5-HT$_{2A}$ receptor activation is necessary for CO$_2$-induced arousal

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¹Department of Neurology, Yale University, New Haven, Connecticut; ⁴Veteran’s Affairs Medical Center, West Haven, Connecticut; ³University of Glasgow School of Medicine, Glasgow, Scotland, United Kingdom; ⁴Department of Neurology, University of Iowa, Iowa City, Iowa; ⁵Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, Iowa; and ⁶Veteran’s Affairs Medical Center, Iowa City, Iowa

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Buchanan GF, Smith HR, MacAskill A, Richerson GB. 5-HT$_{2A}$ receptor activation is necessary for CO$_2$-induced arousal. J Neurophysiol 114: 233–243, 2015. First published April 29, 2015; doi:10.1152/jn.00213.2015.—Hypercapnia-induced arousal is an important protective mechanism pertinent to a number of diseases. Most notably among these are the sudden infant death syndrome, obstructive sleep apnea and sudden unexpected death in epilepsy. Serotonin (5-HT) plays a significant role in hypercapnia-induced arousal. The mechanism of 5-HT’s role in this protective response is unknown. Here we sought to identify the specific 5-HT receptor subtype(s) involved in this response. Wild-type mice were pretreated with antagonists against 5-HT receptor subtypes, as well as antagonists against adrenergic, cholinergic, histaminergic, dopaminergic, and orexinergic receptors before challenge with inspired CO$_2$ or hypoxia. Antagonists of 5-HT$_{2A}$ receptors dose-dependently blocked CO$_2$-induced arousal. The 5-HT$_{3C}$ receptor antagonist, RS-102221, and the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, attenuated but did not completely block CO$_2$-induced arousal. Blockade of non-5-HT receptors did not affect CO$_2$-induced arousal. None of these drugs had any effect on hypoxia-induced arousal. 5-HT$_{2C}$ receptor agonists were given to mice in which 5-HT neurons had been genetically eliminated during embryonic life (Lmx1b$^{\text{floxed}}$) and which are known to lack CO$_2$-induced arousal. Application of agonists to 5-HT$_{2A}$, but not 5-HT$_{2C}$ receptors, dose-dependently restored CO$_2$-induced arousal in these mice. These data identify the 5-HT$_{2A}$ receptor as an important mediator of CO$_2$-induced arousal and suggest that, while 5-HT neurons can be independently activated to drive CO$_2$-induced arousal, in the absence of 5-HT neurons and endogenous 5-HT, 5-HT receptor activation can act in a permissive fashion to facilitate CO$_2$-induced arousal via another as yet unidentified chemosensor system.

SLEEP IS A VITAL HOMEOSTATIC function conserved across a wide range of species from nematodes and fruit flies to mice and men (Campbell and Tobler 1984; Zimmerman et al. 2008). While the reason that sleep is required remains elusive (Brown et al. 2012), without it rats die (Rechtschaffen et al. 1983), and humans have profound neuropsychological, physiological, and behavioral consequences (Killgore 2010). Organisms must strike a delicate balance between achieving adequate amounts of sleep to optimally function during the waking hours and maintaining safety during the sleeping hours by retaining the ability to respond (e.g., with arousal) to a variety of stimuli. One such arousal stimulus is hypercapnia (Berthon-Jones and Sullivan 1984; Phillipson et al. 1977). Hypercapnia encountered during sleep likely signifies a respiratory obstruction of some manner, such as is seen with obstructive sleep apnea (OSA) or from sleeping prone with the face in a pillow. Although hypercapnia has long been recognized as an arousal stimulus, the mechanisms by which it induces arousal are not well understood.

Serotonin (5-HT) neurons located in the rostral brain stem comprise a component of the ascending arousal system that is responsible for regulating sleep-wake transitions (Brown et al. 2012; Ito et al. 2013). 5-HT neurons, including those in this region, are chemosensitive (Richerson 2004; Severson et al. 2003). Mice with a genetic lesion of 5-HT neurons (Lmx1b$^{\text{floxed}}$; Zhao et al. 2006) do not arouse from sleep in response to hypercapnia, but do arouse to other stimuli such as hypoxia, or auditory or tactile stimulation (Buchanan and Richerson 2010). Hypercapnia-induced arousal is thought to be an important factor in diseases such as OSA (Berthon-Jones and Sullivan 1984), panic disorder (Klein 1993), and sudden infant death syndrome (SIDS) (Kinney et al. 2009) and may also be important in sudden unexpected death in epilepsy (SUDEP) (Masseys et al. 2014; Richerson and Buchanan 2011; Sowers et al. 2013). For instance, babies who die of SIDS have abnormalities in their brain stem 5-HT system (Kinney et al. 2009). A leading theory is that these abnormalities result in an inability to sense the CO$_2$ that accumulates when a sleeping infant is in the prone position and, consequently, a lack of arousal (Kinney and Thach 2009). Understanding the specific serotonergic mechanisms involved in CO$_2$-induced arousal will be important for treating and preventing these disorders (Richerson 2013). Here we used a system our laboratory has previously employed for assessing CO$_2$-induced arousal in mice (Buchanan and Richerson 2010) to identify specific 5-HT receptor subtypes underlying CO$_2$ arousal. We also probed for a role of involvement of other ascending arousal system components. Finally, we examined whether 5-HT receptor activation in Lmx1b$^{\text{floxed}}$ mice would be sufficient to recover the arousal response to CO$_2$, which is known to otherwise be absent in these animals (Buchanan and Richerson 2010).

METHODS

Experimental animals. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at Yale University. Adult male wild-type (WT) (28–40 g) and Lmx1b$^{\text{floxed}}$ (24–35 g) littermate pairs and male C57BL/6J mice (26–36 g; Jackson Laboratories, Bar Harbor, ME) were housed in standard cages in a 12:12-h light-dark regimen with food and water available ad libitum. Generation, breeding, and genotyping (Hodges et al. 2009; Zhao et al. 2006) of Lmx1b$^{\text{floxed}}$ mice have been previously described. Briefly, females homozygous for floxed Lmx1b (Lmx1b$^{\text{floxed}}$; phenotypically normal “WT”) were mated with males homozygous for floxed Lmx1b and hemizygous for ePet1-Cre (Lmx1b$^{\text{floxed}}$;ePet1-Cre$^{+}$ or Lmx1b$^{\text{floxed}}$) to pro-
duce progeny of these two genotypes in a slightly less than 1:1 ratio (Hodges et al. 2009). At the conclusion of all experimental trials, the animals were euthanized with an overdose of pentobarbital sodium (150 mg/kg ip). **EEG/EMG headmount and activity/temperature telemeter implants.** ECG/EMG headmounts (8201; Pinnacle Technology; Lawrence, KS) and temperature/activity telemeters (G2 E-Mitter; Mini-Mitter, Bend, OR) were implanted as previously described (Buchanan and Richerson 2010). Briefly, under ketamine (100 mg/kg ip) and xylazine (10 mg/kg ip) anesthesia, the skull was exposed, and the headmount was attached to the skull with two 0.1-in. (anterior) and two 0.125-in. (posterior) stainless steel machine screws (3/64 in. diameter; Pinnacle Technologies, Lawrence, KS). EMG leads emanating from the posterior portion of the headmount were sutured into nuchal muscles bilaterally 3 mm from midline. The base of the headmount, screw heads and EMG leads were anchored with dental acrylic (Jet Acrylic; Lang Dental, Wheeling, IL), and the skin sutured closed leaving only the headmount socket exposed. Telemeters (G2 E-mitter; Mini-Mitter) were implanted intraperitoneally as previously described (Buchanan and Richerson 2010). Animals received pre- and postoperative analgesia with meloxicam (0.3 mg/kg ip pre-op; 0.05 mg·kg\(^{-1}\)·d\(^{-1}\) post-op in drinking water for 7 days) and were allowed to recover for at least 10 days before being studied. **EEG/EMG data acquisition.** Animals were fit with a preamplifier (8202-SL; Pinnacle Technology) attached directly to the implanted headmount, introduced to the recording chamber, and allowed to acclimatize as described below. EEG and EMG leads were then passed through a tether and commutator (8204; Pinnacle Technology) and into an analog conditioning amplifier (model 440 Instrumentation Amplifier; Brownlee Precision, San Jose, CA). Data were digitized with an analog-to-digital converter (PCI-6221 or NI-USB-6009; National Instruments, Austin, TX) in a Dell desktop computer and acquired using software custom written in MATLAB (Mathworks, Natick, MA). EEG signals were amplified by 50,000 and band-pass filtered from 0.3 to 200 Hz. EMG signals were amplified by 50,000 and band-pass filtered from 10 to 1000 Hz. All signals were digitized at 1,000 samples per second. Body temperature and activity were captured by a telemetry receiver (ER-4000, Energizer Receiver; Mini-Mitter) situated directly beneath the recording chamber, transmitted to the computer via a serial port, and sampled every 10 s with the same custom software. **EEG/EMG analysis and sleep state determination.** Data were manually scored in 10-s epochs as waking (W), non-rapid eye movement sleep (NREM) or rapid eye movement sleep (REM) using software custom written in MATLAB. Vigilance state was assigned using a standard approach (Franken et al. 1998) based on the EEG/EMG frequency characteristics as follows: W, low-amplitude, high-frequency (7–13 Hz) EEG with high EMG power; NREM, high-amplitude, low-frequency (0.5–4 Hz) EEG with moderate to low EMG power and lack of voluntary motor activity; REM, moderate-amplitude, moderate-frequency (4.5–8 Hz) EEG with minimal EMG power except for brief bursts and minimal activity correlating with EMG bursts. Fast Fourier transform power spectra were created with MATLAB for each 10-s epoch of data and used along with EEG and EMG characteristics to verify scoring. **Plethysmography.** For quantification of ventilation, the recording chamber was fit with an ultra-low-pressure/high-sensitivity pressure transducer (DC002NDR5; Honeywell International, Minneapolis, MN). The analog output from the pressure transducer was digitized by an A/D converter (PCI-6221; National Instruments), displayed on a computer monitor in real time using an acquisition program custom written in MATLAB and saved on a computer hard drive. The signal was calibrated by delivering metered breaths (300 μl; 150 breaths/ min) via a mechanical ventilator (Mini-VENT; Harvard Apparatus) to the recording chamber. Breathing parameters including respiratory rate (RR), tidal volume and minute ventilation (Ve) were assessed with software custom written in MATLAB using previously described methods (Hodges et al. 2008). **Hypercapnic/hypoxic challenges.** Animals were placed into a 350-cm\(^3\) cast acrylic recording chamber equipped with bedding, food, and water and allowed to acclimate for at least 1 h on 3 consecutive days prior to the actual experimental trial. The gas within the chamber at baseline was room air (RA) 21% O\(_2\), 79% N\(_2\) and was changed to hypercapnia (7% CO\(_2\), 21% O\(_2\), and 72% N\(_2\); 10 min) or hypoxia (5% O\(_2\), 95% N\(_2\); 2 min or 10% O\(_2\), 90% N\(_2\); 10 min), as described previously (Buchanan and Richerson 2010). After a baseline recording period in RA for at least 60 min, animals received an injection (intraperitoneal or subcutaneous) of test substance or vehicle. At least 30 min after the injection, the gas in the chamber was changed to either hypercapnia or hypoxia. At least 20 min after the end of the first gas challenge, the animals were challenged with the other test gas. One-half of the animals were randomly exposed to hypercapnia first, and one-half were exposed to hypoxia first. The gas was changed back to RA between test gas exposures. Each test gas exposure was initiated after mice were noted to be aslepp, as determined by real-time observation of the EEG/EMG recording, eye closure, and absence of motor activity using frequency and amplitude parameters, as described above. It should be noted that systemic application of many 5-HT\(_A\) agonists and antagonists acutely alter sleep architecture; however, animals are able to fall back asleep after drug treatment (Monti et al. 1990), and thus we were able to conduct our studies with the animals aslepp. Flow rates were maintained at 710–760 ml/min with a flow meter (WU-32446-33; Cole-Parmer, Hoffman Estates, IL). All compressed gas containers were obtained from Airgas East (Cheshire, CT). To determine if the responses could be due to auditory, olfactory or tactile (pressure) cues that might be associated with a gas change, control experiments were performed by changing the gas line from one delivering RA to another delivering RA. **Drugs.** Atropine (Hentschke et al. 2007), DOI hydrochloride (Monti et al. 1990), ketanserin tartrate (Kirov and Moyanova 1998), Ro4368554 (Monti and Jantos 2011), and SB-269970 hydrobromide (Westrich et al. 2013) were obtained from Sigma-Aldrich (St. Louis, MO). Cimetidine (Monti et al. 1986), diphenhydramine hydrochloride (Monti et al. 1986), GR-125487 (Galeotti et al. 1998), MDL 11,939 (Dudley et al. 1988), methysergide maleate (Ishida et al. 2007), MK-212 hydrochloride (Hemrick-Luecke and Fuller 1996), nercogline (Ogawa et al. 1993), ondansentron hydrochloride (Ye et al. 2001), propranolol hydrochloride (Zarrindast et al. 2004), RS-102221 hydrochloride (Bonhaus et al. 1998), SB-699551 (Thomas et al. 2006), SKF83566 hydrobromide (Meyer et al. 1993), (S)-(-)-sulpiride (Dimpfel 2008), TCB-2 (Fox et al. 2010), TCS-1102 (Winrow et al. 2010), thioperamide (Monti et al. 1991), and 8-OH-DPAT hydrobromide (Yoshitake and Kehr 2004) were obtained from Tocris Bioscience (Ellissive, MO). Cimetidine (10 mg/kg ip), diphenhydramine (10 mg/kg ip), DOI (0.3–3 mg/kg ip), GR-125487 (1–30 mg/kg ip), methysergide (0.3–10 mg/kg ip), MK-212 (3–30 mg/kg ip), ondansetron (0.3–10 mg/kg ip), propranolol (10 mg/kg ip), SB-269970 (0.3–10 mg/kg ip), SKF83566 (1 mg/kg ip), TCB-2 (0.3–10 mg/kg ip), and 8-OH-DPAT (0.3–3 mg/kg ip) were dissolved in double-distilled H\(_2\)O; atropine (10 mg/kg sc), ketanserin (0.1–3 mg/kg ip), MDL 11,939 (0.3–10 mg/kg ip), sulpiride (10 mg/kg ip), thioperamide (4 mg/kg sc), and TCS-1102 (40 mg/kg sc) were dissolved in ethanol; nercogline (1 mg/kg ip), Ro4368554 (0.3–10 mg/kg ip), RS-102221 (0.3–10 mg/kg ip), and SB-699551 (1–30 mg/kg ip) were dissolved in dimethyl sulfoxide. Drugs were diluted to the appropriate concentration with saline (0.9% NaCl) to ensure all injection volumes were 100 μl. Vehicle treatments consisted of 100-μl injections of saline, 10% ethanol in saline, or 1% dimethyl sulfoxide in saline as appropriate. All drugs tested have been previously shown to affect the central nervous system (CNS) when applied systemically via intraperitoneal or subcutaneous injections in rodents (see cited references listed for each drug above). **Statistical analysis.** Interactions between genotype, gas composition, drug, and vigilance state were analyzed for all physiological
variables using two-way ANOVA, paired t-test, or two-tailed t-test assuming unequal variance as appropriate. The significance threshold was $P < 0.05$ for all conditions. Analyses were accomplished using Microsoft Excel (Redmond, WA), OriginPro 9.0 (OriginLab, Northampton, MA), and Systat 11.0.

RESULTS

Antagonists of 5-HT$_2$ receptor subtypes blocked CO$_2$-induced arousal in WT animals. Consistent with what we have shown previously (Buchanan and Richerson 2010), 7% CO$_2$ by itself (data not shown) or 30 min after vehicle injection rapidly induced arousal from sleep (Fig. 1A). Note that mice normally cycle rapidly between sleep states, so a latency of only 3–5 min from sleep onset to arousal is expected in a control mouse (Buchanan and Richerson 2010). Changing gas flow from one RA tank to another had no effect on sleep state (Fig. 1B). The nonspecific 5-HT receptor antagonist, methysergide; the nonselective 5-HT2 receptor antagonist, ketanserin; and the 5-HT$_{2A}$ selective antagonist, MDL 11,939 all dose-dependently blocked CO$_2$-induced arousal (Fig. 1, C–E). At higher doses, the 5-HT$_{2C}$ selective antagonist, RS-102221, attenuated but did not completely block CO$_2$-induced arousal (Fig. 1F). Note that the representative traces in each panel of these figures are at the highest dose.

Blockade of 5-HT$_3$, 5-HT$_4$, 5-HT$_5$, 5-HT$_6$ or 5-HT$_7$ receptors had no effect on CO$_2$-induced arousal. Given that other 5-HT receptors are also present in the thalamus and cortex and have been implicated in sleep-wake regulation (Monti 2011; Volk et al. 2010), we tested whether blockade of any of these receptor subtypes could impair CO$_2$-induced arousal. The 5-HT$_3$ receptor antagonist, ondansetron; the 5-HT$_4$ receptor antagonist, GR-125487; the 5-HT$_5$ receptor antagonist, SB-699551; the 5-HT$_6$ receptor antagonist, Ro4368554; and the 5-HT$_7$ receptor antagonist SB-269970 had no effect on CO$_2$-induced arousal (Fig. 2). Doses for each drug were within or above the dose range used in the literature to have effects on a variety of behavioral and physiological measures when applied systemically (see references in METHODS).

Antagonists of 5-HT receptors have no effect on hypoxia-induced arousal. To determine whether the effects of 5-HT receptor blockade were specific for hypercapnia-induced arousal, we tested all of the drugs against hypoxia-induced NREM arousal. Figure 1 shows that the latency to arousal was not affected by the 5-HT$_2$ receptor antagonists methysergide (Fig. 1C), ketanserin (Fig. 1D), MDL 11,939 (Fig. 1E), or RS-102221 (Fig. 1F).
arousal, which we previously showed to be unaffected by genetic deletion of 5-HT neurons in Lmx1bf/f/p mice (Buchanan and Richerson 2010). Blockade of 5-HT receptors with the same pharmacological agents tested above did not affect hypoxia-induced arousal at any of the doses tested (Fig. 3, A and B).

To determine whether 5-HT might be involved in arousal to less severe hypoxia, the arousal response to 10% O2 was tested in a separate set of WT and Lmx1bf/f/p mice. Both genotypes aroused equally well to the less severe hypoxic stimulus (Fig. 3C). Similarly, to determine whether 5-HT2A receptor blockade would be sufficient to prevent arousal to the less severe hypoxic stimulus, five WT mice were challenged with 10% O2 following treatment with MDL 11,939 (10 mg/kg ip). Blockade of 5-HT2A receptors in this manner had no effect on the latency to arousal to 10% O2 (Fig. 3D).

Fig. 2. 5-HT3, 5-HT4, 5-HT5, 5-HT6 or 5-HT7 receptor blockade did not affect CO2-induced arousal latency. Data are presented as in Fig. 1. Latency to arousal following CO2 challenge preceded by V or several doses of ondansetron (A), GR-125487 (B), SB-699551 (C), Ro4368554 (D) or SB-269970 (E) are shown. Values are means ± SE; n = 6 for all conditions.

Fig. 3. 5-HT receptor blockade had no effect on hypoxia-induced arousal. A: 90-s EEG, EMG, and chamber [O2] traces depicting arousal from sleep after [O2] within the chamber has reached 8–9% O2. B: bar graphs depicting lack of effect of any concentration of any of the drugs (as labeled on graphs) on latency to hypoxia-induced arousal. Values are means ± SE; n = 6 for each condition. C: latency to arousal following 10% O2 challenge in WT (solid bar) and Lmx1bf/f/p (open bar) mice. Values are means ± SE; n = 6 for each genotype. D: bar graph depicting lack of effect of V (solid bar) or MDL 11,939 (10 mg/kg ip; open bar) on latency to arousal following 10% O2 challenge in WT mice. Values are means ± SE; n = 5 for each condition.
Activation of 5-HT$_{1A}$ receptors attenuates arousal to CO$_2$ but not to hypoxia. The 5-HT$_{1A}$ receptor is an inhibitory somatodendritic autoreceptor on 5-HT neurons as well as on postsynaptic neurons. When activated, these receptors hyperpolarize and thereby inhibit 5-HT neuron firing (Verge et al. 1985). Activation of 5-HT$_{1A}$ receptors with 8-OH-DPAT attenuated but did not completely block CO$_2$-induced arousal (Fig. 4). At the doses tested, 8-OH-DPAT had no effect on hypoxia-induced arousal (Fig. 4C).

The pharmacological profile for CO$_2$-induced arousal is the same in C57BL/6J and phenotypically WT Lmx1b$^{+/+}$ mice. The phenotypically WT Lmx1b$^{+/+}$ mice tested above are littermates of the genetically 5-HT neuron-deficient mice and therefore carry two copies of the loxP-flanked Lmx1b gene (Zhao et al. 2006). To control for possible effects of these transgenes, C57BL/6J mice were tested for their ability to arouse from sleep in response to hypercapnia and hypoxia. C57BL/6J mice were aroused from sleep in response to both hypercapnia and hypoxia with similar latencies (0.99 ± 0.37 min for hypercapnia; 0.90 ± 0.28 min for hypoxia; n = 6 per condition). Vehicle pretreatment had no significant effect on arousal latencies (1.01 ± 0.41 min for hypoxia; 0.92 ± 0.35 min for hypercapnia; n = 6 per condition). MDL 11,939 (1 and 10 mg/kg) blocked CO$_2$-induced arousal (latency 3.86 ± 0.39 min with 1 mg/kg; 4.03 ± 0.46 min with 10 mg/kg; n = 6 per dose). RS-102221 (10 mg/kg) and 8-OH-DPAT (2 mg/kg) attenuated, but did not completely block CO$_2$-induced arousal (latency 2.25 ± 0.29 min with RS-102221; 2.01 ± 0.30 min with 8-OH-DAT; n = 6 per drug). Ondansetron (5 mg/kg) had no effect on CO$_2$-induced arousal latency (0.94 ± 0.42 min; n = 6). None of these drugs had any effect on hypoxia-induced arousal at the doses tested (MDL 11,939, 10 mg/kg: 0.88 ± 0.25 min; RS-102221: 0.84 ± 0.34 min; 8-OH-DPAT: 0.95 ± 0.32 min; ondansetron: 0.91 ± 0.43 min; n = 6 per drug).

Antagonists of other neurotransmitters of the arousal system had no effect on CO$_2$- or hypoxia-induced arousal. Given that there are a number of other neurotransmitters that have been implicated in sleep-wake regulation (Saper et al. 2010) and neurons that contain and release some of these neurotransmitters have been reported to be chemosensitive (Pineda and Aghajanian 1997; Williams et al. 2007), we examined whether systemic blockade of receptors for any of these neurotransmitters could prevent CO$_2$- or hypoxia-induced arousal. Antagonists of receptors for norepinephrine (nicergoline, propranolol), muscarine (atropine), histamine (diphenhydramine, cimetidine, thioperamide), dopamine (SKF83566, sulpiride), or orexin (TCS-1102) had no effect on CO$_2$- or hypoxia-induced arousal (Fig. 5). α$_2$-Receptors were not specifically targeted; however, the 5-HT$_{2A}$ antagonist SB-269970 also has α$_2$ activity (Westrich et al. 2013) and had no effect on CO$_2$- or hypoxia-induced arousal, also arguing against a major role for α$_2$ involvement.

5-HT$_{2A}$ receptor activation restores CO$_2$-induced arousal in Lmx1b$^{+/+}$ mice. Lmx1b$^{+/+}$ mice have an impaired hypercapnic ventilatory response (Hodges et al. 2008) and lack the ability to arouse from sleep in response to CO$_2$ (Buchanan and Richerson 2010). Direct activation of 5-HT receptors by intracerebroventricular application of 5-HT can restore the HCVR in Lmx1b$^{+/+}$ mice (Hodges et al. 2008). To test whether 5-HT agonists can recover hypercapnia-induced arousal, we pretreated Lmx1b$^{+/+}$ mice with the 5-HT$_{2A/2C}$ selective agonist DOI, the 5-HT$_{2A}$ selective agonist TCB-2, or the 5-HT$_{3C}$ agonist MK-212 prior to exposure to 7% CO$_2$ or hypoxia. DOI and TCB-2 recovered CO$_2$-induced arousal in a dose-dependent fashion. MK-212 had no effect on CO$_2$-induced arousal (Fig. 6, A–C). None of these drugs had any effect on the arousal response to hypoxia (Fig. 6, D–F). Application of DOI (1 mg/kg ip) or TCB-2 (10 mg/kg ip) to WT mice had no effect on the latency for CO$_2$-induced arousal (0.95 ± 0.27 min and 0.90 ± 0.38 min, respectively; n = 6) compared with vehicle (0.89 ± 0.33 min and 0.96 ± 0.40 min, respectively; n = 6). There was no significant change in the latency to arousal following the change from one RA tank to another after treatment with DOI (1 mg/kg ip; 3.21 ± 0.29 min; n = 6) or TCB-2 (10 mg/kg ip; 3.37 ± 0.35 min, respectively; n = 6).
compared with vehicle (3.23 ± 0.46 min and 3.48 ± 0.43 min, respectively; *P < 0.05 compared with V. D–F: bars graphs depicting lack of effect of any concentration of DOI (D), TCB-2 (E) or MK-212 (F) on latency to hypoxia-induced arousal.

**Fig. 6.** Systemic application of 5-HT$_{2A}$ receptor agonists recovered CO$_2$-induced arousal in 5-HT neuron-deficient mice. Five-minute EEG and EMG data traces are shown depicting arousal, or lack of arousal, following 7% CO$_2$ challenge preceded by injection of V or of several different doses of DOI (A), TCB-2 (B) or MK-212 (C). Panel layout and abbreviations are as in Fig. 1. Values are means ± SE; n = 6 for all conditions.

**DISCUSSION**

Impairment of CO$_2$-induced arousal contributes significantly to morbidity and mortality associated with OSA and SIDS and may be involved in SUDEP (Kinney et al. 2009; Sowers et al. 2013). The specific mechanisms underlying CO$_2$-induced arousal are uncertain. The data presented here demonstrate that, under normal circumstances, CO$_2$-induced arousal is mediated via the 5-HT$_{2A}$ receptor subtype. Blockade of other 5-HT receptors, or a number of nonserotonergic neurotransmitter receptors, had no effect on CO$_2$-induced arousal at the doses tested. Activation of 5-HT$_{2A}$ receptors in the 5-HT neuron deficient Lmx1b$^{+/+}$ mice, which are known to lack CO$_2$-induced arousal (Buchanan and Richerson 2010), was sufficient to recover the arousal response to CO$_2$ in these animals, suggesting that CO$_2$-induced arousal can be mediated via an alternative chemoreceptive system.

Receptor mechanisms in CO$_2$-induced arousal. Identification of a key role for 5-HT$_{2A}$ receptors in CO$_2$-induced arousal is not surprising. 5-HT$_{2A}$ receptors are found in cortex, thalamus, and locus coeruleus among other sites (Pazos et al. 1985; Pazos and Palacios 1985) and are thought to play a prominent role in sleep-wake regulation with activation increasing wake probability and inactivation increasing slow-wave sleep (Kirov and Moyanova 1998; Monti and Jantos 2006). The thalamus and cortex are final common pathways for arousal; therefore, stimulation of 5-HT$_{2A}$ receptors in these sites could lead to arousal. Similarly, activation of 5-HT$_{2A}$ receptors on neurons in other sites known to contribute to sleep-wake regulation, such as the locus coeruleus, could cause subsequent activation of cortical and thalamic sites, leading to arousal. We screened for involvement of nonserotonergic transmitter systems by testing a single high dose of a number of non-5-HT receptor antagonists. We believe that, based on the literature, we tested sufficiently high doses that these drugs should have at least attenuated CO$_2$-induced arousal if these receptors were prominently involved in the response. This does not eliminate the possibility that other systems may have a minor contribution that becomes more apparent under certain circumstances, such as in the absence of an intact 5-HT system. We did not specifically test this possibility in this study.

Administration of a 5-HT$_{2C}$ antagonist, RS-102221, attenuated but did not completely block CO$_2$-induced arousal. 5-HT$_{2C}$ receptors are not as well distributed in cortical and
thalamic sites as are 5-HT$_{2A}$ receptors; however, 5-HT$_{2C}$ receptors are abundantly expressed in limbic structures (Palacios et al. 1991). Thus the modest effect of RS-102221 may represent a limbic component of CO$_2$-induced arousal. It may also reflect a small contribution of the 5-HT$_{2C}$ receptors that are found in cortical sites. Finally, RS-102221 is a weaker antagonist of 5-HT$_{2A}$ receptors as well (Barnes and Sharp 1999); therefore, the effect on CO$_2$-induced arousal may simply be explained by the off-target effect on 5-HT$_{2A}$ receptors.

Along with 5-HT$_{2A}$ receptors, 5-HT$_3$, 5-HT$_6$, and 5-HT$_7$ are found in thalamic and cortical loci and have been implicated in sleep-wake regulation (Monti 2011). However, blockade of these receptors, and of 5-HT$_4$ and 5-HT$_5$ receptors, had no effect on the arousal responses to hypercapnia or hypoxia. The doses tested in these experiments have been shown to have CNS effects in mice when applied systemically. It is intriguing that there is such a clear effect when 5-HT$_{2A}$ receptors are blocked, but not any effect upon blockade of all the other tested receptors (except maybe 5-HT$_{2C}$). Not all neurons within the raphe are chemosensitive. It may be that the subset of raphe 5-HT neurons that are activated by acidosis (Richerson 2004) project to target areas with high density of 5-HT$_{2A}$ receptor expression, or activation of the other receptors is not sufficient to induce arousal from sleep.

Raphe subnuclei in CO$_2$-induced arousal. There are two major populations of 5-HT neurons in the CNS: a midbrain group (dorsal and median raphe) that projects primarily rostrally, and a medullary group (raphe magnus, pallidus, and obscuris) that projects primarily caudally. While subsets of 5-HT neurons in both the rostral (Severson et al. 2003; Veasey et al. 1997) and caudal sites (Bernard et al. 1996; Bradley et al. 2002; Wang et al. 2001) sites can sense changes in serum pH and modulate their firing frequency in response to these changes, it is primarily the rostral 5-HT neuronal groups that are associated with sleep-wake regulation (Saper et al. 2010). Therefore, we postulate that, under normal circumstances, stimulation of these groups is most likely to be responsible for causing arousal in response to inspired CO$_2$. In this study, drugs were applied systemically and thus could also activate the caudal 5-HT neurons. A subset of these neurons are thought...
to be primarily responsible for modulating breathing in response to hypercapnia and not necessarily involved in CO₂-gated sleep-wake modulation (Richerson 2004). Disruption of certain populations of medullary 5-HT neurons in piglets, for example in the raphe paragigantocellularis, alters sleep architecture and may play a role in hypoxia-induced arousal (Darnall et al. 2005).

Activation of medullary raphe 5-HT neurons rapidly causes an increase in ventilation (Depuy et al. 2011). This suggests a primary role of these neurons in regulating breathing and not simply a modulatory one, as might otherwise be expected for a monoaminergic mechanism. Activation of midbrain 5-HT neurons would be expected to similarly rapidly cause arousal.

Profound respiratory stimuli, such as those causing augmented breaths, can activate respiratory mechanoreceptors which can cause arousal (Gleeson et al. 1990). 5-HT₂A receptors are present throughout respiratory networks, and 5-HT₂A agonists increase breathing frequency and ventilation (Cayetanot et al. 2002). Hypercapnia is thought to cause arousal independent of its effect on breathing (Buchanan and Richerson 2010). In our study, increases in breathing in response to CO₂ were observed even in animals that did not arouse to CO₂. This suggests that, if the respiratory component contributes to arousal, the degree of respiratory enhancement caused by 7% CO₂ is not sufficient to cause arousal. Our studies do not address whether arousal can occur without the breathing change, because the state change from NREM to wake is associated with increased breathing, making it difficult to separate these two intertwined responses. Mechanoreceptor activation may play a larger role in hypoxia-induced arousal, as hypoxia leads to a greater occurrence of augmented breaths and a greater degree of mechanoreceptor activation (Bell and Haouzi 2010).

Beyond 5-HT in CO₂-induced arousal. CO₂-induced arousal could be recovered in Lmx1b<sup>−/−</sup> mice that do not have 5-HT neurons in the CNS by pretreatment with agonists of the 5-HT₂A receptor (DOI and TCB-2), but not the 5-HT₂C receptor (MK-212). A similar phenomenon was seen in these mice for the HCVR (Hodges et al. 2008). This adds a layer of complexity to our previous finding that CO₂-induced arousal is lost in Lmx1b<sup>−/−</sup> mice. One plausible explanation is that activation of 5-HT₂A receptors on some other nonserotonergic neurons enhances their CO₂ sensitivity. At baseline (i.e., in the presence of normal 5-HT neurons and endogenous 5-HT), although the alternative CO₂-sensing system is present and available to be activated, the effect of activation of 5-HT neurons by CO₂ might be sufficiently robust that the effect of activation of the other chemosensors by CO₂ is less important.

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**Fig. 8.** Proposed model of CO₂-induced arousal. A: under normal conditions, elevations in the concentration of CO₂ inspired into the lungs are transmitted as increases in H<sup>+</sup> ion concentration (decreases in pH) via the bloodstream to midbrain 5-HT neurons, thereby activating these neurons and causing release of 5-HT (green circles) onto arousal centers (presumably thalamic and cortical neurons), where it activates 5-HT₂A receptors on these neurons to induce arousal from sleep. There is also lesser stimulation of non-5-HT chemosensitive neurons. B: in the absence of 5-HT neurons, there is no 5-HT release following decrease in pH, and no subsequent 5-HT₂A receptor activation, no arousal center stimulation, and no arousal from sleep. Stimulation of non-5-HT neurons fails to induce arousal in the absence of 5-HT₂A receptor activation. C: in the absence of 5-HT neurons, there is still activation of non-5-HT chemosensitive neurons. Ca: activation of 5-HT₂A receptors on non-SHT neurons by exogenously applied 5-HT₂A receptor agonists (e.g., DOI, TCB-2; purple circles) could enhance the chemosensitivity of these neurons and lead to arousal center stimulation. Cb: alternatively, 5-HT₂A receptors could be activated directly at the arousal centers, and receptor binding permits stimulation by the non-5-HT neurons, leading to arousal.

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In the absence of 5-HT neurons (and endogenous 5-HT), these neurons do not sense CO₂ sufficiently to induce arousal from sleep (Buchanan and Richerson 2010); however, with 5-HT₂A receptor activation such as by DOI or TCB-2, the mechanism for sensing CO₂ is positively modulated, allowing those neurons to cause arousal from sleep (Fig. 8). A similar mechanism was proposed for the HCVR (Hodges et al. 2008). Such enhancement of chemosensitivity could occur via activation of 5-HT₂A receptors directly on these alternate chemosensitive neurons, or on neurons within downstream target structures. DOI is known to alter sleep architecture (Monti and Jantos 2006). It is possible that the perceived recovery of CO₂-induced arousal with DOI and TCB-2 in the Lmx1b<sup>−/−</sup> mice could simply have been a reflection of an increased wake probability caused by the 5-HT₂A agonists. However, if this were the case, we would have expected to see a reduced arousal latency during the RA-to-RA control experiments. A final possibility is that the “alternate” chemosensor discussed here is the primary sensor but requires 5-HT and more specifically 5-HT₂A receptor activation to mediate arousal to CO₂.

There are a number of possibilities for which nonserotonergic neurons could have their chemosensitivity enhanced, either directly or indirectly, by 5-HT₂A receptor activation. Other neuronal populations involved in sleep-wake regulation have chemoreceptive capabilities. These include noradrenergic neurons of the locus coeruleus (Pineda and Aghajanian 1997), histaminergic neurons of the tuberomammillary nucleus (Johnson et al. 2005), and orexinergic neurons in the lateral hypothalamus (Johnson et al. 2010, 2012; Williams et al. 2007). Another chemosensitive region, the retrotrapezoid nucleus, may be involved in sleep-wake regulation (Abbott et al. 2013; Guyenet and Abbott 2013). Glutamatergic neurons of the parabrachial nucleus have also been implicated in CO₂-induced arousal (Kaur et al. 2013), although these neurons are not known to be intrinsically chemosensitive.

In this study, we tested a wide variety of antagonists against receptors for neurotransmitters that are released from the aforementioned putative chemoreceptive nuclei. None of these drugs had any effect on CO₂-induced arousal at the doses tested. This may suggest that we did not target the appropriate transmitter systems. For instance, we used nicergoline, which has catecholaminergic activity beyond α₁-adrenergic activity, and we did not test glutamatergic or GABAergic receptor antagonists, largely because systemic application of these agents would have widespread consequences. Given the possible role for glutamatergic inputs from regions such as retrotrapezoid nucleus and parabrachial nucleus, attempting to target contributions of these regions with a targeted approach will likely prove informative in the future. We relied on the literature to guide the doses we tested. Therefore, it is possible that we did not test high enough doses; however, we believe we should have at least seen some attenuation of the arousal response if these other neurotransmitter systems played a prominent role.

Another possibility is that 5-HT₂A receptor activation leads to increased excitability of nonserotonergic neurons. Finally, 5-HT₂A receptor activation could cause upregulation of some other chemosensors, such as the ubiquitously expressed acid sensing ion channels. Although, under normal conditions acid sensing ion channels 1 and 2 are not involved in CO₂-induced arousal or the HCVR (Price et al. 2014).

The ability to arouse from sleep in response to hypercapnia is an important protective reflex. This reflex may be lost in diseases such as SIDS (Kinney et al. 2009) and SUDEP (Richerson and Buchanan 2011) and may be important in OSA apnea (Berthon-Jones and Sullivan 1984; Kaur et al. 2013). In all of these diseases, hypoxia is encountered along with hypercapnia. The relative contributions of each of these aberrations to the morbidity and mortality associated with these diseases in unclear. 5-HT appears to be more prominently involved in CO₂-induced arousal with little, if any, role in hypoxia-induced arousal in our hands. Understanding the specific mechanisms underlying CO₂- and hypoxia-induced arousal may lead to pharmacotherapy that could be employed in susceptible individuals to reduce morbidity and mortality from these diseases.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

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