-amino-5-phosphonopentanoic acid (APS; 5 mM, 700 nl).[9] Animals were selected to match the control respiratory rate. (A) Phrenic neurogram tracings from one individual animal. (B–E) Pooled data for changes in respiratory rate and other respiratory parameters. Respiratory rate depression was similar between [D-Ala,²N-MePhe,⁴Gly-ol]-enkephalin (n = 6) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione/2-amino-5-phosphonopentanoic acid injection (n = 13), as was the effect on peak phrenic activity. (F, G) Values for inputs to inspiratory on- and off-switch were derived from the values for inspiratory and expiratory duration and are presented as percentages of apneic threshold with an apneic threshold of 100% (appendix). *Black brackets* indicate that the difference (Δ) between [D-Ala,²N-MePhe,⁴Gly-ol]-enkephalin injection and control was tested against no change (Wilcoxon signed rank test). *Blue brackets* indicate comparison of the Δ values from [D-Ala,²N-MePhe,⁴Gly-ol]-enkephalin injection *versus* control with 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione/2-amino-5-phosphonopentanoic acid injection *versus* control (Mann–Whitney rank sum test). Levels of significance below the critical $P = 0.025$ are highlighted in *red*.

(0.90 to 1.04) to 5.03 (4.45 to 6.66) s ($P = 0.031$; fig. 7D, left), which was less than the increase from the glutamate antagonists (0.81 [0.77 to 0.90] to 17.5 [11.4 to 22.6] s; $P = 0.020$; fig. 7D, blue bracket). DAMGO increased expiratory duration from 1.32 (1.08 to 1.64) to 5.41 (4.53 to 9.83) s ($P = 0.031$; fig. 7E, left), which was less than the increase from the glutamate antagonists (1.16 [0.98 to 1.59] to 22.1 [17.1 to 29.3] s; $P = 0.007$; fig. 7E, blue bracket). Similar to inspiratory duration, the inputs to inspiratory off-switch were decreased by DAMGO from 161 (155 to 169) to 101 (100 to 101)% of apneic threshold ($P = 0.031$; fig. 7F, left), which was less than the decrease from the glutamate antagonists (180 [170 to 188] to 100 [100]% of apneic threshold; $P = 0.010$; fig. 7F, blue bracket). DAMGO decreased inputs to inspiratory on-switch from 151 (130 to 154) to 101 (100 to 101)% of apneic threshold ($P = 0.031$; fig. 7G, left), which was similar to the decrease from glutamate antagonists (146 [126 to 162] to 100 [100 to 100]%; $P = 0.965$; fig. 7G, blue bracket). The discrepancy between the significant difference in effect on expiratory duration and no difference in effect on inputs to inspiratory on-switch may have been because with very long respiratory phases, large differences in phase duration can be caused by only very small differences in inputs to phase duration (appendix).

The prominent respiratory rate depression by DAMGO injection into the parabrachial nucleus/Kölliker–Fuse complex that is similar to the effects of glutamate antagonists in scale and pattern (*i.e.*, significant prolongation of inspiratory and expiratory duration) suggests that many of the parabrachial nucleus/Kölliker–Fuse complex neurons that contribute to respiratory phase timing are opioid-sensitive. As described under "Effects of Supraclinical Opioid Concentrations, Compared to Glutamate Receptor Blockade in the Parabrachial Nucleus and Kölliker–Fuse Nucleus," this may apply only for animals with baseline respiratory rates between 20 and 37 breaths/min.

Control Studies: Effects of Naloxone or Artificial Cerebrospinal Fluid Injections; Potential Desensitization to Remifentanil

To rule out endogenous opioid receptor activation, in four animals, naloxone was microinjected into the bilateral parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex without remifentanil infusion. Naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex or pre-Bötzinger complex, respectively, did not have any effect on respiratory rate (*vs.* control, $P > 0.999$ or $P > 0.999$, respectively; data not shown), inspiratory duration ($P = 0.625$ or $P = 0.625$, respectively), expiratory duration ($P = 0.250$ and $P = 0.250$, respectively), or peak phrenic activity ($P = 0.375$ and $P = 0.250$, respectively). At high concentrations (more than 100 μM), naloxone can act as a GABA$_A$ receptor antagonist.[28] However, we did not observe any changes in respiratory rate with any naloxone injections into the parabrachial nucleus/Kölliker–Fuse complex or pre-Bötzinger complex (*i.e.*, respiratory nuclei, which are under GABA$_A$-mediated control). Therefore, naloxone-mediated GABA$_A$ receptor antagonism did not appear to be a confounder in our experiments.

Injection of artificial cerebrospinal fluid, which was used as solvent, into the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex did not affect respiratory rate (parabrachial nucleus/Kölliker–Fuse complex *vs.* control, $P = 0.500$; pre-Bötzinger complex *vs.* control, $P > 0.999$; n = 3; data not shown), inspiratory duration ($P = 0.250$ and $P = 0.750$, respectively), expiratory duration ($P = 0.250$ and 0.500, respectively), or peak phrenic activity ($P = 0.500$ and $P = 0.500$, respectively).

Post hoc, we reviewed all of our experimental data for signs of desensitization to the remifentanil effect caused by prolonged remifentanil infusion and repeated bolus injections. We found that even after long remifentanil infusions when multiple adjustments (*i.e.*, increases or decreases), in infusion rate were required to achieve 50% respiratory rate depression, the respiratory rate did not change during the 15 min of steady state before the first naloxone injection. After apneic bolus injections of remifentanil that followed naloxone injections into the parabrachial nucleus/Kölliker–Fuse complex or pre-Bötzinger complex, the respiratory rate reliably returned to prebolus values (difference pre- to postbolus rate 0 [−1 to 1] breaths/min, n = 26), including after the last remifentanil bolus injection in Cohort A, which was the third remifentanil bolus per animal (difference 0 [−1 to 1] breaths/min, n = 8). Other authors have shown a decrease in respiratory rate depression during continuous remifentanil infusion starting after 90 min and resulting in only ∼60% of the maximal depression after 300 min in freely behaving rabbits with variable partial pressure of carbon dioxide and decreasing sedation levels.[24] However, in our decerebrate rabbit model with controlled partial pressure of carbon dioxide and partial pressure of oxygen, there was no obvious attenuation of the respiratory rate depression after prolonged remifentanil infusion and repeated bolus injections. We conclude that desensitization to the respiratory depressant effects of remifentanil was not a relevant confounding factor in our experiments. The complete recovery of the respiratory rate to control values with intravenous naloxone injection at the end of the experiment confirmed that there was also no systematic decrease in the respiratory rate caused by a deterioration of the preparation.

Discussion

This study explored the contributions of the parabrachial nucleus/Kölliker–Fuse complex and the pre-Bötzinger complex to opioid-induced respiratory depression in an acute, *in vivo* rabbit model. Remifentanil decreased the
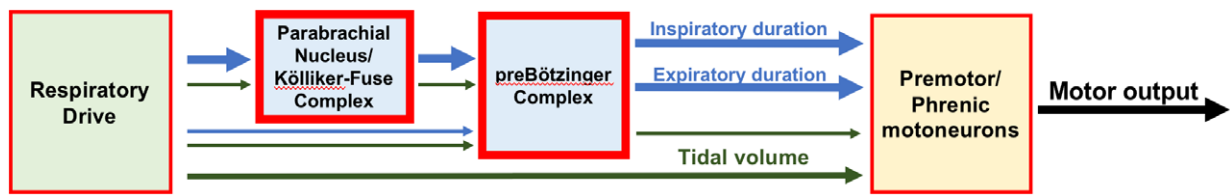
Fig. 8. Schematic of opioid effects on respiratory rate and tidal volume. Tonic respiratory drive determines the activity of the parabrachial nucleus/Kölliker–Fuse complex, the pre-Bötzinger complex, and inspiratory premotor and motoneurons. Part of the drive that determines respiratory phase duration (*blue*) is relayed by the parabrachial nucleus/Kölliker–Fuse complex, whereas other drive projects directly to the respiratory rhythm generator in the pre-Bötzinger complex. Drive that determines the magnitude of the tidal volume (*green*) is partially relayed through the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex but also directly projects to respiratory premotor neurons and phrenic motoneurons. Expiratory motoneurons were not recorded in this study. Analgesic opioid doses depress mostly parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex activity (*bold red frames*) and thus the respiratory rate. The magnitude of the opioid effect that can be reversed in each area is presented in table 2. Higher opioid doses directly affect respiratory drive and premotor and motoneurons, resulting in an additional decrease in tidal volume.

respiratory rate by depressing inspiratory on-switch through effects in the parabrachial nucleus/Kölliker–Fuse complex and the pre-Bötzinger complex. Remifentanil also depressed inspiratory off-switch through effects on the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex. However, apneic concentrations decreased inspiratory duration through an effect in the pre-Bötzinger complex. Sequential naloxone injection into the Kölliker–Fuse nucleus, parabrachial nucleus, and pre-Bötzinger complex could not completely reverse the respiratory depression from analgesic remifentanil concentrations and even less so from apneic doses (table 2). This suggests that opioids significantly depressed respiratory drive to the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex and that the depression of drive reduced the activity of these areas, especially at high opioid concentrations (fig. 8).

Opioids Depress Drive to the Parabrachial Nucleus/Kölliker–Fuse Complex and pre-Bötzinger Complex

We used changes in inspiratory and expiratory duration with naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex or pre-Bötzinger complex to calculate the relative inputs to inspiratory on- and off-switch from these areas (table 2). Two observations stood out: naloxone injection into the parabrachial nucleus/Kölliker–Fuse complex or pre-Bötzinger complex restored inputs more at analgesic than apneic remifentanil concentrations (table 2), suggesting that the level of parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex activity that can be recovered with naloxone injection depends on respiratory drive to these areas and that this drive is opioid-sensitive (fig. 8).

Second, naloxone reversal of the parabrachial nucleus/Kölliker–Fuse complex did not always prevent apnea from the apneic remifentanil bolus, and reversal

of the pre-Bötzinger complex rarely did. However, reversal of both areas together reliably prevented apnea, and respiratory rhythm persisted even after very high remifentanil bolus doses, albeit with a decreased rate. This suggests that the inputs from both areas to inspiratory on-switch are additive and that respiratory drive to these areas is opioid-sensitive but still sufficient to sustain respiratory rhythm even at very high remifentanil concentrations (see "Effects of Very High Remifentanil Concentrations on Areas outside the Parabrachial Nucleus/Kölliker–Fuse Complex and Pre-Bötzinger Complex"; fig. 6).

In a freely behaving mouse model, morphine dose-dependently depressed the respiratory rate. μ-Opioid receptor deletion in the Kölliker–Fuse nucleus prevented respiratory rate depression by ~20% of baseline rate at every dose, and μ-opioid receptor deletion in the pre-Bötzinger complex did not reduce respiratory depression at doses of 30 mg/kg or more (see fig. 3 in the study by Varga *et al.*[11]), suggesting that the increasing depression was due to a reduction in respiratory drive. Apneic doses were not tested. A similar study found no additional reduction of respiratory depression after μ-opioid receptor deletion in the pre-Bötzinger complex and parabrachial nucleus/Kölliker–Fuse complex in the same animals; however, the study was likely underpowered (see fig. 3 in the study by Bachmutsky *et al.*[13]). Species differences may exist between mammals; for example, in dogs naloxone injection into the parabrachial nucleus fully reversed respiratory rate depression,[5] whereas no reversal was observed in the pre-Bötzinger complex.[4]

Potential Sources of Opioid-sensitive Respiratory Drive

In our decerebrate, hyperoxic, and moderately hypercapnic preparation, both supratentorial "awake" drive[32] and

carotid body inputs were eliminated. This left the prevailing hypercapnia (tissue carbon dioxide tension) and the medullary and pontine arousal centers of the raphe as the main source of respiratory drive (for review, see the article by Palkovic *et al.*[1]). Chemosensitive neurons in the retrotrapezoid nucleus, considered the main source and integrator of respiratory chemodrive,[33,34] were unaffected even by apneic morphine doses.[35] However, in rats, injection of the opioid antagonist D–Phe–Cys–Tyr–D–Typ–Arg–Thr–Pen–Thr–NH2 (CTAP) into the caudal medullary raphe reduced respiratory rate depression by intravenous DAMGO from 30 to 15%,[36] making this area a promising candidate for future research.

Opioid Effects on Postinspiratory Activity

Vagal nerve recordings in rats display prominent postinspiratory activity, which controls motor output to airway muscles.[31,37] It also allows gauging the contribution of postinspiratory activity to respiratory phase timing. Volumetric mapping of the brainstem respiratory network showed highly synchronized activity during respiratory phase transitions in the areas relevant for phase timing.[31] Prominent activity during inspiratory on- and off-switch was observed in the pre-Bötzinger complex, and the main postinspiratory–expiratory phase transition was in the dorsal respiratory group.[31] In the *in-situ* rat model, vagal postinspiratory activity was completely abolished by systemic opioids, suggesting a mechanism for the observed increase in inspiratory duration.[10] In rabbits, vagal activity is mostly inspiratory, and postinspiratory activity, although present in the pre-Bötzinger complex, cannot be determined from vagal recordings. We thus limit our discussion of opioid effects to inspiratory–expiratory phase timing.

Potential Neuronal Targets for Respiratory Opioid Effects

Opioids prolong expiratory duration, and apnea always results from a failure of inspiratory on-switch.[1] *In vitro*, μ-opioid receptor agonists depressed more than 60% of Kölliker–Fuse complex neurons.[9,11] In our preparation, DAMGO injection into the parabrachial nucleus/Kölliker–Fuse complex resulted in severe depression of inspiratory on-switch (fig. 7), suggesting that opioids depressed neurons that promote inspiratory on-switch in the pre-Bötzinger complex.[7] DAMGO also directly inhibited Dbx+ pre-Bötzinger complex neurons in medullary slices[38] (*i.e.*, pre-inspiratory and inspiratory neurons whose stimulation generates inspiratory bursts *in vivo*).[39] Both mechanisms, depression of pontine inputs to the pre-Bötzinger complex and direct inhibition of pre-Bötzinger complex neurons, result in prolonged expiratory duration.

DAMGO injection into the parabrachial nucleus/Kölliker–Fuse complex increased inspiratory duration (fig. 7D), and naloxone in the parabrachial nucleus/Kölliker–Fuse complex reversed the increase in inspiratory duration from systemic remifentanil, suggesting that remifentanil depressed inputs to pre-Bötzinger complex neurons that promote inspiratory off-switch[7,8] (*e.g.*, SST+ postinspiratory neurons).[39] Naloxone injection into the pre-Bötzinger complex decreased inspiratory duration (fig. 3), as did μ-opioid receptor deletion in the pre-Bötzinger complex in mice,[11] pointing to additional, direct inhibition of postinspiratory neurons.

In contrast, at apneic remifentanil concentrations, naloxone injection solely into the pre-Bötzinger complex *increased* inspiratory duration (fig. 4). Opioids directly depressed inspiratory pre-Bötzinger complex neurons *in vitro*,[29] and DAMGO injection into the pre-Bötzinger complex shortened inspiratory duration in rabbits *in vivo*.[26,40] We hypothesize that the moderate increase in inspiratory duration at apneic remifentanil doses (fig. 4) was the net effect of depressed pontine inputs to inspiratory off-switch and direct inhibition of pre-Bötzinger complex inspiratory neurons. All neuron types have been described in the pre-Bötzinger complex in multiple species *in vivo*,[7,39,41–43] but opioid effects on individual neuron types have yet to be systematically investigated.

Remifentanil Effects on Phrenic Nerve Motor Output

We found that depression of peak phrenic activity did not closely correlate with changes in respiratory phase timing (fig. 5). After opioid reversal in the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex, very high remifentanil doses depressed phrenic motor output completely, while rhythmic respiratory activity continued (fig. 6). Single neuron recordings *in vivo* showed no direct depression of respiratory premotor neurons at analgesic systemic opioid concentrations[26,44] but neurons were directly depressed at near-apneic fentanyl doses.[44] Direct depression of spinal motoneurons was observed *in vitro* at "clinical" DAMGO concentrations (100 nM).[45] Direct depression of motor output from very high opioid doses may reduce the effectiveness of drugs designed specifically to stimulate respiratory rhythm.

Methodologic Considerations

Naloxone Injections. Modeling suggests that the spread of our injection volume (700 nl) resulted in an effective naloxone concentration of 50 μM (5% of 1 mM barrel concentration) within a spherical radius of 1 and 1.2 mm at 5 min after injection.[22] We believe that this concentration was sufficient to fully antagonize the remifentanil effect in the entire parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex area at analgesic (~50 nM[25]) and apneic plasma concentrations. We have discussed elsewhere that the neuronal population of the parabrachial nucleus/Kölliker–Fuse complex that

contributes to phase timing may be located between our injection sites, *i.e.*, in the medial parabrachial nucleus,[8,46] which matches other studies.[13] Projections from this area to the pre-Bötzinger complex were shown functionally and histologically.[7,47,48] Pilot data showed an unchanged naloxone effect 2 h after microinjection, suggesting that sequential injections completely blocked the remifentanil effect in all areas. The lack of change in respiratory rate starting 3 to 5 min after naloxone injection indicated that the area of effective naloxone concentration did not increase after that point. In a subset of nine animals in the current study, naloxone injection 0.5 mm caudal to the pre-Bötzinger complex caused minor increases in respiratory rate (1 [0 to 3] breaths/min), whereas more caudal injections did not, suggesting that our pre-Bötzinger complex injections did not reach the rostral ventral respiratory group.

Opioid Dosing. We chose a 50% depression of respiratory rate as surrogate for an analgesic remifentanil dose because veterinary[14] and respiratory studies[13,24] showed a ~50% rate depression at opioid doses that suppressed pain responses in spontaneously breathing animals. These doses are likely higher than analgesic doses in humans where analgesics can be dosed to "acceptable" pain levels, whereas animal studies generally measure complete lack of movement to pain stimulus. However, a 40 to 60% depression of minute ventilation was observed in human volunteers after 0.15 mg/kg[49] or 0.2 mg/kg[50] morphine (*i.e.*, a dose sufficient to provide effective analgesia for patients after major surgery).

Conclusions

Opioid reversal in the parabrachial nucleus/Kölliker–Fuse complex and pre-Bötzinger complex does not completely reverse opioid-induced respiratory depression, suggesting that depression of respiratory drive limits the activity that can be recovered in these areas. This mechanism must be taken into account during the development of drugs designed to stimulate the respiratory rhythm generator.

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## Competing Interests

The authors declare no competing interests.

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Appendix: Calculation of Inputs to Respiratory Phase Duration

The determinants of inspiratory and expiratory phase duration can be understood using a "leaky integrator" model.[1,51,52] In short, populations of phase switching neurons located in the pre-Bötzinger complex promote the switch from expiration to inspiration (preinspiratory neurons)[53,54] and, to a degree, the switch from inspiration to expiration (postinspiratory neurons).[39] The sum of inputs to these neuron populations determines the time to phase switch threshold and thus the duration of the preceding phase. Using the actual values for inspiratory duration the sum of inputs (σ) to inspiratory off-switch and for expiratory duration, the sum of inputs to inspiratory on-switch can be calculated from $LI(\sigma) = \sigma \star (1 - e^{-t/\tau})$, where $LI(\sigma)$ is the leaky integrator function, σ is the sum of inputs to $LI(\sigma)$, $e = 2.71828$ is the base of the natural logarithm, and τ is the time constant of $LI(\sigma)$. Phase switching occurs when leaky integrator output $LI(\sigma)$ = threshold, where the threshold is set at 1.0 input units or as a percent = 100. The time constant of the leaky integrator determines the rate of rise of the sum of inputs (σ) toward the threshold for phase off-switch. Conversely, the inputs can be calculated from $\sigma = 1/(1 - e^{-T/\tau})$, where T is the time of threshold crossing.

Time constants for inspiratory on-switch have been determined for rabbits ($\tau = 1.07\,s$[52]) and dogs ($\tau = 0.8\,s$[51]). D'Angelo[55] described a τ of 0.21 s for inspiratory duration; however, the latter appeared very different from our present results, where a decrease in central (parabrachial nucleus/Kölliker–Fuse) input to phase duration changed inspiratory and expiratory duration similarly (fig. 2). Bradley *et al.*[56] stated a τ of 1.0 s for inspiratory duration for cats. To simplify calculation and comparison between inspiratory and expiratory duration, we used a τ of 1.0 s to calculate inputs to inspiratory on- and off-switch.
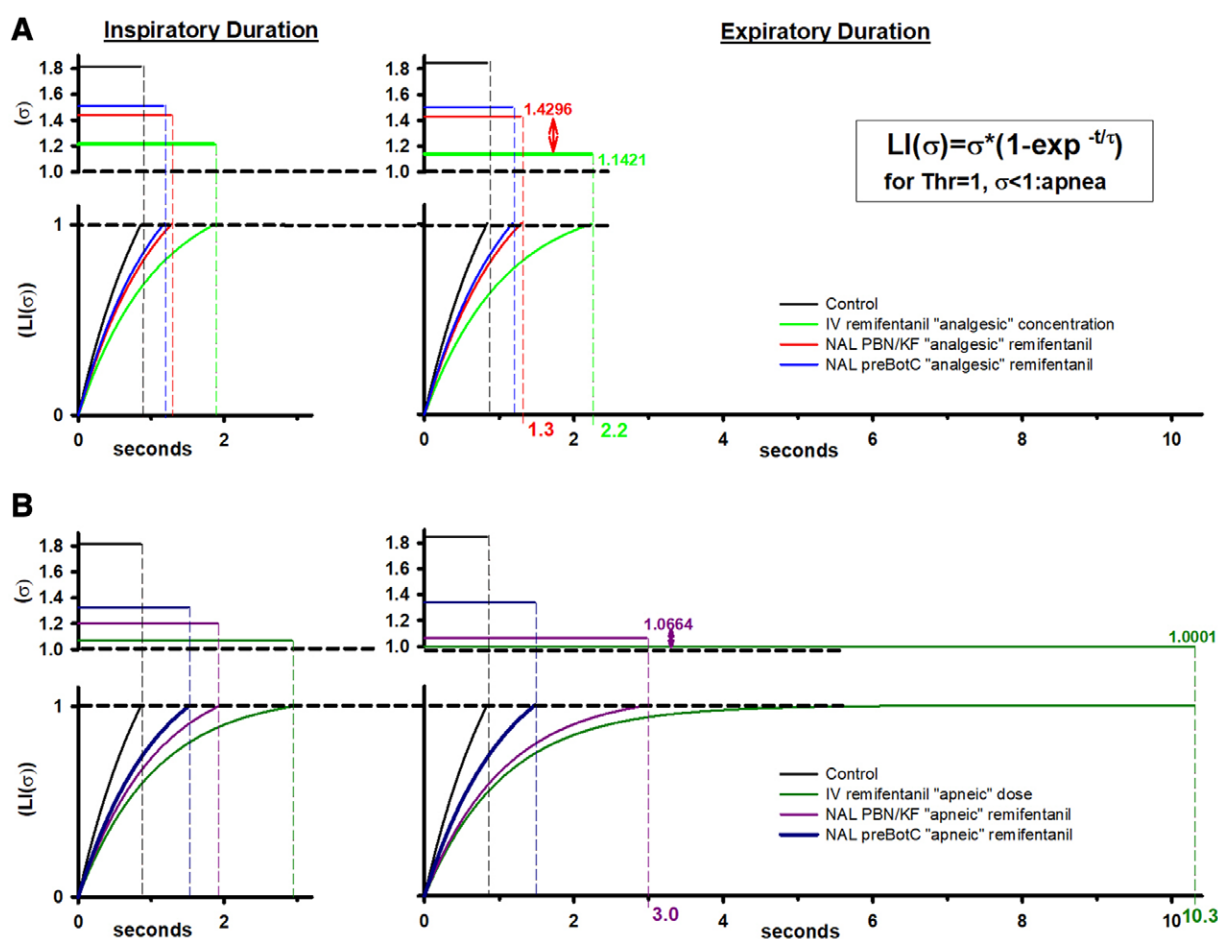
Fig. A1. Actual values for inspiratory and expiratory duration measured at (*A*) analgesic and (*B*) apneic remifentanil concentrations before and after local naloxone injections for the data presented in fig. 3A (analgesic) and fig. 4A (apneic) and their corresponding input values; naloxone injection into the parabrachial nucleus/Kölliker–Fuse decreased expiratory duration after apneic remifentanil bolus from 10.3 to 3 s, which reflected an increase in inputs to expiratory duration from 100.01 to 107% (a 7% increase) of the apneic threshold (*purple arrow*). σ is a step input pattern with onset at t = 0, and LI(σ) is the output step response of the leaky integrator. In contrast, naloxone injection into the parabrachial nucleus/Kölliker–Fuse decreased expiratory duration during analgesic remifentanil concentrations from 2.2 to 1.3 s, reflecting an increase in inputs to expiratory duration from 114 to 143% of apneic threshold (*red arrow*). This illustrates that at high input levels (short respiratory phase durations), the same change in the sum of inputs results in a much smaller change in phase duration than at low input levels, for which small changes in the sum of inputs can change phase duration by several seconds. Calculating the inputs to phase duration allows comparison of opioid effects, as determined by the amount of naloxone reversal, from multiple injections without bias resulting from the respiratory rate at the time of injection. NAL, naloxone; PBN/KF, parabrachial nucleus/Kölliker–Fuse complex; preBotC, pre-Bötzinger complex.

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