5-Hydroxytryptamine 1A/7 and 4α Receptors Differentially Prevent Opioid-Induced Inhibition of Brain Stem Cardiorespiratory Function

Xin Wang, Olga Dergacheva, Harriet Kamendi, Christopher Gorini, David Mendelowitz

**Abstract**—Opioids evoke respiratory depression, bradycardia, and reduced respiratory sinus arrhythmia, whereas serotonin (5-HT) agonists stimulate respiration and cardiorespiratory interactions. This study tested whether serotonin agonists can prevent the inhibitory effects of opioids on cardiorespiratory function. Spontaneous and rhythmic inspiratory-related activity and γ-aminobutyric acid (GABA) neurotransmission to premotor parasympathetic cardioinhibitory neurons in the nucleus ambiguus were recorded simultaneously in an in vitro thick slice preparation. The μ-opioid agonist fentanyl inhibited respiratory frequency. The 5-hydroxytryptamine 1A/7 receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin increased respiratory frequency by itself and also prevented the fentanyl-induced respiratory depression. The 5-hydroxytryptamine 4α agonist BIMU-8 did not by itself change inspiratory activity but prevented the μ-opioid-mediated respiratory depression. Both spontaneous and inspiratory-evoked GABAergic neurotransmission to cardiac vagal neurons were inhibited by fentanyl. 8-Hydroxy-2-(di-n-propylamino)tetralin inhibited spontaneous but not inspiratory-evoked GABAergic activity to parasympathetic cardiac neurons. However, 8-hydroxy-2-(di-n-propylamino)tetralin differentially altered the opioid-mediated depression of inspiratory-evoked GABAergic activity but did not change the opioid-induced reduction in spontaneous GABAergic neurotransmission. In contrast, BIMU-8 did not alter GABAergic neurotransmission to cardiac vagal neurons by itself but prevented the fentanyl depression of both spontaneous and inspiratory-evoked GABAergic neurotransmission to cardiac vagal neurons. In the presence of tetrodotoxin, the inhibition of GABAergic inhibitory post synaptic currents with fentanyl is prevented by coapplication of BIMU-8, indicating that BIMU-8 acts at presynaptic GABAergic terminals to prevent fentanyl-induced depression. These results suggest that activation of 5-hydroxytryptamine receptors, particularly 5-hydroxytryptamine 4α agonists, may be a useful therapeutic approach in preventing opioid-evoked cardiorespiratory depression. **(Hypertension. 2007;50:368-376.)**

**Key Words:** heart rate, parasympathetic, serotonin, opioid, ambiguus, GABA

Opiate exposure causes cardiorespiratory depression, including a centrally mediated bradycardia, respiratory inhibition, and a reduction in respiratory sinus arrhythmia. Different opioid receptor agonists differentially enhance the activity of cardioinhibitory cardiac vagal neurons (CVNs) through disinhibition. More specifically, μ-opioid receptor agonists and norepinephrine inhibit both spontaneous γ-aminobutyric acid (GABA) and glycinergic synaptic inputs, whereas a κ-opioid agonist diminishes spontaneous glycinergic but not GABAergic neurotransmission to CVNs. In vitro studies from both brain stem slice and brain stem-spinal cord preparations demonstrate that opioids decrease the frequency of respiratory activity. This opioid-induced depression of breathing is caused by μ- and κ-opioid-receptor activation and by direct inhibition of rhythm-generating respiratory neurons in the pre-Botzinger complex. Respiratory neurons, including neurons in the pre-Botzinger complex, as well as CVNs, are modulated by serotonin. Serotonin enhances activity in respiratory neurons through its action on 5-hydroxytryptamine (5-HT)1A/7, 5-HT4, and 5-HT3 serotonin receptors. The contrasting actions of opioids and serotonin on respiratory neurons allow for the possibility that serotonergic receptor agonists could alleviate the depressive action of opioids. In recent neurophysiological investigations, the serotonergic ligands; 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), an agonist of 5-HT1A/7 and 5-HT3 serotonin receptors; and buspirone, a partial agonist at the 5-HT1A receptor, reversed morphine-induced depression of respiratory neurons in anesthetized rats and goats. The 5-HT3 receptor agonists BIMU-8 and zacopride reversed opioid-induced depression of respiratory function and maintained fentanyl analgesia in anesthetized rats and immobilization in goats. Central 5-HT1A receptors have also been shown to be involved in mediating both cardiopulmonary- and baroreceptor reflex-evoked changes in cardiac vagal activity but may
not be involved in chemoreceptor-elicited responses in CVNs. However, the sites of activation of 5-HT receptors in the brain stem that mediate the changes in parasympathetic cardiac activity was not determined. Microinjection or iontophoretic application of different 5-HT agonists into the nucleus ambiguus has provided mixed responses. Although application of the 5-HT1A receptor agonist 8-OH-DPAT generally inhibit CVNs at a low dose of application, higher application doses elicited an excitation of CVNs. Other work has shown that microinjection of the 5-HT1A receptor agonist 8-OH-DPAT excited CVNs to evoke a bradycardia. Because little is known concerning the sites and mechanisms of action of 5-HT receptors on CVNs, and 5-HT1A and 5-HT4 receptor activation show different effects on cardiorespiratory function and opioid depression, this study examines whether 5-HT1A and 5-HT4 receptor agonists alter GABAergic neurotransmission to CVNs, as well as whether the opioid-mediated changes in spontaneous and respiratory-evoked neurotransmission to parasympathetic cardiac activity can be prevented by activation of 5-HT1A and 5-HT4 receptors.

Materials and Methods

Animal procedures and methodologies for identifying CVNs and recording from them in whole-cell patch clamp configuration with spontaneous rhythmic respiratory network activity are published as online supplemental data (http://hyper.ahajournals.org) and are identical to previously published methodologies. All of the animal procedures were performed with the approval of the animal care and use committee of George Washington University in accordance with the recommendations of the panel on euthanasia of the American Veterinary Medical Association and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Focal drug application was performed with a PV830 Pneumatic PicoPump pressure delivery system (WPI), and GABAergic neurotransmission was isolated as described previously.

μ-Opioid, 5-HT1A, and 5-HT4 Receptor Agonist Administration

Rhythmic inspiratory-related activity and inspiratory-evoked GABAergic synaptic inputs in CVNs were recorded simultaneously for 15 minutes in artificial cerebrospinal fluid. Slices were then exposed to the μ-opioid receptor agonist fentanyl by inclusion in the perfusate for 15 minutes. The exposure to fentanyl was then terminated, and the slice was perfused with the original artificial cerebrospinal fluid for 30 minutes.

Fentanyl was applied at a concentration of 1 or 5 μmol/L; these doses were chosen to be similar to the concentrations used in other studies of brain stem cardiorespiratory function. Each slice was exposed to 1 dose of fentanyl only. At the end of each experiment, respiratory evoked GABAergic activity was reversibly blocked with gabazine (25 μmol/L).

The 5-HT1A receptor agonist 8-OH-DPAT was used at a dose of 5 μmol/L either alone or in combination with fentanyl. In a separate series of experiments, the 5-HT4 receptor agonist BIMU-8 was administered either alone or in combination with fentanyl at a concentration of 10 μmol/L. This drug was a generous gift from the Boehringer Ingelhein Pharma GmbH & Co.

In a separate series of experiments, miniature GABAergic inhibitory postsynaptic currents (mIPSCs) were isolated by inclusion of tetrodotoxin (1 μmol/L) in the perfusate to block action potential events. mIPSCs were recorded in the presence and absence of fentanyl (10 mmol/L) or coapplication of fentanyl (10 mmol/L) and BIMU-8 (10 μmol/L).
Synaptic Inputs to CVNs

Effect of 5-HT1A/7 Receptor Agonist 8-OH-DPAT and 5-HT4 Agonist BIMU-8 on Spontaneous and Inspiratory-Evoked GABAergic Inhibitory Synaptic Inputs to CVNs

Application of 5-HT1A/7 receptor agonist 8-OH-DPAT (5 μm; n = 10) significantly increased inspiratory bursting frequency by 35 ± 5.8% and 40 ± 7.3%, 1 nmol/L (n = 8) and 5 nmol/L (n = 22), respectively. In contrast, the 5-HT1A/7 receptor agonist 8-OH-DPAT (5 μmol/L; n = 10) significantly increased inspiratory bursting frequency by 25.5 ± 7.8% (P < 0.001). Administration of the 5-HT4 receptor agonist BIMU-8 (10 μmol/L; n = 12) induced a slight increase in inspiratory bursting frequency (16 ± 5.3%; P = 0.05). Coapplication of either 8-OH-DPAT (5 μmol/L; n = 9) or BIMU-8 (10 μmol/L; n = 17) with fentanyl (5 nmol/L) prevented the fentanyl-induced depression of respiration and maintained a stable respiratory bursting frequency during the 60-minute experimental period. Data were analyzed with ANOVA with repeated measures, and any significant changes in the sequential responses are noted with asterisks, in this and all subsequent figures: *P < 0.05, **P ≤ 0.01, and ***P ≤ 0.001. There were no significant differences between the groups before drug application (time 0).

under these recording conditions were blocked by the application of the GABA_A antagonist gabazine (data not shown).

Discussion

There are 4 major findings in this study. First, whereas the μ-opioid receptor agonist fentanyl induces a dose-dependent and reversible decrease in the frequency of respiratory bursting rate in the thick in vitro brainstem slice, the 5-HT1A/7 receptor agonist, 8-OH-DPAT, enhances respiratory activity, and both 8-OH-DPAT and the 5-HT4 receptor agonist, BIMU-8, prevent the μ-opioid–mediated respiratory-related depression. Second, both spontaneous and inspiratory-induced GABAergic neurotransmission to CVNs is inhibited by fentanyl. The 5-HT1A/7 receptor agonist, 8-OH-DPAT, also evokes a decrease in spontaneous but not inspiratory-evoked GABAergic postsynaptic neurotransmission to parasympathetic CVNs. Although coadministration of 8-OH-DPAT with fentanyl reversed the opioid-induced respiration-related depression and inspiratory-evoked GABAergic neurotransmitter input to parasympathetic CVNs, spontaneous GABAergic neurotransmission remained diminished with coapplication of 8-OH-DPAT and fentanyl treatment. Third, application of the 5-HT4 receptor agonist, BIMU-8, does not significantly alter either spontaneous or inspiratory-elicited GABAergic inhibitory postsynaptic inputs to cardiac parasympathetic neurons. However, BIMU-8 completely prevented the fentanyl-induced depression of both spontaneous and inspiratory-evoked GABAergic neurotransmission to CVNs. Our results also show that the BIMU-8 prevention of opioid inhibition can occur by interactions at the presynaptic GABAergic synaptic terminals.
The results in this study demonstrate that activation of
\( \mu \)-opioid receptors induces a reversible decrease in the
frequency of inspiratory bursting rate in the thick in vitro
brain stem slice. These data are consistent with previous
findings both in vivo and in vitro. Opioids suppress the
frequency of neonatal rat respiration by likely acting on
\( \mu \)-opioid receptors located within regions of the ventral
medulla containing respiratory rhythm-generating neurons
localized to the pre-Botzinger complex. Although both
inspiratory and preinspiratory neurons have been shown to
be sufficient for respiratory rhythmogenesis, preinspira-
tory neurons are opioid insensitive.\(^2\) Because opioids
inhibit the generation of inspiratory but not expiratory
events, it has been proposed that pre-Botzinger neurons are
responsible for inspiratory activity, whereas preinspiratory
opioid-insensitive neurons may mediate primarily expira-
tory activity and/or the termination of inspiration.\(^2\)\(^5\) Our
results are consistent with this hypothesis, because opioid
receptor agonists decrease the inspiratory frequency, but
not inspiratory amplitude or duration. Although the results
in this study demonstrate that both 8-OH-DPAT and
BIMU-8 can prevent \( \mu \)-opioid–mediated respiratory de-
pression on coapplication with fentanyl, future studies are
warranted to examine whether 8-OH-DPAT and/or

Figure 2. Fentanyl diminished spontaneous and inspiratory-evoked GABAergic inhibitory postsynaptic inputs to CVNs. Inspiratory-
related bursting activity was recorded from the hypoglossal rootlet (XII) and electronically integrated ([XII]) in this and all of the
subsequent figures where appropriate, and GABAergic neurotransmission was isolated by focal application of N-methyl-D-aspartate,
non-N-methyl-D-aspartate, and glycineric receptor antagonists. In control conditions, the frequency of GABAergic IPSCs significantly
increased during inspiratory bursts. After continuous and focal application of fentanyl (5 nmol/L; n=9), both spontaneous and
inspiratory-evoked GABAergic IPSC frequency was significantly depressed. +++ denotes a statistically significant difference of
\( P<0.001 \) between responses during control (no drug) and fentanyl exposure.
BIMU-8 can reverse pre-existing opioid-evoked respiratory depression.

In normal eupneic respiration, inspiration evokes an increase in the inhibitory GABAergic neurotransmission to CVNs, which likely mediates respiratory sinus arrhythmia. Spontaneous GABAergic activity to cardioinhibitory parasympathetic neurons originates, at least in part, from neurons located in the nucleus tractus solitarius. This spontaneous GABAergic activity is likely involved in cardiovascular reflexes, such as the baroreflex. The current study demonstrates that the μ-opioid receptor agonist fentanyl inhibits both spontaneous and inspiratory-evoked GABAergic synaptic inputs to CVNs. This may be a cellular mechanism responsible for opioid-induced bradycardia, blunted baroreceptor reflex, and decrease in respiratory sinus arrhythmia.

Central injections of 5-HT₁A agonists evoke a bradycardia, which is accompanied by an increase in respiration. Because 5-HT₁A receptors are likely inhibitory, this suggests the 5-HT₁A receptor–mediated decrease in heart rate is likely caused by disinhibition of parasympathetic cardioin-
hibitory neurons. Our results strongly support this hypothesis, because the 5-HT1A/7 receptor agonist 8-OH-DPAT inhibited spontaneous GABAergic inhibitory postsynaptic neurotransmission to parasympathetic CVNs. A major beneficial effect of 8-OH-DPAT is increased oxygen diffusion, likely by increased ventilation perfusion ratios.17 Our data provide a cellular basis for the beneficial effect of 8-OH-DPAT on cardiorespiratory ventilation-perfusion function, because 8-OH-DPAT differentially inhibits spontaneous GABAergic activity but does not change the increase in respiratory-evoked GABAergic inhibitory neurotransmission to cardiac parasympathetic neurons during inspiration that is responsible for respiratory sinus arrhythmia. Although activation of 5-HT1A receptors by 8-OH-DPAT could reverse opioid-induced inhibition of respiration, it also produces a disinhibition of CVNs, similar to the effect of opioids; therefore, activation of both μ-opioid receptors and 5-HT1A receptors in the brain stem depress spontaneous GABAergic neuro-

Figure 4. The 5-HT1A/7 receptor agonist 8-OH-DPAT prevented fentanyl-induced decrease in inspiratory-evoked GABAergic neurotransmitter release to CVNs. Coapplication of 8-OH-DPAT and fentanyl elicited a significant decrease in spontaneous GABAergic neurotransmitter release to CVNs. However, inspiratory-evoked GABAergic IPSC frequency was stable during the 60-minute experimental period with coapplication of 8-OH-DPAT (5 μmol/L) and fentanyl (5 nmol/L; n=9 cells). *P<0.05, ++P<0.01 denotes statistical comparison between control and 8-OH-DPAT/fentanyl responses.
transmission to CVNs, likely resulting in increased activity of cardiac parasympathetic neurons in the nucleus ambiguus and a bradycardia.

A potentially clinically important finding of this study is that both depressed respiratory function and cardiorespiratory interactions by μ-opioid agonists can be prevented by administration of the 5-HT$_4$ receptor agonist. Co-administration of the 5-HT$_4$ receptor agonist BIMU-8 prevented the opioid-mediated depression of respiration and both spontaneous and respiratory related GABAergic neurotransmission to CVNs. Although it cannot be ruled out that BIMU-8 recruits an independent GABAergic pathway to CVNs that compensates for the reduced GABAergic transmission inhibited by opioids, our results examining GABAergic mIPSCs indicate that a more likely mechanism of action is that BIMU-8 occludes the effects of fentanyl at the presynaptic GABAergic synaptic terminal. This preventive effect is likely via competition of intracellular signaling pathways. It is possible that stimulation of the μ-opioid receptors decreases cAMP in GABAergic synaptic terminals, as well as inspiratory neurons, and consequently decreases GABAergic neurotransmission to CVNs and inspiratory drive, whereas stimulation of the 5-HT$_4$ receptors would be predicted to increase cAMP and, thus, increase and restore GABAergic neurotransmission to CVNs and inspiratory drive.

Figure 5. The 5-HT$_4$ agonist BIMU-8 prevented the fentanyl-induced decrease in spontaneous and inspiratory-evoked GABAergic neurotransmitter release to CVNs. Application of BIMU-8 (10 μm; n=12 cells) alone did not significantly alter either spontaneous or inspiratory-evoked GABAergic IPSCs vs control responses before BIMU-8 application. In addition, both spontaneous and inspiratory-evoked GABAergic IPSC frequency was stable during the 60-minute experiment period with coapplication of BIMU-8 (10 μmol/L) and fentanyl (5 nmol/L; n=17 cells).
Figure 6. BIMU-8 prevented the \(\mu\)-opioid depression of GABAergic synaptic function. Application of the \(\mu\)-opioid receptor agonist fentanyl significantly inhibited the frequency of GABAergic mIPSCs that were isolated in the presence of tetrodotoxin (1 \(\mu\)mol/L; left). In a second series of experiments, coapplication of the 5-HT\(_{4}\) receptor agonist BIMU-8 (10 \(\mu\)mol/L; \(n=7\)) prevented the opiate-mediated depression of GABAergic neurotransmission (right).

**Perspectives**

The results in this study show that the 5-HT\(_{4}\) agonist BIMU-8 did not by itself enhance either respiration or GABAergic neurotransmission to CVNs; however, BIMU-8 did prevent the \(\mu\)-opioid depression of both respiration and spontaneous and inspiratory-evoked GABAergic neurotransmission to CVNs. In contrast, the 5-HT\(_{1A/7}\) receptor agonist 8-OH-DPAT directly reduced spontaneous inhibitory postsynaptic GABAergic neurotransmitter input to parasympathetic CVNs and could not prevent the opioid-evoked inhibition of spontaneous GABAergic activity to CVNs. 5-HT receptor activation, especially 5-HT\(_{4}\) receptor agonists, may be a useful target to prevent respiratory depression and inhibition of respiratory sinus arrhythmia that occurs with \(\mu\)-opioids.

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**Disclosures**

None.

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MATERIALS AND METHODS

All animal procedures were performed with the approval of the Animal Care and Use Committee of The George Washington University in accordance with the recommendations of the panel on euthanasia of the American Veterinary Medical Association and the NIH publication, “Guide for the Care and Use of Laboratory Animals.”

Fluorescent labeling of CVNs and medullary slice preparation

Neonatal Sprague-Dawley rats (P3-P7; Hilltop, Scottdale, PA) were anesthetized with ketamine/xylazine (87/13 mg/Kg IP) and cooled to approximately 4º C to slow the heart rate. A right thoractomy was performed, and the retrograde fluorescent tracer X-rhodamine-5-(and-6)-isothiocyanate (Molecular Probes, Eugene, OR) was injected into the fat pads at the base of the heart. After 24 - 48 hours recovery, animals were anesthetized with halothane or isoflurane and sacrificed by cervical dislocation. The brain was removed and immediately transferred into ice-cold (4º C) physiological saline solution containing (in mmol/L): 140 NaCl, 5 KCl, 2 CaCl₂, 5 Glucose, and 10 HEPES and oxygenated with 100% O₂, pH 7.4. The medulla was removed with care to preserve the hypoglossal cranial nerve rootlet. The brain stem was fixed on an agar block and secured in a vibrotome (Leica, Nussloch, Germany) with the rostral end up. Thin slices were sectioned serially in a rostro-caudal progression until the inferior olives and the nucleus ambiguus could be visualized on the rostral surface of the tissue. A single thick (800µm) section that included CVNs,
the hypoglossal nerve rootlet, the pre-Bötzinger complex and the rostral portion of the hypoglossal nucleus was cut and submerged in a recording chamber which allowed perfusion (10 ml/min) of artificial cerebrospinal fluid (aCSF) containing (in mmol/L) 125 NaCl, 3 KCl, 2 CaCl₂, 26 NaHCO₃, 5 Glucose, and 5 HEPES equilibrated with carbogen (95% O₂-5% CO₂, pH = 7.4).

**Recording Respiratory Network Activity**

The thick medullary slice preparation contains the pre-Bötzinger complex, local circuits for motor output generation, and respiratory hypoglossal motorneurons¹. Spontaneous respiratory-related activity was recorded by monitoring motorneuron population activity from hypoglossal nerve rootlets using a suction electrode. Signals recorded from hypoglossal rootlet activity were amplified 50,000 times, band pass filtered (low-pass 10Hz, high-pass 300Hz, CWE inc., Ardmore, PA) and electronically integrated (τ = 50ms; CWE inc., Ardmore, PA).

**Patch Clamp Techniques**

CVNs in the nucleus ambiguus (NA) were identified by the presence of the fluorescent tracer². Patch pipettes (2.5-3.5 MΩ) were visually guided to the surface of individual CVNs using differential interference optics and infrared illumination (Zeiss, Oberkochen, Germany). Patch pipettes contained (in mmol/L) 150 KCl, 4 MgCl₂, 2 EGTA, 2 Na-ATP, 10 HEPES; pH = 7.4. This pipette solution causes the Cl⁻ current induced by the activation of GABA
receptors to be recorded as an inward current (calculated reversal potential of Cl\(^-\) = + 4mV). Voltage clamp recordings were made with an Axopatch 200B, and pClamp 8 software (Axon Instruments, Union City, CA). All synaptic activity in CVNs was recorded at -80mV. Only one experiment was conducted per preparation.

**Data analysis**

*Electrophysiology:* Synaptic events were detected using MiniAnalysis version 5.6.12 (Synaptosoft, Decatur, GA). Inhibitory postsynaptic currents frequency was cross-correlated to the beginning of the inspiratory burst, including from 5 seconds before to 5 seconds after the onset of inspiratory activity. All data are presented as averages ± SEM. Software used for statistics was Graphpad Prism 4.01 (Graphpad Software, San Diego, CA), Microcal Origin 6.0 (OriginLabs Corp., Northhampton, MA), and Microsoft Excel (Microsoft Corp., Redmond, WA).

**References**