
Figures and figure supplements

Opioid suppression of an excitatory pontomedullary respiratory circuit by convergent mechanisms

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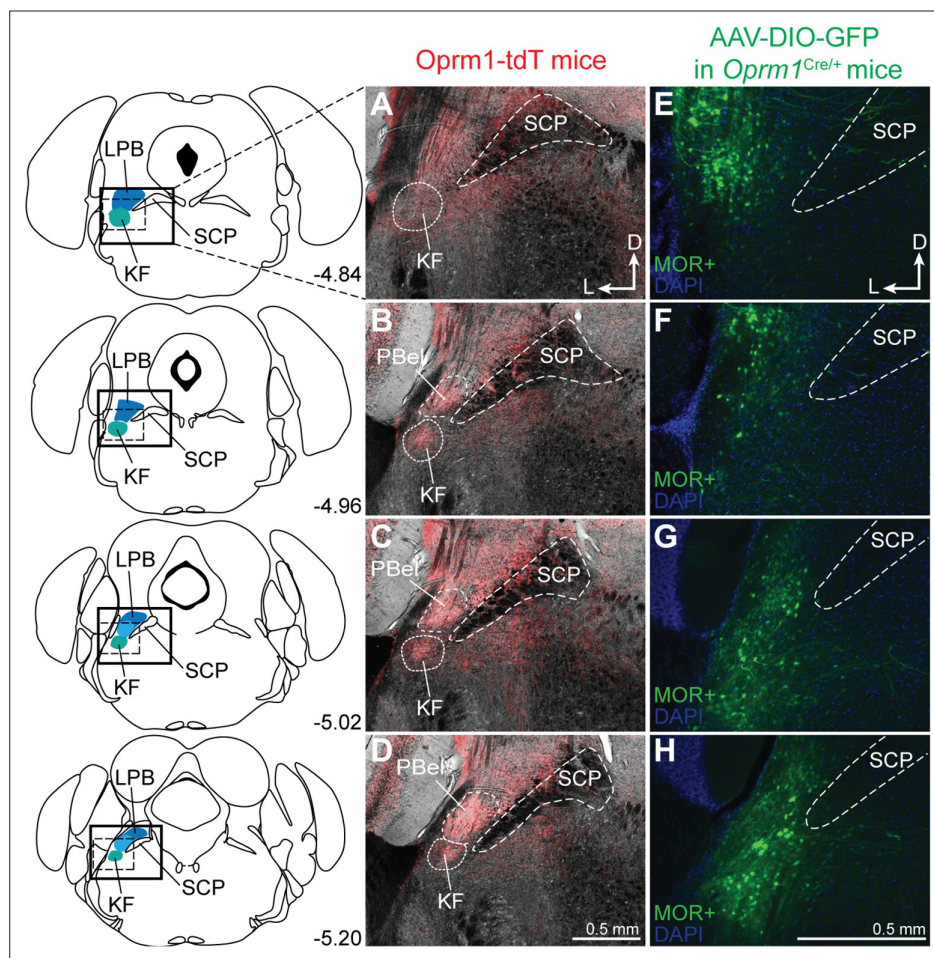


Figure 1. Dorsolateral pontine neurons express mu opioid receptors (MORs). (A–D) Representative images of tdTomato expression, as an indicator of MOR expression, in coronal dorsolateral pontine slices from *Oprm1*-tdT mice ($n = 3$) across the rostral to caudal Kölliker-Fuse/lateral parabrachial area (KF/LPB) axis. Fluorescent tdTomato image is overlaid onto brightfield image to show landmarks. (E–H) Representative images of GFP expression, as an indicator of MOR expression, following injection of virus encoding Cre-dependent GFP into KF/LPB to label MOR+ neurons in adult *Oprm1*^{Cre/+} mice ($n = 5$). Left column are slice schematics corresponding to each row. The approximate levels caudal to bregma (in mm) are to the right of each schematic. The images correspond to the solid boxed area (A–D) or the dotted boxed area (E–H) of the slice schematic. The scale bar in (D) applies to images (A–D). The scale bar in (H) applies to images (E–H). PBel, external lateral subdivision of parabrachial; SCP, superior cerebellar peduncle.

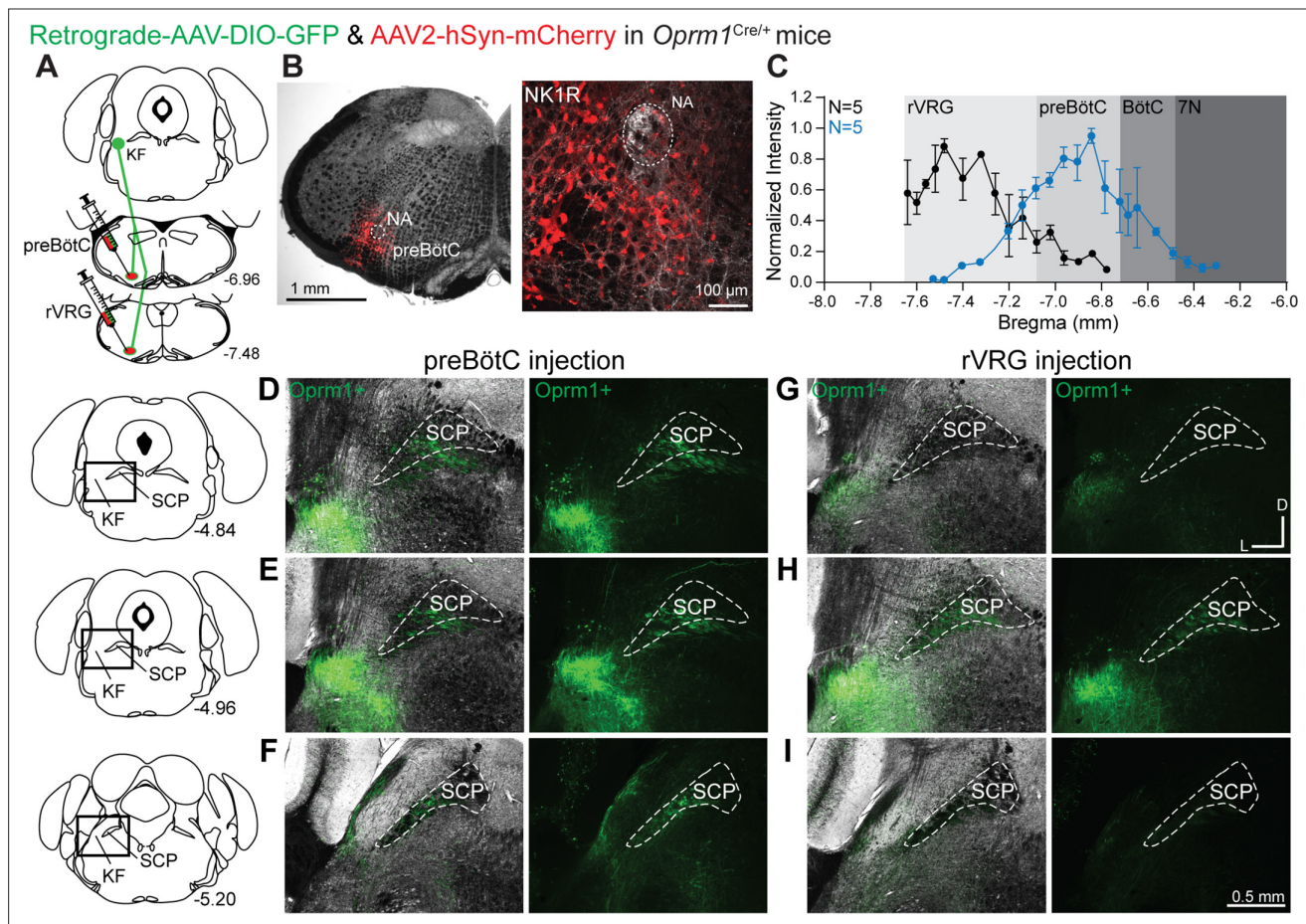


Figure 2. *Oprm1*⁺ Kölliker-Fuse (KF) neurons and neurites retrogradely labeled from the preBötzinger complex (preBötC) and rostral ventral respiratory group (rVRG). **(A)** Schematic illustrating the approach to retrogradely label *Oprm1*⁺ KF neurons and neurites projecting to the preBötC or rVRG. **(B)** Images of coronal slices from the medulla with a control injection of AAV2-hSyn-mCherry into the preBötC of an *Oprm1*^{Cre/+} mouse to mark the injection site. Immunohistochemistry for the neurokinin 1 receptor (NK1R) was used as a marker for the preBötC and the nucleus ambiguus (NA). **(C)** Quantification of normalized AAV2-hSyn-mCherry fluorescence intensity along the rostral to caudal axis in the ventrolateral medulla of preBötC (n = 5) and rVRG (n = 5). Anatomical level relative to Bregma is indicated on the x-axis. **(D–I)** Representative images of GFP expression, as an indicator of retrograde-labeled *Oprm1*-expressing neurons and neurites, following injections into the preBötC (**D–F**) or the rVRG (**G–I**) across three levels of the dorsolateral pons. The bregma level is indicated on the schematics to the left of each row. The scale bar in **(I)** applies to all images **(D–I)**. Higher magnification images of bregma –4.84 are shown in **Figure 2—figure supplement 2**.

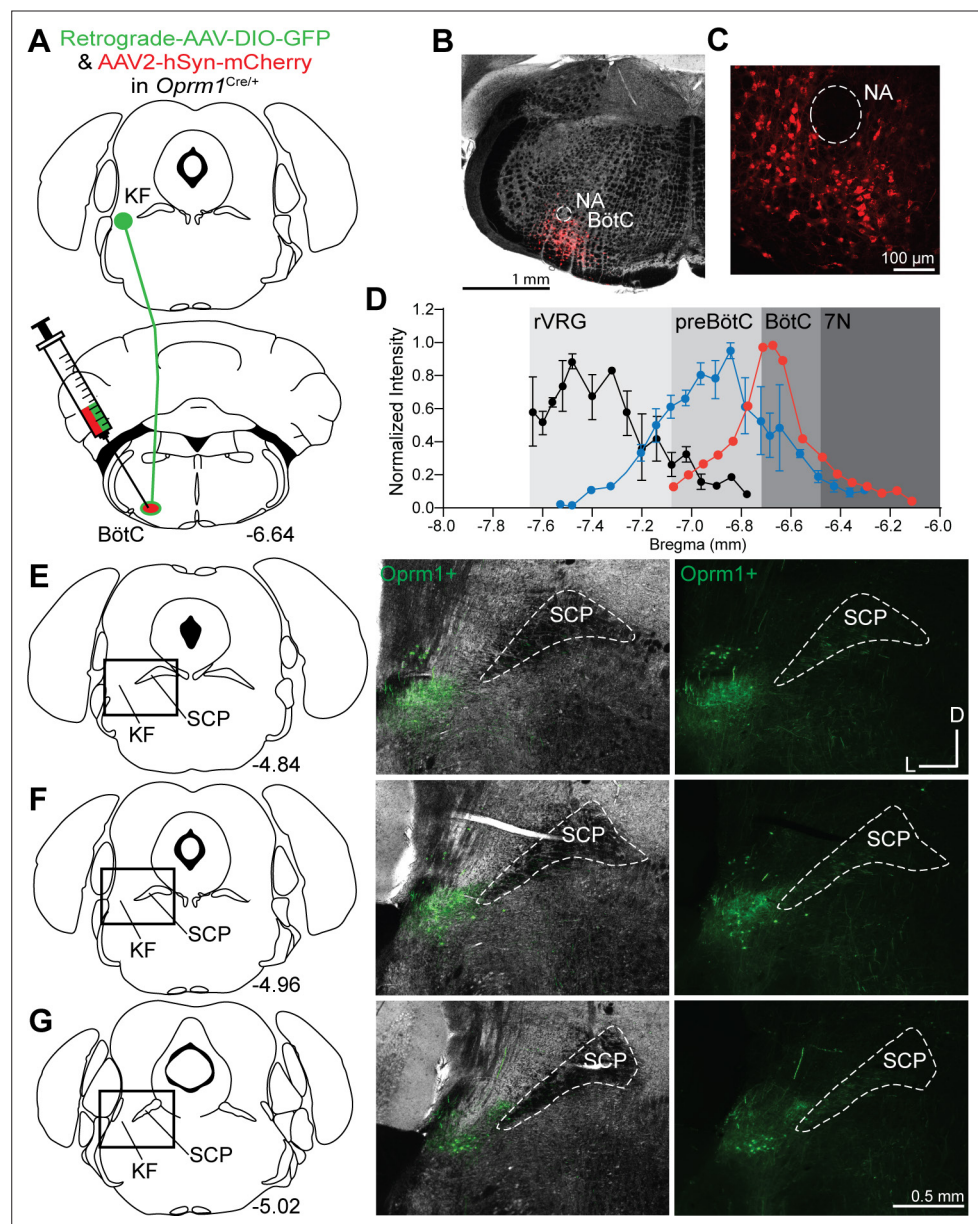


Figure 2—figure supplement 1. *Oprm1*⁺ Kölliker-Fuse (KF) neurons project to the Bötzinger complex (BötC). (A) Schematic illustrating the approach to retrogradely label *Oprm1*⁺ KF neurons projecting to the BötC. (B–, C) Images of coronal slices from the medulla with a control injection of AAV2-hSyn-mCherry into the BötC of an *Oprm1*^{Cre/+} mouse to mark injection spread. The nucleus ambiguus (NA) was used as a medullary marker in both images. (D) Quantification of normalized AAV2-hSyn-mCherry spread along the rostral to caudal axis in the ventrolateral medulla of representative injection into BötC. Data from injections into preBötC (n = 5) and rostral ventral respiratory group (rVRG) (n = 5) are duplicated from **Figure 2** for comparison. (E–G) Images of *Oprm1*⁺ expression following a BötC injection across three levels of the KF/lateral parabrachial area (LPB). The bregma coordinates and approximate location of KF/LPB are indicated. The scale bar in (G) applies to all images in (E–G).

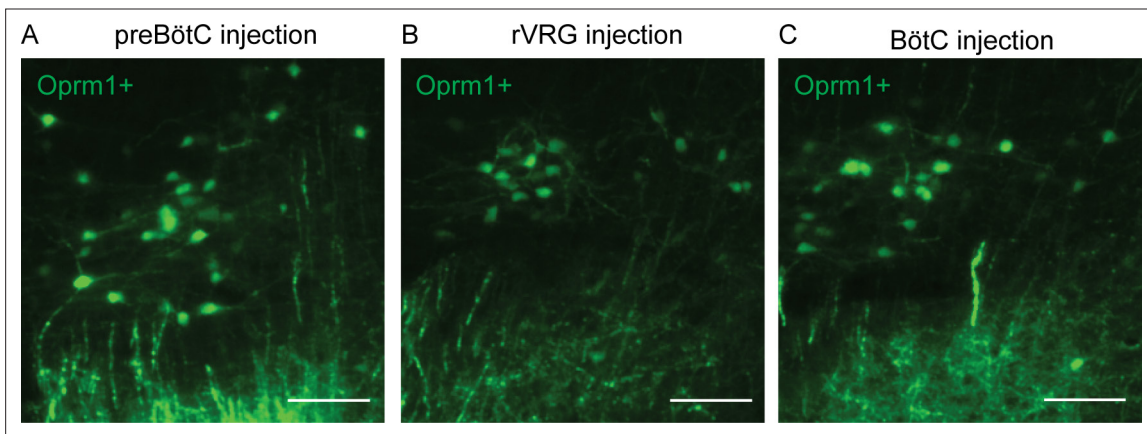


Figure 2—figure supplement 2. Higher magnification images of retrogradely labeled Kölliker-Fuse (KF) neurons. Images of GFP expression, as an indicator of retrograde-labeled *Oprm1*-expressing neurons, following injections into preBötzinger complex (preBötC) (**A**), rostral ventral respiratory group (rVRG) (**B**), or BötC at bregma -4.84 of the dorsolateral pons. Scale bar is $100\ \mu\text{m}$.

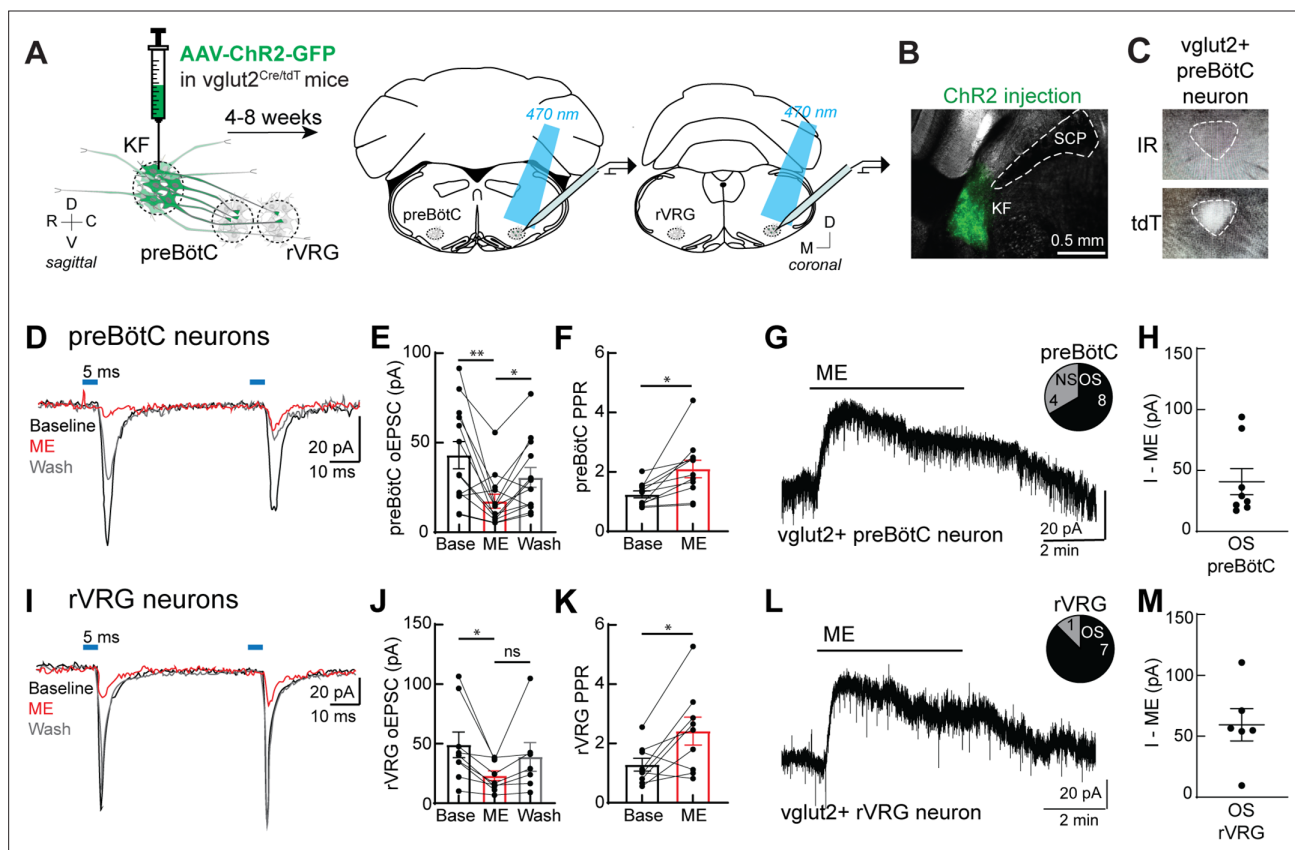


Figure 3. Presynaptic opioid receptors inhibit glutamate release from Kölliker-Fuse (KF) terminals onto excitatory preBötzinger complex (preBötC) and rostral ventral respiratory group (rVRG) neurons. **(A)** Schematic of approach to optogenetically stimulate KF terminals and drive optogenetically evoke excitatory postsynaptic currents (oEPSCs) in excitatory preBötC and rVRG neurons in an acute brain slice. **(B)** Representative image of ChR2-GFP expression in the KF (injection area) of vglut2-tdT mouse. **(C)** tdTomato-expressing, excitatory vglut2-expressing preBötC (or rVRG) neurons were identified in acute brain slices. **(D)** Recording of pairs of oEPSCs (5 ms stimulation, 50 ms inter-stimulus interval) from an excitatory preBötC neuron in an acute brain slice at baseline (black), during perfusion of Met-enkephalin (ME, 3 μ M) (red), and after wash (gray). **(E)** ME decreased oEPSC amplitude in preBötC neurons ($n = 13$; ** $p=0.007$, * $p=0.013$ by one-way ANOVA and Tukey's post-test). **(F)** ME increased the paired-pulse ratio (P2/P1) in preBötC neurons ($n = 11$; * $p=0.001$ paired t -test). **(G)** ME (3 μ M) induced outward currents in 8 of 12 preBötC neurons. OS, opioid-sensitive; NS, non-opioid-sensitive. **(H)** The amplitude of the outward current (I-ME, pA) in OS preBötC neurons. **(I)** Recording of pairs of oEPSCs (5 ms stimulation, 50 ms inter-stimulus interval) from an excitatory rVRG neuron in an acute brain slice at baseline (black), during perfusion of ME (3 μ M) (red), and after wash (gray). **(J)** ME decreased oEPSC amplitude in rVRG neurons ($n = 9$; * $p=0.027$ by one-way ANOVA and Tukey's post-test). **(K)** ME increased the paired-pulse ratio (P2/P1) in rVRG neurons ($n = 9$; * $p=0.043$ by paired t -test). **(L)** ME-mediated outward currents were observed in 7 of 8 rVRG neurons. **(M)** The amplitude of the outward current (I-ME, pA) in OS rVRG neurons. For all graphs, bar/line and error represent mean \pm SEM. Individual data points are from individual neurons.

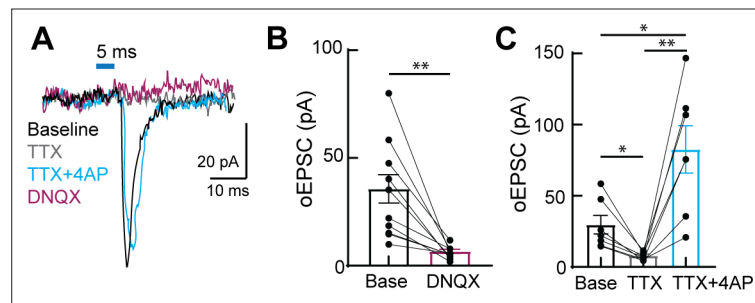


Figure 3—figure supplement 1. Kölliker-Fuse (KF) neurons send monosynaptic, glutamatergic projections to excitatory ventrolateral medullary neurons. **(A)** Recording of optogenetically evoke excitatory postsynaptic currents (oEPSCs) from an excitatory (vglut2+) preBötzinger complex (preBötC) neuron in an acute brain slice at baseline (black), during perfusion of tetrodotoxin (TTX) (1 μ M) (gray), during perfusion of TTX (1 μ M) + 4-aminopyridine (4-AP) (100 μ M) (cyan), and during perfusion of 6,7-dinitroquinoxaline-2,3-dione (DNQX) (10 μ M) (purple). **(B)** KF synapses onto medullary respiratory neurons are monosynaptic. TTX blocked oEPSCs ($n = 7$; $*p=0.0207$ by one-way ANOVA and Tukey's post-test), which were restored by perfusion of TTX + 4-AP ($n = 7$; $**p=0.0086$, $*p=0.042$ by one-way ANOVA and Tukey's post-test). **(C)** KF synapses onto medullary respiratory neurons are glutamatergic. AMPA-type glutamate receptor antagonist DNQX (10 μ M) blocked oEPSCs ($n = 11$; $**p=0.001$ paired t -test).

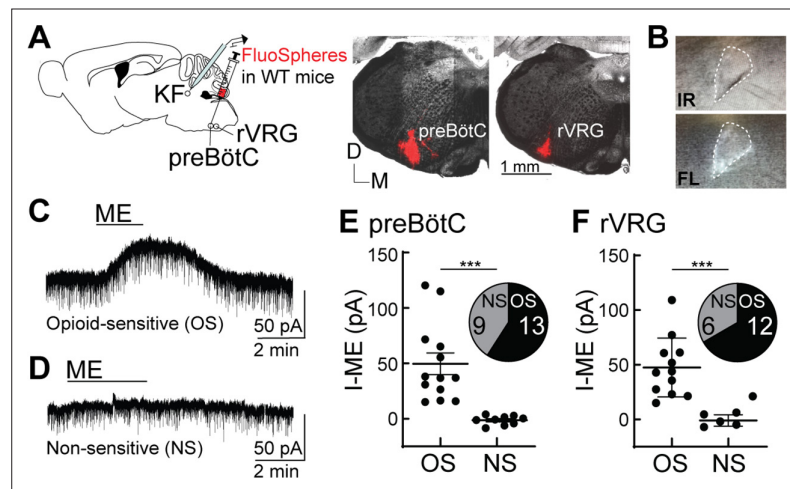


Figure 4. Opioids hyperpolarize Kölliker-Fuse (KF) neurons that project to the preBötzinger complex (preBötC) and rostral ventral respiratory group (rVRG). **(A)** Schematic (left) of approach to retrogradely label KF neurons that project to the preBötC or rVRG with FluoSpheres in wild-type mice. Images (right) of FluoSpheres in the injection area (preBötC or rVRG). The scale bar applies to both injection images. **(B)** A KF neuron retrogradely labeled by FluoSpheres shown with IR-Dodt and epifluorescent (FL) illumination. **(C, D)** Whole-cell voltage-clamp recordings from opioid-sensitive ('OS') and non-opioid-sensitive ('NS') retrogradely labeled KF neurons. Met-enkephalin (ME) (1 μ M) induced an outward current in the opioid-sensitive (OS) neuron **(C)**, but not the non-opioid-sensitive (NS) neuron **(D)**. **(E, F)** Quantification of the amplitude of the ME-mediated current (I-ME [pA]) in OS and NS KF neurons that project to the preBötC **(E)** ($n = 22$; *** $p=0.0005$; unpaired t -test) or the rVRG **(F)** ($n = 18$; *** $p=0.0007$; unpaired t -test). ME induced an outward current in 13 of 22 KF neurons that project to the preBötC and 12 of 18 KF neurons that project to the rVRG. Individual data points are from individual neurons in separate slices. Line and error are mean \pm SEM.

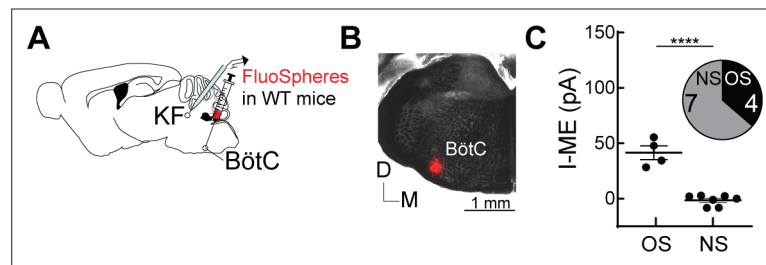


Figure 4—figure supplement 1. Opioids hyperpolarize Kölliker-Fuse (KF) neurons that project to the Bötzing complex (BötC). (A) Schematic of approach to retrogradely label KF neurons that project to the BötC with FluoSpheres in wild-type mice. (B) Image of FluoSpheres injection into the BötC. (C) Quantification of Met-enkephalin (ME)-mediated current in opioid-sensitive (OS) and non-opioid-sensitive (NS) KF neurons that project to the BötC ($n = 11$; $p = 0.0001$; unpaired t -test). ME ($1 \mu\text{M}$) induced an outward current in 4 of 11 KF neurons that project to the BötC. Individual data points are from individual neurons in separate slices. Line and error are mean \pm SEM.

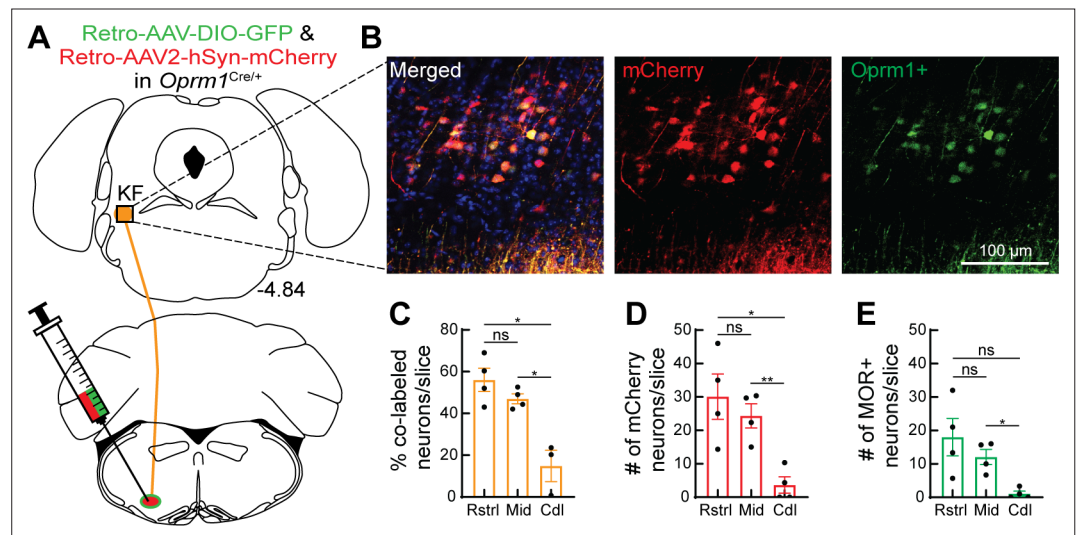


Figure 5. *Oprm1*⁺ and *Oprm1*⁻ dorsolateral pontine neurons project to the ventrolateral medulla. **(A)** Schematic of approach injecting retrograde virus encoding Cre-dependent GFP expression and a retrograde virus encoding mCherry expression into the ventrolateral medulla of *Oprm1*^{Cre/+} mice to label *Oprm1*⁺ and *Oprm1*⁻ dorsolateral pontine neurons that project to these respiratory nuclei. **(B)** Representative images of mCherry expression (retrogradely labels neurons regardless of *Oprm1* expression) and GFP expression (retrogradely labels *Oprm1*⁺ neurons) in a rostral dorsolateral pontine slice (bregma -4.84 mm). **(C)** Summary of percentage of retrograde-labeled neurons that were *Oprm1*⁺ (co-labeled with mCherry and GFP) in rostral (Rstrl, bregma -4.84 mm), mid-rostral (Mid, bregma -4.96 mm), and caudal (Cdl, bregma -5.2 mm) slices. **(D, E)** Summary of the average number of mCherry-expressing **(D)** or GFP-expressing MOR⁺ **(E)** dorsolateral pontine neurons per slice in rostral, mid-rostral, and caudal slices. Bar and error are mean ± SEM. Individual data points are from individual mice. N = 4 mice, three slices per region per mouse. *p<0.05, **p<0.01, ns = p>0.05 by one-way ANOVA and Tukey's post-test.

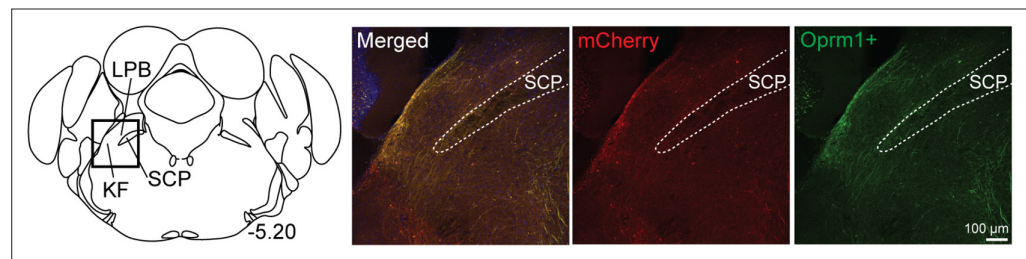


Figure 5—figure supplement 1. Medullary-projecting *Oprm1*⁺ neurons are mostly absent from the caudal Kölliker-Fuse (KF) and lateral parabrachial areas. Retrograde-labeled neurons (both *Oprm1*⁺ and *Oprm1*⁻) were mostly lacking in caudal KF or lateral parabrachial area. The bregma coordinate and approximate location in KF/lateral parabrachial area (LPB) are indicated. The scale bar applies to all images.

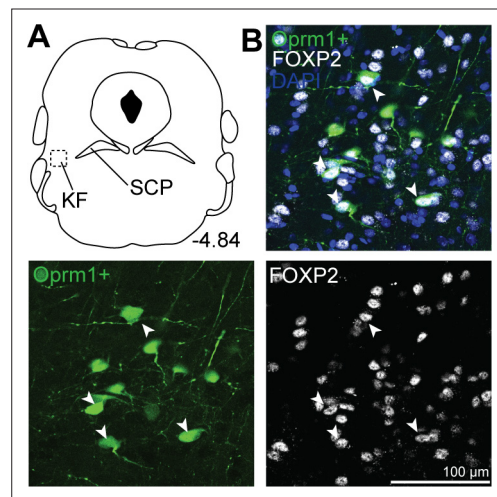


Figure 6. *Oprm1*⁺, medullary-projecting Kölliker-Fuse (KF) neurons express Forkhead box protein P2 (FoxP2). *Oprm1*⁺ neurons that project to the ventrolateral medulla were retrogradely labeled by injection of retrograde AAV-DIO-GFP into *Oprm1*^{Cre/+} mice. Immunohistochemistry was used to label FoxP2. (A, B) In rostral slices (bregma −4.84), FoxP2 is expressed in *Oprm1*⁺ KF neurons that project to the ventrolateral medulla. Schematic (A) depicts the approximate bregma level and imaging area (dotted boxed area). The scale bar applies to all images. SCP, superior cerebellar peduncle.

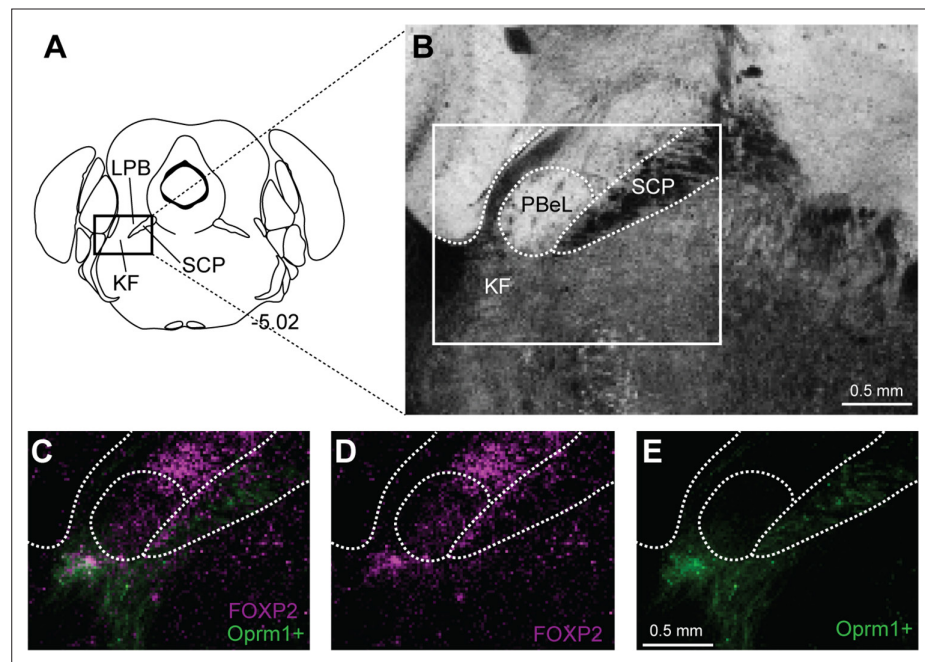


Figure 6—figure supplement 1. Forkhead box protein P2 (FoxP2) expression in caudal Kölliker-Fuse (KF), but not external lateral parabrachial subnucleus. *Oprm1*⁺ neurons that project to the ventrolateral medulla were retrogradely labeled by injection of retrograde AAV-DIO-GFP into *Oprm1*^{Cre/+} mice. Immunohistochemistry was used to label FoxP2. **(A)** Schematic of approximate bregma level of the images. **(B)** Brightfield image of coronal slice used for imaging. **(C–E)** In caudal slices, FoxP2 is expressed in *Oprm1*⁺ KF neurons that project to the ventrolateral medulla and excluded from the outer portion of the external lateral parabrachial subnucleus (PBeL). Images in **(C–E)** are of the boxed area in **(B)**. The scale bars apply to all images in each row. SCP, superior cerebellar peduncle.

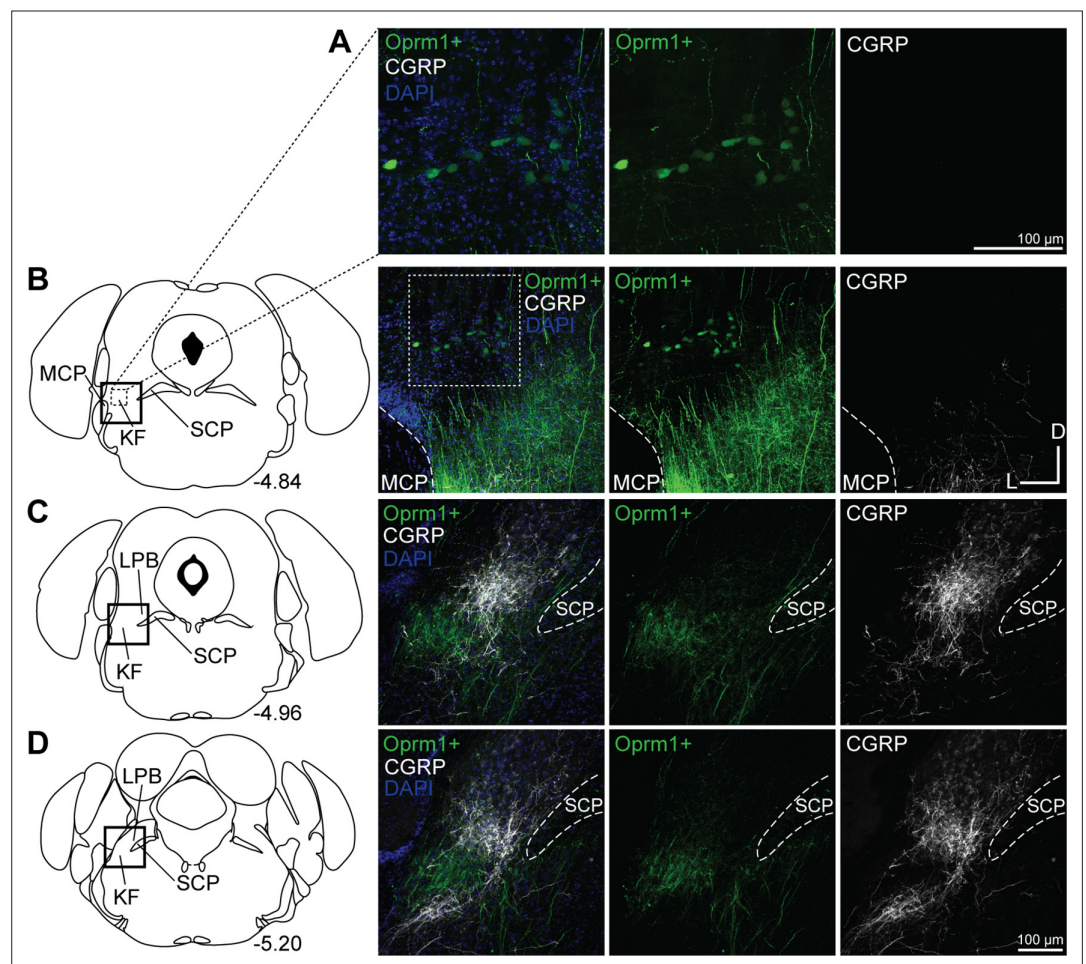


Figure 7. *Oprm1*+, medullary-projecting Kölliker-Fuse (KF) neurons do not express calcitonin gene-related peptide (CGRP). *Oprm1*+ neurons that project to the ventrolateral medulla were retrogradely labeled by injection of retrograde AAV-DIO-GFP into *Oprm1*^{Cre/+} mice. Immunohistochemistry was used to label CGRP. (A, B) CGRP is absent from rostral KF and *Oprm1*+ KF neurons that project to the ventrolateral medulla (*Oprm1*+). (C, D) CGRP marks lateral parabrachial area (LPB) neurons and their axon fiber projections, but is absent from retrograde-labeled *Oprm1*+ axon fiber projections in mid-rostral (C) and caudal (D) slices. The approximate bregma levels are to the right of each schematic. The images correspond to the dotted boxed area (row A) or the solid boxed area (rows B–D) of the slice schematic. The images in (A) are zoomed into the dotted boxed area of the image in (B). The scale bar in (A) applies to the images in row (A). The scale bar in (D) applies to images in rows (B–D). SCP, superior cerebellar peduncle; MCP, medial cerebellar peduncle.

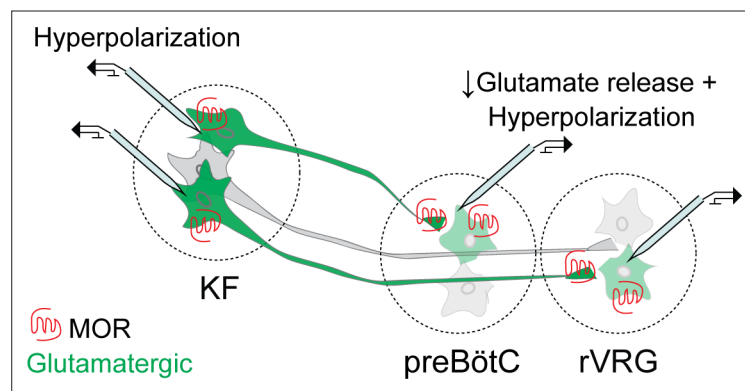


Figure 8. Summary schematic of mu opioid receptor (MOR) regulation of excitatory pontomedullary circuitry. Kölliker-Fuse (KF): Somatodendritic MORs hyperpolarize KF neurons that project to the ventrolateral medulla. Ventrolateral medulla: presynaptic MORs inhibit glutamate release from KF axon terminals onto glutamatergic preBötzing complex (preBötC) and rostral ventral respiratory group (rVRG) neurons. Somatodendritic MORs hyperpolarize glutamatergic preBötC and rVRG neurons that receive KF input. Glutamatergic neurons are in green.