We wanted to understand the brain circuitry that awakens the individual when there is elevated CO₂ or low O₂ (e.g., during sleep apnea or asphyxia). The sensory signals for high CO₂ and low O₂ all converge on the parabrachial nucleus (PB) of the pons, which contains neurons that project to the forebrain. So, we first deleted the vesicular glutamate transporter 2, necessary to load glutamate into synaptic vesicles, from neurons in the PB, and showed that this prevents awakening to high CO₂ or low O₂. We then showed that PB neurons that express calcitonin gene-related peptide (CGRP) show cFos staining during high CO₂. Using CGRP-Cre-ER mice, we expressed the inhibitory opsin archaerhodopsin just in the PB-CGRP neurons. Photoinhibition of the PB-CGRP neurons effectively prevented awakening to high CO₂, as did photoinhibition of their terminals in the basal forebrain, amygdala, and lateral hypothalamus. The PB-CGRP neurons are a key mediator of the waking response to apnea.

As a person falls asleep, there is a gradual loss of muscle tone in the airway dilator muscles. In individuals who have obstructive sleep apnea (OSA), usually due to narrow airways, this may cause the tongue to extend back, occluding the airway (White and Younes 2012; White 2016). The person continues to try to breathe with greater and greater efforts but no airflow, until at some point there is an arousal, with electroencephalogram (EEG) desynchronization indicative of waking up. At this point, there is a sudden increase in tone in the airway dilator muscles, reopening the airway. The OSA patient then continues to breathe, falls asleep again, and the cycle repeats. In a typical OSA patient this may occur hundreds of times over the course of the night, leading to fragmented sleep as well as deleterious cognitive, metabolic, and cardiovascular side effects (Frannes and Arble 2018; Gaines et al. 2018; Olaithe et al. 2018).

OSA is often treated with continuous positive airway pressure (CPAP), which stents the airway open. Although this is effective, it requires sleeping with a mask and a machine that causes continuous airway flow (and noise). Many unfortunate OSA patients cannot tolerate the CPAP apparatus, and it is cumbersome to take when traveling (White 2016). Hence, a way to treat OSA that manipulated the brain circuitry that controls airway musculature and arousal during sleep would be of value.

Unfortunately, until quite recently not much was known about this circuitry. During apnea, there are three main sensory stimuli that alert the brain (Fig. 1; Gleeson et al. 1990). Hypoxia is sensed primarily by the carotid body, which transmits that information to the nucleus of the solitary tract (NTS) via the carotid sinus branch of the glossopharyngeal nerve (Lindsey et al. 2018). The carotid body also recognizes hypercarbia (elevated CO₂) via the same mechanism and is sensitized to this during hypoxia (Nurse and Piskuric 2013). In addition, there are a number of neuronal types in the CNS that also sense high CO₂ directly. One of these is the retrotrapezoid nucleus in the base of the pons (Guyenet et al. 2016). The retrotrapezoid nucleus and the NTS send major axonal outputs to the ventrolateral medulla (VLM), which contains the pattern generator for breathing, and the ventrolateral part of the parabrachial nucleus (PB), an important relay for visceral sensory information from the brainstem to the forebrain and in the orexin neurons in the lateral hypothalamus (Severson et al. 2003; Corcoran et al. 2009; Burdakov et al. 2013). Interestingly, these neurons also project to the NTS, the VLM, and the PB. In fact, in animals that have been exposed to elevated CO₂ or to hypoxia or to intermittent airway occlusion, there is characteristic distribution of expression of cFos, an immediate early gene marking neurons that have been activated. In these animals, cFos is expressed in the retrotrapezoid nucleus, the NTS, the VLM, and the ventrolateral PB (Teppema et al. 1997; Berquin et al. 2000; Ferreira et al. 2015).

A third source of sensory input during apnea is generated by the hypoxia and hypercarbia causing increased respiratory effort, which produces negative pressure in the airway. This is registered by mechanoreceptor fibers in the vagus nerve, which also terminate in the same part of the NTS as the chemoreceptor inputs. cFos studies in animals with stimulation of the airway mechanoreceptors shows the same pattern of cFos expression as in hypoxia or hypercarbia, involving the NTS, VLM, and ventrolateral PB (Gestreau et al. 1997). In humans with OSA, the timing of arousal correlates most closely with the airway negative pressure, a bit less well with the degree of hypercarbia, and least well with the level of hypoxia (Gleeson et al. 1990). However, the degree of ventilatory effort is a complex function of the hypercarbia and hypoxia, and all
three measures converge in the same brain locations. So, studying these connections is likely to yield important information about the brain response to apnea.

The PB is a particularly likely spot to integrate these signals and control both the respiratory and arousal responses to apnea. The PB was identified as early as the 1920s as a key area for respiratory control (Lumsden 1923), and the ventrolateral part of it has been called the “pneumotaxic center” or the “pontine respiratory group” (Feldman 1986). It contains two main components: (1) neurons in the medial PB (PBm) and the external lateral PB (PBel) subnuclei project to the forebrain and we hypothesized that they may have a role in arousal during apnea; and (2) neurons in the far lateral PB, including the lateral crescent (PBlc) and Kölliker-Fuse (KF) nucleus project to the NTS, VLM, and respiratory motor groups such as the hypoglossal nucleus (necessary to control tongue motor tone that keeps the airway open), and phrenic motor nucleus (which controls the diaphragm) (Saper and Loewy 1980; Fulwiler and Saper 1984; Herbert et al. 1990; Moga et al. 1990).

To test the effects of manipulating this circuitry on arousal during apnea, we developed a mouse model. Because rodents do not have occlusion of the airway during sleep, we placed the mice in a plethysmographic chamber, where we could also record their EEG and electromyography (EMG) to monitor wake-sleep (Fig. 1B,C). Every 5.5 min, we changed the gas mixture in the chamber.

Figure 1. (A) Schematic representation showing the convergence of the major three sensory stimuli during apnea on the parabrachial area (PB). The carotid body senses both hypoxia and hypercapnia, and relays to the nucleus of the solitary tract (NTS) via the glossopharyngeal nerve. The NTS also receives mechanosensory input such as negative inspiratory pressure from the airways. Increased pCO2 also activates central chemoreceptors in the retrotrapezoid nucleus (RTN), as well as raphe serotoninergic neurons and hypothalamic orexin neurons. All of these outputs converge on the ventrolateral corner of the parabrachial nucleus (PB), which includes three subnuclei. The external lateral subnucleus projects to the forebrain to cause cortical arousal through projections to the lateral hypothalamus, basal forebrain, and amygdala. The lateral crescent and Kölliker-Fuse (KF) nuclei project to respiratory and autonomic control sites in the brainstem and spinal cord. (B, C) A mouse model of apnea. A mouse is allowed to sleep in a plethysmograph chamber (B) while EEG, EMG and breathing are recorded (C). The mouse is intermittently exposed to bouts of 10% CO2 (hypercapnia), hypoxia, or both given every 5 min for 30 sec. During these trials, the arousals are judged by EEG desynchronization (loss of delta waves and appearance of low voltage, fast EEG), which is usually accompanied by EMG activation. Mice undergo spontaneous periods of sleep and wake; however, only trials where the mouse is in NREM sleep for at least 30 sec before onset of the CO2 are used to examine arousal. (Adapted and modified from Kaur et al. 2013.)
ber, either to hypoxic (10% O₂), hypercarbic (10% CO₂), or both (Fig. 1C), for 30 sec (Kaur et al. 2013). It took ∼10 sec for the gas mixture to asymptotically approach the target level, and mice would consistently awaken after 10–20 sec of exposure to the new gas mixture.

As most neurons in the PB are glutamatergic, we initially used mice with flanking loxP sites around the second exon of the vesicular glutamate transporter 2 gene (Vglut2flox/flox mice), and injected the PB with an AAV that expressed both Cre recombinase and Venus green protein (Kaur et al. 2013). We found that injections that removed functional Vglut2 from the far ventrolateral PB, including the PBel, PBlc, and KF, dramatically lengthened the time it took animals to arouse to either hypoxia, hypercarbia, or both, to well beyond the 30-sec gas exposure (indicating that most of their eventual arousals were spontaneous, not gas-induced) (Fig. 2). Because human awakening during apnea mainly corresponds to hypercarbia (Gleeson et al. 1990), subsequent experiments were done using just hypercarbia as a stimulus (Kaur et al. 2013).

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**Figure 2.** Testing the role of glutamatergic signaling from the PBel in hypercapnia-induced arousal. (A) The numbers of the Cre-transduced neurons in the external lateral parabrachial nucleus (PBel) region after injection of AAV-Cre in Vglut2flox/flox mice. The groups coded by red, purple, and dark blue in the rest of the figure had by far the largest numbers of neurons in which Vglut2 was deleted from the PBel. (B) A photomicrograph of a brain section immunostained for Neu-N, a neuronal marker after bilateral injection of AAV-DTA killed Vglut2+ neurons into the lateral PB of a Vglut2-Cre mouse. (C) A representative trial from a mouse with deletion of the Vglut2 gene in the LPB that failed to wake up to hypercapnia but showed an intact ventilatory response as can be seen by gradual increase in respiration with increasing CO₂ levels. (D) Graph showing that the latency to arousal with CO₂ was much longer in the groups where there was deletion of glutamatergic signaling in the PBel (shown by blue box in A and are represented by red, blue, and magenta in graph D). (E) Compares the percentage amount of sleep in different groups of mice during the light, dark period and then compared for average over 24 h. Delta power during the NREM, which is an indicator of the depth of sleep, is compared in F. Mice with deletions of the Vglut2 gene in the medial PB (cyan, magenta, and blue) had significantly increased NREM sleep and EEG delta power during NREM. (scp) Superior cerebellar peduncle, (LPB) lateral parabrachial nucleus. Scale = 100 µm. ** and * represent P < 0.01 and P < 0.05 respectively, compared to the control group (AAV-GFP) and (#) P < 0.05, compared to the AAV-CreWT group. (Adapted and modified from Kaur et al. 2013.)
these neurons played a part in CO2 arousal. To inhibit CGRP (calcitonin gene-related peptide) (Kaur et al. 2017), we next use CGRP-Cre-ER mice to determine if CGRP-expressing, glutamatergic neurons in the PBel appear to be the critical link between the brain sensory systems that detect apnea and the forebrain arousal circuitry that causes EEG arousal.

The serotonin system appears, in contrast, to play a modulatory role, in that it is not necessary for sensing hypercapnia, but is normally required for the PB^CGRP neurons to respond to CO2. However, the ability to replace the effect of the serotonin neurons on EEG arousal to CO2 with a 5HT2a agonist drug suggests that the serotonin system is facilitatory, but it is not required to carry the signal for the CO2 level to achieve EEG arousal.

Better understanding of these parallel pathways that underlie the EEG arousal and airway dilator muscle response to CO2 will be useful in allowing us to design pharmaco-
Figure 3. Selective silencing of the PBCGRP neurons and the terminal fields using optogenetics. (A) The strategy to inhibit the PBC\textsuperscript{CGRP} cells that express the inhibitory opsin archaerhodopsin (ArchT) by orange-yellow laser light (593 nm) on one side of the brain of a CGRP-CreER mouse injected with AAV-Flex-ArchT, while deleting the PBC\textsuperscript{CGRP} neurons on the contralateral by injecting a diphtheria toxin viral vector that kills the cells in a Cre-dependent manner (AAV-Flex-DTA). (B) The in vitro validation of the silencing of the PBC\textsuperscript{CGRP} neurons expressing ArchT. The same laser light produced no changes in the firing rate of PBC\textsuperscript{CGRP} neurons that expressed GFP (mice injected with AAV-Flex-GFP). (C) A representative recording of EEG, EMG, and respiration during the 10% CO\textsubscript{2} stimulus in a CGRP-CreER mouse with no-laser photoinhibition (Laser-OFF, where mouse wakes up in 18 sec), whereas in D, the same mouse is exposed to the yellow laser light during the CO\textsubscript{2} trial (Laser-ON), and the mouse did not wake up to CO\textsubscript{2}, but had intact ventilatory-response to CO\textsubscript{2}, similar to the control with Laser-OFF. (E) A comparison of the effects of PBC\textsuperscript{CGRP} soma inhibition to that of inhibiting PBC\textsuperscript{CGRP} terminals in the CeA, BF, and LH. (Top) Latency of arousal to CO\textsubscript{2} (mean ± SEM) with Laser-OFF or during Laser-ON. (Bottom) Survival of sleep curves during and after a hypercapnic stimulus shown with and without laser. (***) \( P < 0.0001; (**) \( P < 0.001; (*) \( P < 0.05, \) one-way or repeated measures ANOVA followed by Holm-Sidak for multiple comparison. (Adapted and modified from Kaur et al. 2017.)
Figure 4. Selective silencing of the PBCGRP neurons did not change the ventilatory kinetics in response to CO₂. (A) Graphs showing respiratory rate (RR) and tidal volume ($V_T$) for every breath, 30 sec before the onset of CO₂ and for 30 sec during the CO₂ trials, in Laser-OFF and Laser-ON conditions, for the mice in which the PBCGRP neurons were injected with the inhibitory opsin ArchT to photoinhibit the PBCGRP neurons. (B) Graphs comparing the RR and $V_T$ for three breaths before CO₂ (Pre CO₂) and for three breaths during CO₂ just before waking up in Laser-OFF and then at the same time point in trials in the same animal with Laser-ON (in which the animals did not awaken). (Adapted and modified from Kaur et al. 2017.)

Figure 5. Neural circuit for arousal from apnea: PBeCGRP neurons receive CO₂, O₂, and airway mechanoreceptor inputs via the nucleus of solitary tract (NTS) and retrotrapezoid nucleus (RTN). The PBeCGRP neurons in turn project extensively to the lateral hypothalamus (LH), basal forebrain (BF), and central nucleus of amygdala (CeA) and bed nucleus of the stria terminalis (BST). Based on our findings, PBeCGRP neurons mainly cause cortical arousal by projections to the BF, which has more potent waking effects than PBCGRP inputs to the CeA and LH. The serotonergic dorsal raphe neurons (DR) modulate the arousal through their input to the PBeCGRP neurons.
logical approaches that can suppress the EEG arousal, while sensitizing the ventilatory response and perhaps preventing the airway closure altogether.

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