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Decreased noradrenergic excitation of hypoglossal motoneurons during sleep causing hypotonia of pharyngeal dilator muscles is a major contributor to the pathogenesis of obstructive sleep apnea (OSA), a widespread disease for which treatment options are limited. Previous OSA drug candidates targeting various excitatory/inhibitory receptors on hypoglossal motoneurons have proved unviable in reactivating these neurons, particularly during rapid-eye-movement (REM) sleep. To identify a viable drug target, we show that the repurposed  $\alpha_2$ -adrenergic antagonist yohimbine potently reversed the depressant effect of REM sleep on baseline hypoglossal motoneuron activity (a first-line motor defense against OSA) in rats. Remarkably, yohimbine also restored the obstructive apnea-induced long-term facilitation of hypoglossal motoneuron activity (hLTF), a much-neglected form of noradrenergic-dependent neuroplasticity that could provide a second-line motor defense against OSA but was also depressed during REM sleep. Corroborating immunohistologic, optogenetic, and pharmacologic evidence confirmed that yohimbine's beneficial effects on baseline hypoglossal motoneuron activity and hLTF were mediated mainly through activation of pontine A7 and A5 noradrenergic neurons. Our results suggest a 2-tier (impaired first- and second-line motor defense) mechanism of noradrenergic-dependent pathogenesis of OSA and a promising pharmacotherapy for rescuing both these intrinsic defenses against OSA through disinhibition of A7 and A5 neurons by  $\alpha_2$ -adrenergic blockade.

## Introduction

Obstructive sleep apnea (OSA), which affects greater than 3%–7% of the general adult population and 1%–4% of the pediatric population worldwide, is a leading cause of excessive daytime sleepiness and an independent risk factor for wide-ranging cardiovascular, metabolic, cognitive, and neuropsychiatric abnormalities causing decreased quality of life and life expectancy (1–3). A pivotal step in the pathogenesis of OSA is sleep state-dependent hypotonia of upper airway dilator muscles (particularly the genioglossus [GG]) causing pharyngeal collapse, an effect that is exacerbated in patients with narrowed upper airways (4). Current standard treatment using continuous positive airway pressure (CPAP) to restore upper airway patency is effective but has a poor adherence rate (5), whereas alternative treatments such as oral-appliance therapy, weight loss, upper-airway surgery, and hypoglossal nerve stimulation are not always effective (6, 7). Pharmacotherapy is currently an unmet need for the management of patients with OSA (8–10).

Previous OSA drug candidates have generally targeted various excitatory receptors on hypoglossal motoneurons (HMs, also known as XII neurons) supplying the upper airway dilator muscles (11–13). However, related preclinical and clinical studies to date have been disappointing (8–10). Moreover, many candidate drugs have side effects such as drowsiness, weight gain, and suppression of REM sleep, which are counterproductive for OSA (14–17). Other drugs that target certain inhibitory receptors (such as muscarinic receptors) on HMs have well-known cardiovascular liabilities (18). Certain potassium channels expressed on HMs are thought to provide more specific drug targets for OSA, but selective blockers for these inhibitory channels are presently lacking (18, 19).

Among the excitatory neuromodulators of HM activity that set the resultant GG muscle tone, norepinephrine is one of the most potent (10, 20, 21), but a noradrenergic-based drug therapy for OSA remains elusive. In a recent study, administration of a norepinephrine reuptake inhibitor desipramine in healthy

**Conflict of interest:** The authors have declared that no conflict of interest exists.

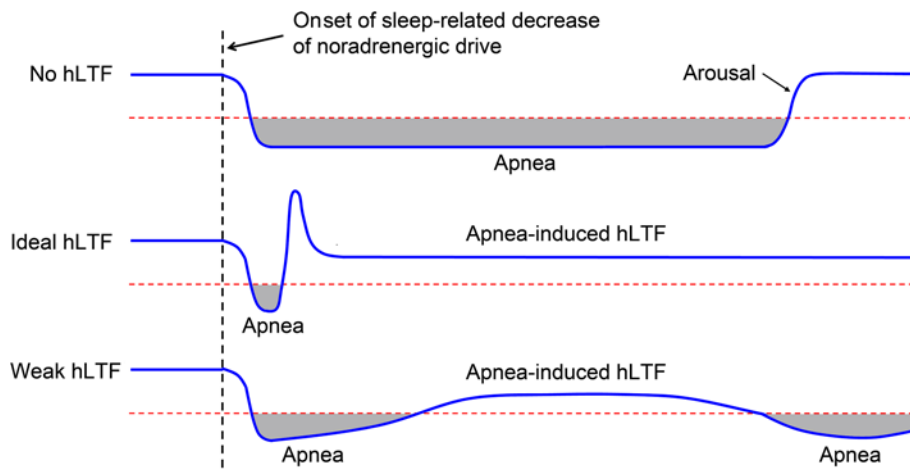
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**Figure 1. Stylized illustration of the roles of baseline HM activity and hLTF as first- and second-line motor defenses against OSA.** Top: In the worst-case scenario where hLTF (hypoglossal long-term facilitation) is lacking, sustained decline of baseline HM activity (hypoglossal nerve activity, tracing in blue) secondary to decreased noradrenergic drive during sleep would result in protracted unremitting obstructive apnea (interval shaded in gray) until arousal. Horizontal dotted line (in red) denotes the hypothetical threshold of HM activity, below which obstructive apnea occurs. Middle: In the best-case scenario where hLTF is in full force, any lapse of baseline HM activity during sleep would be promptly reversed (<10 sec) by reflexive facilitation of HM activity and further apnea prevented by the ensuing hLTF memory, which (ideally) may be long-lasting after even a brief airway obstruction episode. Lower: In a real-life scenario where hLTF is in effect but significantly depressed secondary to decreased noradrenergic drive during sleep, a corresponding decline of baseline HM activity would be reversed much more slowly (>10 sec) due to weakened and delayed reflexive facilitation of HM activity in response to obstructive apnea. Additionally, the reversal (if at all prior to arousal) would be relatively short-lived (<12 min) due to a much shortened hLTF memory following each apnea episode, hence allowing full-blown obstructive apnea to occur and recur. In patients with severe OSA, the weakened hLTF in sleep is manifested as a short-term potentiation of genioglossus activity during an obstructive apnea episode with an “afterdischarge” afterward (25).

subjects reportedly increased the tonic component of GG activity during non-REM sleep (22). However, a similar effect was not demonstrated during REM sleep; indeed, desipramine is known to inhibit REM sleep (23). Moreover, desipramine failed to increase the inspiratory-phasic component of GG activity, which is typically larger than the tonic component and, hence, figures more prominently in protecting against OSA, particularly during the inspiratory phase of the breathing rhythm — when airway obstruction is most prone to occur.

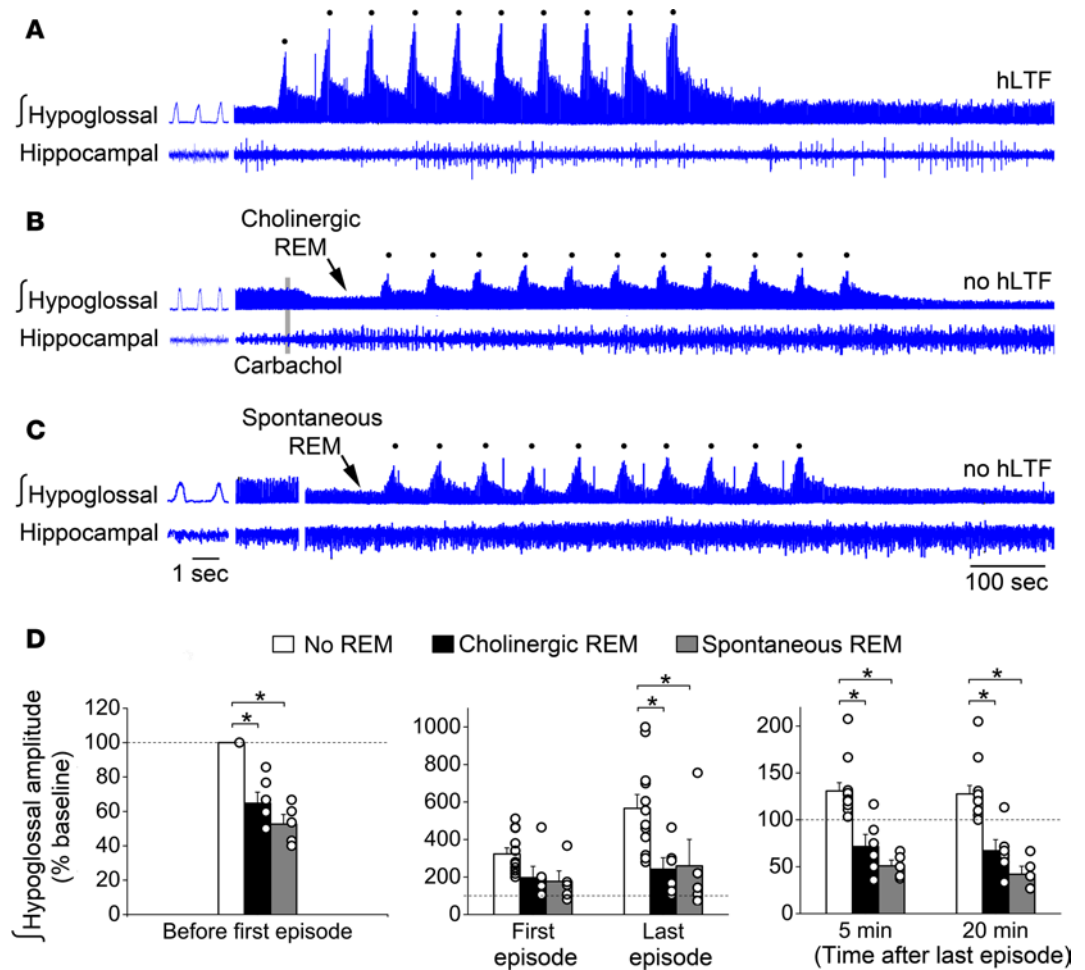
One major difficulty with a noradrenergic-based therapy for OSA is that the influence of endogenous noradrenergic drive on HM activity has proved highly complex and cannot be mimicked by direct activation of HMs with exogenous adrenergic drugs, particularly during REM sleep (12, 13). In particular, recent studies reveal that repetitive obstructive apnea per se may elicit persistent facilitation of rat GG activity, a form of vagally mediated neuroplasticity (referred to as hypoglossal long-term facilitation, hLTF) that requires the activation of  $\alpha_1$ -adrenoceptors on HMs for its expression (24). However, because hLTF is an effect rather than cause of

OSA, its possible role in the pathogenesis of OSA has been poorly understood. In theory, any hLTF resultant from obstructive apnea would serve to promptly reopen the upper airway and avert further persistence or recurrence of OSA (Figure 1). We reasoned that, in order for repetitive OSA to occur, not only baseline HM activity but also hLTF (first- and second-line motor defenses against OSA, respectively) must be depressed during sleep. Indeed, obstructive apnea has been reported to elicit short-term potentiation of GG activity (an abridged form of hLTF) in patients with severe OSA (25).

Because baseline HM activity and hLTF are both noradrenergic dependent, we hypothesized that therapeutic drugs targeting endogenous noradrenergic drive rather than HMs per se may prove more effective in restoring baseline HM activity and hLTF in defense against OSA, especially during REM sleep. Accordingly, we propose a drug therapy for OSA through antagonism of  $\alpha_2$ -adrenoceptors, a set of  $G_i$  protein-coupled receptors that regulates the release of norepinephrine from central noradrenergic neurons (26). In support of this proposition, we show that the classic  $\alpha_2$ -adrenergic blocker yohimbine effectively reversed the depressant effects of REM sleep on baseline HM activity and obstructive apnea-induced hLTF in rats, thereby rescuing these first- and second-line motor defenses against OSA. We present immunohistologic, optogenetic, and pharmacologic evidence indicating that induction of hLTF was mediated by episodic activation of pontine A7 and A5 noradrenergic neurons and was enhanced by blockade of  $\alpha_2$ -adrenoceptors on these neurons. Collectively, our results suggested that disinhibition of A7 and A5 neurons by  $\alpha_2$ -adrenergic blockade effectively restored and augmented baseline HM activity and obstructive apnea-induced hLTF during sleep, hence providing a promising drug therapy for OSA.

## Results

*hLTF-mediated motor defense against obstructive apnea is depressed during REM sleep.* During quiet breathing, XII nerve activity (collectively referred to as HM activity here) represents the compound activities of the



**Figure 2. Obstructive apnea-induced hLTF was depressed during REM sleep.** (A) Obstructive apnea (lasting 10–15 seconds) in a urethane-anesthetized, paralyzed, and mechanically ventilated rat elicited time-dependent (reflexive) facilitation of the amplitude of integrated hypoglossal nerve activity ( $\int$ Hypoglossal, which comprised predominantly the inspiratory-phasic component) during each apnea episode (denoted by dots above the  $\int$ Hypoglossal recording). Such obstructive apnea when applied repeatedly for 10–12 episodes induced sustained facilitation of  $\int$ Hypoglossal amplitude with a long-term memory afterward, evidencing hypoglossal long-term facilitation (hLTF). (B) After microinjection of the cholinergic agonist carbachol at dorsomedial pons to induce a REM-like sleep state (indicated by increased hippocampal activity and decreased baseline  $\int$ Hypoglossal amplitude) in one rat, baseline  $\int$ Hypoglossal amplitude was markedly depressed, and obstructive apnea elicited relatively weak time-dependent facilitation of  $\int$ Hypoglossal amplitude compared with the control state, seen in A. Also, episodic obstructive apnea no longer induced sustained hLTF. (C) Similar effects were seen during spontaneously occurring REM sleep (under urethane anesthesia, ref. 29) in another rat. (D) Bar graphs (overlaid with individual data points) showing the depressant effects ( $P < 0.05$ , 2-way ANOVA with repeated measures) of cholinergic-induced REM-like sleep ( $n = 6$ ) and spontaneous REM sleep ( $n = 5$ ) vs. no REM sleep ( $n = 12$ ) on baseline  $\int$ Hypoglossal amplitude before episodic obstructive apnea (left panel), as well as the facilitation of  $\int$ Hypoglossal amplitude during the first and last apnea episodes (middle panel) and at 5 and 20 minutes after the last apnea episode (right panel). Values are normalized to control baseline at 100% (dashed line) and shown as means  $\pm$  SEM. \* $P < 0.05$  between values as indicated, Tukey post-hoc test.

protrudor GG and retractors hyoglossus and styloglossus; these agonist-antagonist muscles pairs have been shown to be coactivated during the inspiratory phase in rats in order to reduce pharyngeal collapsibility (27). Therefore, the inspiratory-phasic component of HM activity (or GG electromyogram [GG EMG]) is a useful index of the changes in pharyngeal collapsibility in the inspiratory phase, when airway obstruction is most prone to occur. In Figure 2, resting HM activity in urethane-anesthetized, paralyzed, and mechanically ventilated rats with vagi intact is seen to be dominated by the inspiratory-phasic component with negligible tonic component. Episodic obstructive apnea (simulated by stopping the ventilator at end-expiration to prevent lung inflation, see Methods) robustly elicited a time-dependent (reflexive) facilitation of HM activity during each apnea episode (average  $353.5 \pm 73.8\%$  of baseline level;  $P < 0.05$ )

followed by a long-lasting (> 20 min) memory trace after the last episode, evidencing hLTF (Figure 2, A and D). Indeed, a memory trace of the reflexive facilitation of HM activity is also seen after the first apnea episode, although it was relatively short-term (Figure 2A).

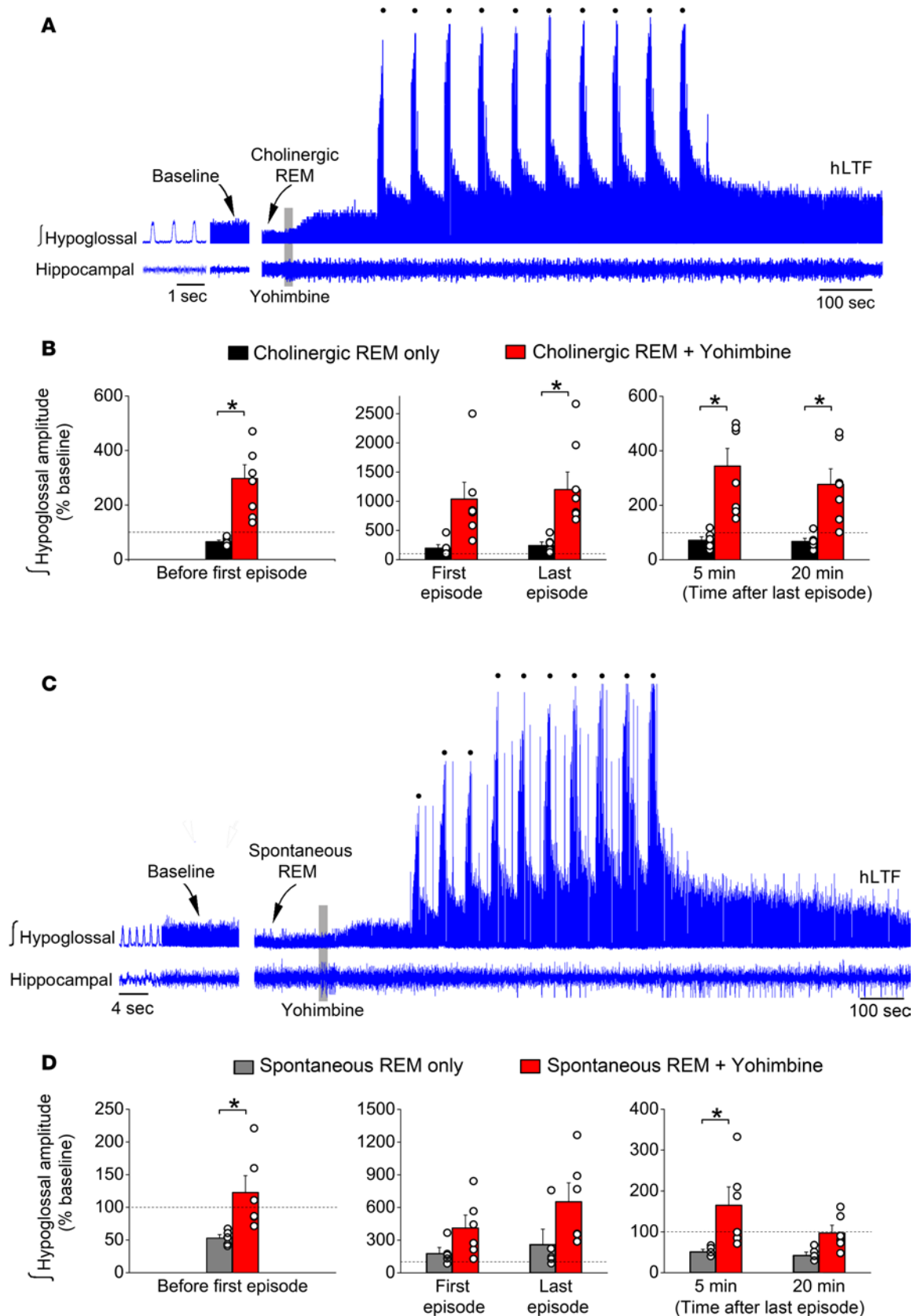
To test whether obstructive apnea-induced hLTF, like baseline HM activity, was also depressed during sleep, we injected the cholinergic agonist carbachol at pontine dorsomedial reticular formation to induce REM-like sleep (as indicated by the appearance of hippocampal theta discharge and corresponding decrease of baseline HM activity; ref. 28). Cholinergic-induced REM-like sleep precipitated a decrease of baseline HM activity in our vagi-intact rats ( $n = 6$ ) (Figure 2, B and D) as with vagotomized rats reported previously (11). Remarkably, throughout the period of cholinergic-induced REM-like sleep, hLTF was also greatly attenuated, as indicated by a marked decrease in HM activity (compared with corresponding HM activities before REM-like sleep) both during the apnea episodes and long afterward (> 20 min). Despite this, episodic obstructive apnea still elicited a small but measurable reflexive facilitation of HM activity during each apnea episode (average  $136.9 \pm 67.1\%$  of pre-REM baseline level,  $P < 0.05$ ) with a brief memory effect (< 2 min) after the last episode (Figure 2, B and D). Hence, hLTF was greatly weakened and shortened but not abolished under cholinergic-induced REM-like sleep.

To verify that the depressant effect of cholinergic-induced REM-like sleep on hLTF was not an artifact of the cholinergic agent per se, similar measurements were made in a separate group of rats ( $n = 5$ ) after transition to spontaneous REM sleep, a condition that is attainable in rats under urethane anesthesia (29). Again, spontaneous REM sleep resulted in marked decreases in not only baseline HM activity (29), but also obstructive apnea-induced hLTF (Figure 2, C and D), severely blunting (but not abolishing) both these first- and second-line motor defenses against OSA. Indeed, compared with cholinergic-induced REM-like, spontaneous REM sleep exerted even greater and more persistent depressant effects on HM activity and hLTF, lasting up to >20 minutes after episodic obstructive apnea (Figure 2D).

The demonstrated adverse effects of REM sleep on hLTF during and following episodic obstructive apnea (Figure 2) provide the first preclinical evidence in support of the hypothesis that sleep state-dependent depression of hLTF could contribute importantly to the pathogenesis of OSA (Figure 1). Because the hLTF so induced has been shown to be mediated mainly by repetitive interruption of lung volume feedback independently of 5-HT<sub>2</sub> receptors and without accompanying long-term facilitation of diaphragm activity (24), it was distinct from the 5-HT<sub>2</sub> receptor-dependent hLTF induced by repetitive hypoxia (30), although a minor contribution of the latter effect cannot be ruled out. Given that patients with OSA often experience only hypopnea (with partial airway obstruction and less severe resultant decreases in blood oxygen saturation and increases in hypoxic stimulation), the hLTF attained in this case may be weaker than that resulting from complete obstructive apnea, making this second-line motor defense against OSA even less effective during sleep.

*Yohimbine rescues baseline HM activity and hLTF from depression during REM sleep.* Because baseline HM activity and obstructive apnea-induced hLTF are both noradrenergic dependent (24), we tested whether the depressions of baseline HM activity and hLTF during REM sleep could be reversed by disinhibition of central noradrenergic neurons with  $\alpha_2$ -adrenergic blockade. Indeed, systemic yohimbine (0.5–1.0 mg/kg i.v.) promptly reversed the decrease in HM activity under cholinergic-induced REM-like sleep, as evidenced by a resultant rise of baseline HM activity above the control level ( $P < 0.05$ ,  $n = 7$ ; Figure 3). Systemic yohimbine also potentially reversed the blunting of obstructive apnea-induced hLTF under cholinergic-induced REM-like sleep, with HM activity remaining well above the corresponding control levels up to 20 minutes after episodic obstructive apnea (Figure 3, A and B). Systemic yohimbine at a lower dose (0.25–0.5 mg/kg i.v.) had similar therapeutic effects on baseline HM activity and hLTF under spontaneous REM sleep, albeit with smaller (dose-dependent) effect sizes (Figure 3, C and D). Hence, yohimbine at a dose > 0.25 mg/kg i.v. in rats effectively rescued both the first- and second-line motor defenses against OSA under REM sleep, when baseline HM activity was lowest.

The demonstrated ability of yohimbine in reversing the depressant effects of REM sleep on HM activity under baseline conditions, as well as during and after episodic obstructive apnea, provide strong preclinical evidence of the likely efficacy of this drug therapy for patients with not only hypopnea, but also apnea (complete airway obstruction) during sleep. On the other hand, by strongly reversing the depressant effect of REM sleep on baseline HM activity prior to the onset of obstructive apnea, yohimbine therapy should effectively avert the development of full-blown repetitive obstructive apnea. Thus, the beneficial effect of yohimbine therapy on hLTF (especially its memory phase) as a second-line fail-safe mechanism in defense against OSA (Figure 1) may not be always fully manifested or necessary in a clinical setting.



**Figure 3. Systemic yohimbine reversed the depressions of baseline hypoglossal activity and obstructive apnea-induced hLTF during REM sleep.** (A) Depression of baseline  $\int$ Hypoglossal amplitude (amplitude of integrated hypoglossal nerve activity) during cholinergic-induced REM-like sleep was promptly reversed by systemic administration of yohimbine (0.75 mg/kg i.v.) in one rat. Systemic yohimbine also restored the facilitation of  $\int$ Hypoglossal amplitude during and after episodic obstructive apnea (compare Figure 2, A and B). (B) Bar graphs (overlaid with individual data points) showing the

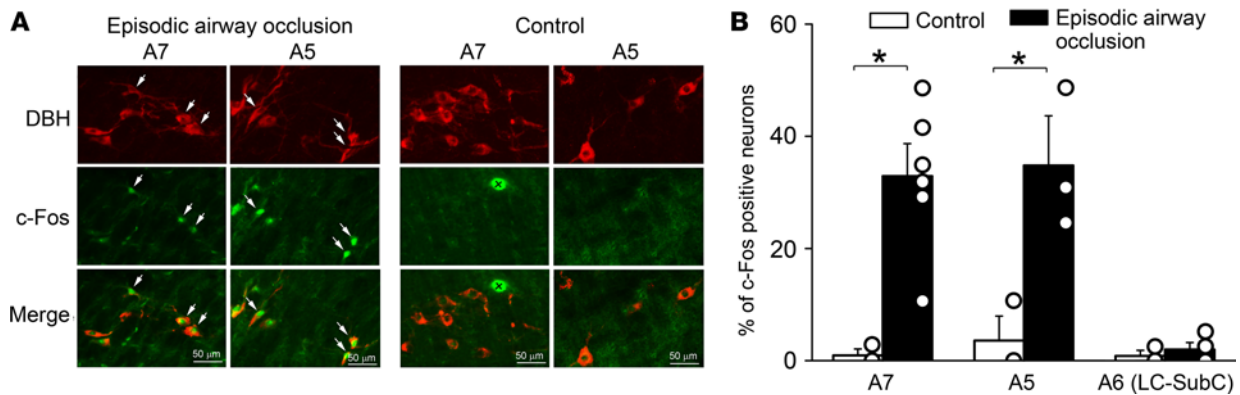
efficacy ( $P < 0.05$ , 2-way ANOVA with repeated measures) of systemic yohimbine (0.75–1 mg/kg i.v.,  $n = 7$ ) in reversing the depressant effects of cholinergic-induced REM-like sleep (vs. cholinergic REM only,  $n = 6$ ) on baseline  $\int$ Hypoglossal amplitude before episodic obstructive apnea (left panel), as well as the facilitation of  $\int$ Hypoglossal amplitude during the first and last apnea episodes (middle panel) and at 5 and 20 minutes after the last apnea episode (right panel). \* $P < 0.05$  between values as indicated, Tukey post-hoc test. (C and D) Similar to A and B showing efficacy of system yohimbine ( $P < 0.05$ , 2-way ANOVA with repeated measures) but with a lower dose of 0.5 mg/kg i.v. ( $n = 6$ ) under spontaneous REM sleep (vs. spontaneous REM only,  $n = 5$ ). \* $P < 0.05$  between values as indicated, Tukey post-hoc test.

*Pontine A7/A5 noradrenergic neurons are activated by episodic obstructive apnea.* To identify the sites of action of yohimbine therapy for OSA, we first investigated whether activities of pontine noradrenergic neurons known to affect breathing were influenced by obstructive apnea. In rats exposed to similar episodic obstructive apnea (experimental group), c-Fos expression was markedly increased in pontine A7 and A5 neurons (identified by dopamine  $\beta$ -hydroxylase immunofluorescent labeling) compared with corresponding control levels, indicating obstructive apnea-induced excitation of these noradrenergic neurons (Figure 4, A and B). In contrast, c-Fos expressions in pontine A6 (locus coeruleus and subcoeruleus) noradrenergic neurons were not significantly different in the experimental vs. control groups (Figure 4B). These data suggested that induction of noradrenergic-dependent hLTF likely involved the activation of A7 and A5 neurons.

*Episodic optogenetic activation of pontine A7/A5 neurons induces hLTF memory.* To confirm the roles of pontine noradrenergic neurons in the induction of hLTF, episodic optogenetic stimulation of A7 and A5 neurons was performed in transgenic rats that expressed Cre recombinase under the control of the endogenous tyrosine hydroxylase promoter (Figure 5A). After 8–10 episodes of 15-second photostimulation of A7 or A5 neurons pretreated with a Cre-inducible viral vector encoding channelrhodopsin 2-enhanced yellow fluorescent protein (ChR2-EYFP), HM activity in these transgenic rats (urethane anesthetized, paralyzed, and mechanically ventilated with vagi-intact) gradually increased to a peak at approximately 5 minutes after stimulation before returning to its prestimulation baseline level in 10–20 minutes (Figure 5, B, C, and E). After administration of the  $\alpha_1$ -adrenoceptor antagonist prazosin (0.5 mg/kg, i.v.), HM activity was decreased, and similar episodic optogenetic stimulation of A7 neurons no longer resulted in hLTF (Figure 5D). Taken together, these data suggested that episodic optogenetic activation of A7/A5 noradrenergic neurons could induce long-lasting hLTF memory afterward. The latter effect confirmed that the optical stimuli did not cause inadvertent depolarization block of these neurons, consistent with previous finding that excitatory neurons (unlike small interneurons) in the brain are resistant to laser light-induced depolarization block (31).

*A7/A5 neurons mediate yohimbine's beneficial effects on baseline HM activity and hLTF.* Unlike the hLTF induced by episodic activation of A7 and A5 neurons with obstructive apnea (Figures 2–4), hLTF induced by direct episodic optogenetic stimulation of A7 or A5 neurons was manifested only after (but not during) stimulation and for a shorter period (Figure 5, B, C, and E). Thus, induction of hLTF by episodic obstructive apnea likely also involved other processes, such as simultaneous activation of noradrenergic neurons in bilateral A7 and A5 regions and/or modulation of  $\alpha_2$ -adrenoceptor activities therein, which could not be reproduced optogenetically. To test this, we injected yohimbine focally at the bilateral A7 and A5 regions in rats (urethane anesthetized, paralyzed, and mechanically ventilated with vagi-intact) before inducing hLTF with episodic obstructive apnea. In Figure 6, yohimbine injection at the bilateral A7 and A5 regions resulted in a significant increase of baseline HM activity ( $P < 0.05$ ,  $n = 5$ ) as well as enhancement of hLTF both during the obstructive apnea episodes ( $P < 0.05$ ) and up to 20 minutes afterward ( $P < 0.05$ ). These beneficial effects were similar to those resulting from systemic yohimbine, albeit to a lesser extent (Figure 6). The differences in effect sizes are likely due to more complete coverage of all noradrenergic neurons by systemic than by focal yohimbine administration. These pharmacological data (Figure 6), when taken together with the immunohistological (Figure 4) and optogenetics data (Figure 5), provide strong evidence that  $\alpha_2$ -adrenoceptors on A7 and A5 noradrenergic neurons (26) were among the sites of action of yohimbine therapy in enhancing baseline HM activity and obstructive apnea-induced hLTF.

*A7/A5 neurons mediate clonidine's adverse effects on baseline HM activity and hLTF.* In a separate group of vagi-intact, nonparalyzed and spontaneously breathing (nonventilated) rats, focal injection of the  $\alpha_2$ -adrenoceptor agonist clonidine at the bilateral A7 region resulted in a significant decrease of baseline GG activity ( $n = 8$ , Figure 7, A and D), in agreement with similar findings in vagotomized rats reported previously (32). In addition, clonidine injection at the bilateral A7 region also markedly suppressed hLTF (in



**Figure 4. Pontine A7 and A5 noradrenergic neuronal groups were activated by episodic obstructive apnea.** (A) Photomicrographs showing A7 and A5 noradrenergic neurons identified by dopamine  $\beta$ -hydroxylase (DBH) immunofluorescent labeling that were immunopositive to c-Fos in a rat exposed to episodic obstructive apnea, compared with one that was not exposed (control). “x” indicates artifacts; arrows indicate doubly labeled neurons. (B) Percentage of c-Fos immunopositive neurons among A7, A5, or A6 noradrenergic neurons in the experimental group ( $n = 6$ ) and control group ( $n = 3$ ). LC, locus coeruleus; SubC, subcoeruleus. \* $P < 0.05$  between values as indicated, 2-tailed Student's  $t$  test.

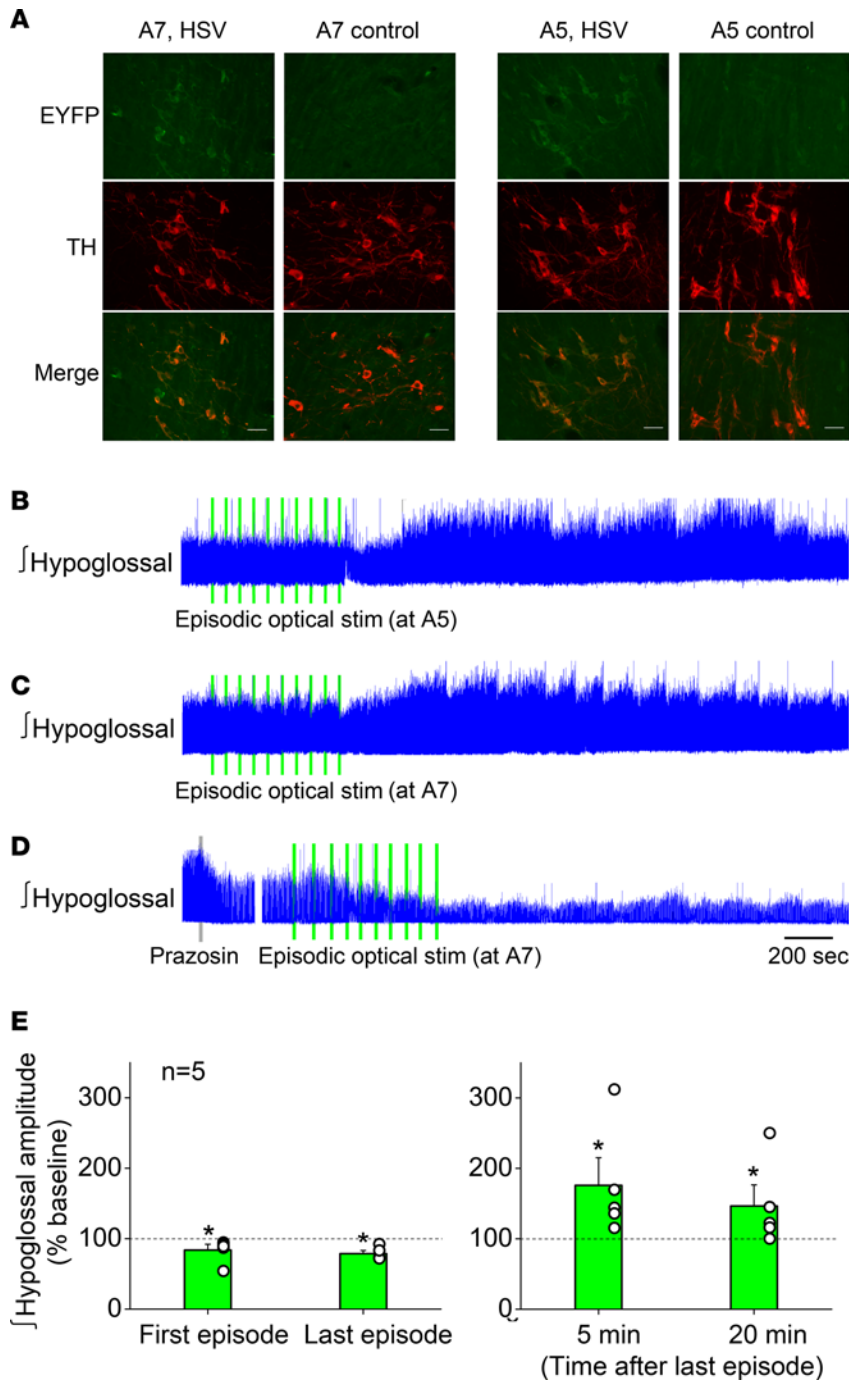
terms of GG activity) both during and after the obstructive apnea episodes (simulated by repetitive artificial airway occlusions at end-expiration to prevent lung inflation; see Methods) compared with corresponding levels after complete washout of the clonidine, significantly shortening the memory phase of hLTF to  $< 1$  minute (Figure 7, B–D). In contrast, baseline GG activity was not significantly affected after focal injection of clonidine at the bilateral A5 region (Figure 7D), in agreement with similar findings in vagotomized rats reported previously (33). Despite the lack of effect on baseline GG activity, however, clonidine injection at the bilateral A5 region still markedly suppressed hLTF both during and after the obstructive apnea episodes as with injection at bilateral A7 (Figure 7D). Thus, even partial decrease of central noradrenergic activity with relatively modest or negligible resultant decreases in baseline GG muscle activity may greatly impair both the reflexive and memory phases of obstructive apnea-induced hLTF.

*Obstructive apnea recruits tonic component of GG activity during spontaneous breathing.* Comparison of the above data for paralyzed and mechanically ventilated rats (Figures 2, 3, 5, and 6) vs. nonparalyzed and spontaneously breathing rats (Figure 7) showed that hLTF was effectively induced by repetitive no-lung-inflations (by stopping the ventilator or by airway occlusion) in both cases. This is consistent with the previous finding that repetitive interruption of vagally mediated lung volume feedback alone could induce hLTF (24). However, in spontaneously breathing rats (with vagi intact), GG activity also exhibited a significant tonic (respiratory phase-spanning) component during (but not before) airway obstruction (Figure 7, B–D). As with the inspiratory-phasic component of GG activity, the tonic component recruited by obstructive apnea was also strongly suppressed by clonidine application at the bilateral A7 or A5 regions. Unlike the inspiratory-phasic component, however, the tonic component so recruited decayed rapidly ( $< 1$  min) after episodic obstructive apnea, hence contributing only to the early memory phase of hLTF (Figure 7, B–D). The presence of a tonic component of GG activity during and shortly after airway obstruction in nonparalyzed, spontaneously breathing rats is consistent with the reported short-term potentiation of GG activity in patients with OSA (25). This effect was likely mediated by the pharyngeal negative pressure reflex (34), since a similar tonic component was absent in the HM activity in paralyzed and mechanically ventilated rats in which airway pressure remained nonnegative throughout (Figures 2 and 3).

## Discussion

Transcending the rich body of literature in the field, the present study suggests several conceptual advances that are critical for successful translation into a viable noradrenergic-based drug therapy for OSA. Rather than targeting HMs in the XII nucleus for potential treatment of OSA as in previous studies, the forgoing results demonstrate that yohimbine blockade of  $\alpha_2$ -adrenoceptors on central noradrenergic neurons effectively reversed the depression of the inspiratory-phasic component of baseline HM activity during REM sleep. Equally important, yohimbine also restored hLTF of the inspiratory-phasic component of HM activity in defense against obstructive apnea during REM sleep. More specifically, our immunohistologic, optogenetic,





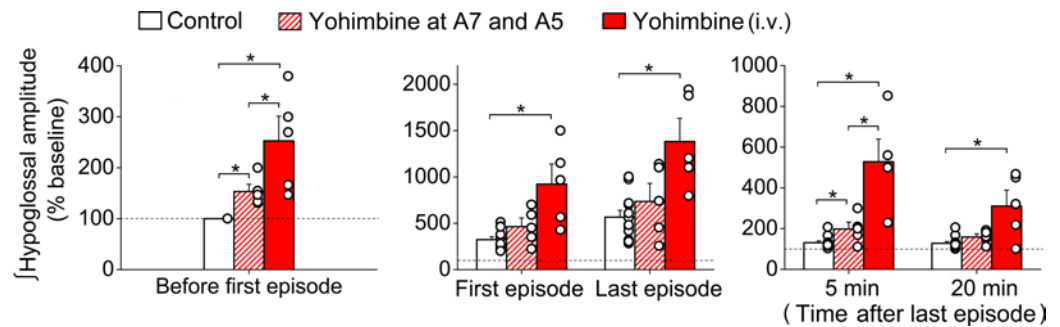
**Figure 5. Episodic optogenetic stimulation at the A7 or A5 regions induced hLTF after stimulation.** (A) Immunohistological imaging showing that most A7 and A5 neurons that expressed Enhanced Yellow Fluorescent Protein (EYFP) after transduction with herpes simplex virus (HSV) were also immunopositive to the catecholamine marker tyrosine hydroxylase (TH). Similar selectivity of Channelrhodopsin-2 (ChR2-EYFP) expression in pontine noradrenergic neurons after transduction with traditional Cre-dependent AAV vector has been previously reported (66). (B) Episodic photostimulation (10 square-wave light pulses at 1 pulse per minute, each lasting 15 seconds) at the A5 region expressing ChR2-EYFP induced hLTF (hypoglossal long-term facilitation) after stimulation. (C) Similar to B but with episodic photostimulation at the A7 region. (D) After systemic application of the  $\alpha_2$ -adrenergic antagonist prazosin, episodic photostimulation at the A7 region no longer induced hLTF after stimulation. (E) Bar graphs (overlaid with individual data points) showing corresponding responses during (left panel) and after (right panel) episodic photostimulation of A7 in 5 rats. Optogenetics data for the HSV and AAV vectors were similar and were merged for statistical analysis.  $\int$ Hypoglossal amplitude (amplitude of integrated hypoglossal nerve activity) is normalized relative to baseline (dashed line). \* $P < 0.05$  vs. baseline, 2-tailed Student's *t* test.

and pharmacologic data confirm that yohimbine's therapeutic actions on HM activity and obstructive apnea-induced hLTF were mediated mainly through blockade of  $\alpha_2$ -adrenoceptors on pontine A7 and A5 noradrenergic neurons. These findings support the hypothesis that, in addition to noradrenergic-dependent depression of baseline HM activity, noradrenergic-dependent depression of hLTF may also contribute to the pathogenesis of OSA (Figure 1). Most importantly, our results provide the first preclinical proof-of-concept to our knowledge of a promising drug therapy for OSA based on  $\alpha_2$ -adrenergic blockade.

*Mechanism of action of  $\alpha_2$ -adrenergic blocker therapy for OSA.* A dilemma is why direct activation of  $\alpha_1$ -adrenoceptors on HMs did not yield similar beneficial effects under REM sleep, as demonstrated here with  $\alpha_2$ -adrenergic blockade (12, 13).

The present study provides valuable clues. Specif-

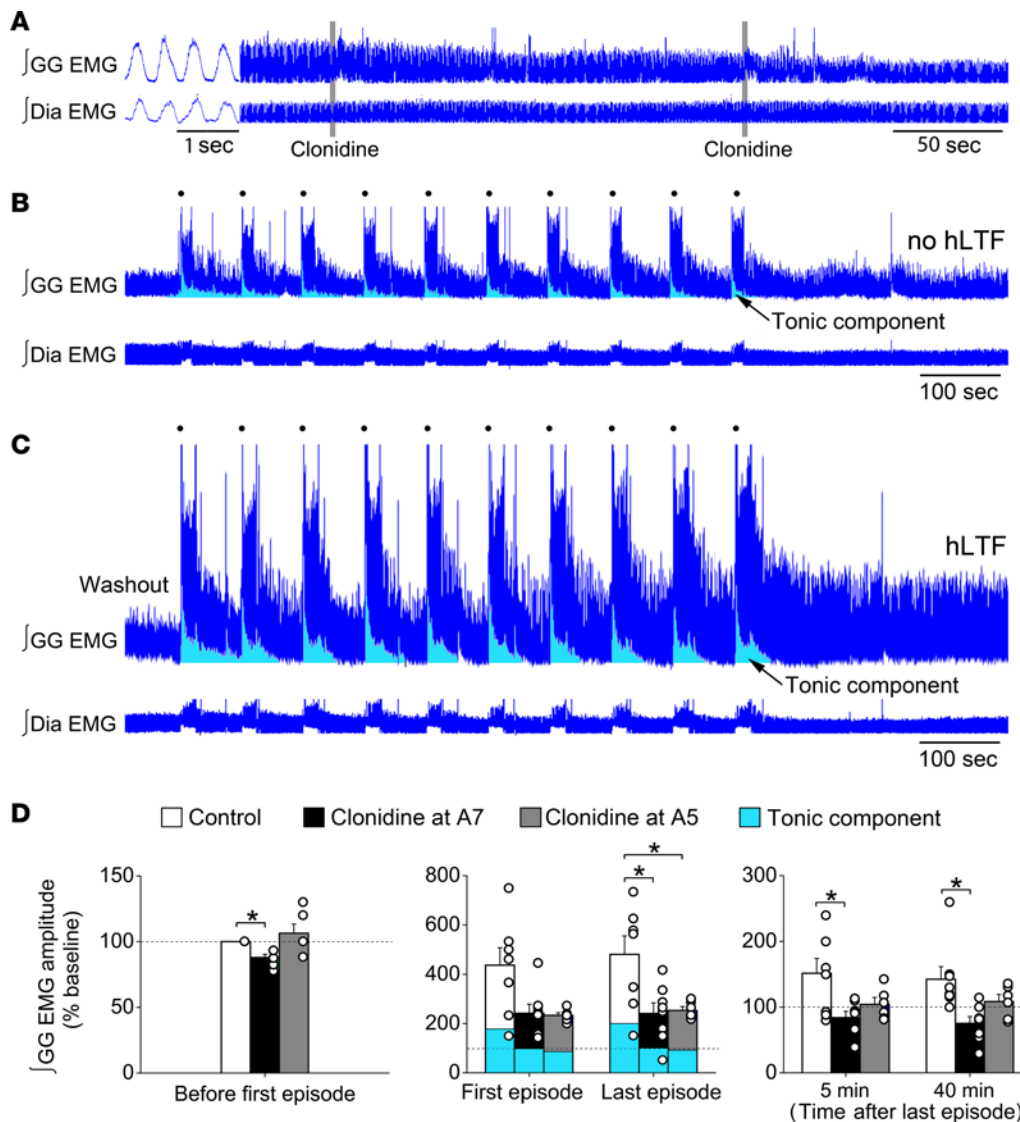
ically, our optogenetics data (Figure 6) show that the influence of central noradrenergic drive on HM activity is plastic (rather than purely reflexive), such that episodic optogenetic stimulation of A7 and A5 neurons could lead to the expression of hLTF after stimulation. Thus, excitatory inputs to A7 and A5 neurons may interact with  $\alpha_2$ -autoreceptor activity to modulate norepinephrine release in a time-dependent manner that long outlasts the duration of these inputs. Because HM activity was not increased during optogenetic stimulation of these noradrenergic neurons, induction of the memory phase of hLTF did not require reflexive facilitation of HM activity during stimulation of A7 or A5 neurons. This finding implies that the induction mechanism for the memory phase of hLTF was likely localized in the A7 and A5 noradrenergic pathways presynaptic to the HMs, independent of HM activity. Such episodic activity-dependent neuroplasticity in the A7 and A5 noradrenergic-HM pathways cannot be reproduced by sustained pharmacologic activation of  $\alpha_1$ -adrenoceptors or other excitatory receptors on HMs.



**Figure 6. Yohimbine applied systemically or at bilateral A7 and A5 regions increased baseline hypoglossal activity and enhanced obstructive apnea-induced hLTF.** Bar graphs (overlaid with individual data points) showing the efficacy of yohimbine microinjected at bilateral A7 and A5 regions (50 nl at 2.5 mM at each injection site,  $n = 5$ ) or administered systemically (0.75 mg/kg i.v.,  $n = 5$ ) versus control ( $n = 12$ ) in augmenting baseline  $\int$ Hypoglossal amplitude (amplitude of integrated hypoglossal nerve activity) before episodic obstructive apnea (left panel), as well as facilitation of  $\int$ Hypoglossal amplitude during the first and last apnea episodes (middle panel) and at 5 and 20 minutes after the last apnea episode (right panel). \* $P < 0.05$  between values as indicated, 2-way ANOVA with repeated measures and Tukey post-hoc test. See Supplemental Figure 1 for microinjection loci.

The suggested expression of episodic activity-dependent neuroplasticity in the A7 and A5 noradrenergic-HM pathways independent of HM activity is further supported by our finding that the memory phase of obstructive apnea-induced hLTF was abolished after inhibition of A7 or A5 neurons with clonidine (Figure 7). Thus, integrity of both A7 and A5 noradrenergic cell groups was requisite for the expression of the memory phase of hLTF. Of particular significance, inhibition of A7 or A5 neurons by clonidine significantly depressed the reflexive facilitation of both the inspiratory-phasic and tonic components of HM activity during obstructive apnea and abolished the memory phase of hLTF afterward, even though baseline GG activity was only slightly decreased (after inhibition of A7) or virtually unchanged (after inhibition of A5) (Figure 7D). The demonstrated suppression of both the reflexive phase and memory phase of hLTF after even partial inhibition of central noradrenergic activity with minimal resultant decreases in baseline GG activity provides a possible mechanistic explanation as to why OSA is generally as prevalent in non-REM sleep as in REM sleep in adult patients (35), despite the fact that GG muscle hypotonia is typically less severe in non-REM sleep than in REM sleep (36, 37). Collectively, our findings support the concept that noradrenergic-dependent depression of obstructive apnea-induced hLTF may contribute importantly to the pathogenesis of OSA as much as decreases in baseline HM and GG activities per se (Figure 1).

Another difficulty with previous attempts of direct pharmacologic activation of HMs to counter OSA is that the resultant excitatory effects may be offset by concurrent inhibitory inputs during REM sleep. Given that HMs are strongly inhibited during cholinergic-induced REM-like sleep (38), as in natural REM sleep (18, 39), it is remarkable that  $\alpha_2$ -adrenergic blockade with systemic yohimbine alone was sufficient to simultaneously excite and disinhibit HMs during both cholinergic-induced REM-like sleep and spontaneous REM sleep (Figure 3). Because endogenous inhibition of HMs is generally most prominent in REM sleep when central noradrenergic activity is lowest, we speculate that inhibitory inputs to HMs may be inversely gated by endogenous noradrenergic drive (or directly gated by  $\alpha_2$ -adrenoceptor activity), perhaps in a manner similar to that seen in some brain systems (40–42). Furthermore, given that central noradrenergic neurons are generally presumed silent during REM sleep (33, 43), it is surprising that systemic yohimbine alone was sufficient to reactivate central noradrenergic drive during both cholinergic-induced REM-like sleep and spontaneous REM sleep (Figure 3) when the  $\alpha_2$ -autoreceptors were supposedly already quiescent. We speculate that inhibition of A7 and A5 neurons during REM sleep may be mediated by the activation of  $\alpha_2$ -adrenoceptors expressed on these neurons in response to inputs from other (extrapontine) noradrenergic or adrenergic cell groups that are active during REM sleep. If so,  $\alpha_2$ -adrenergic blockade in A7 and A5 neurons by yohimbine would relieve these neurons from the inhibitory influences of extrapontine noradrenergic/adrenergic neurons during REM sleep. This is supported by our finding that focal injection of yohimbine at bilateral A7 and A5 neurons produced similar beneficial effects on HM activity and obstructive apnea-induced hLTF, as did systemic yohimbine (Figure 6), suggesting that postsynaptic  $\alpha_2$ -adrenoceptors on the dendrites and/or cell bodies of these pontine noradrenergic neurons (rather than  $\alpha_2$ -autoreceptors on their distal axonal terminals presynaptic to the HMs) were likely the sites of action of yohimbine therapy.



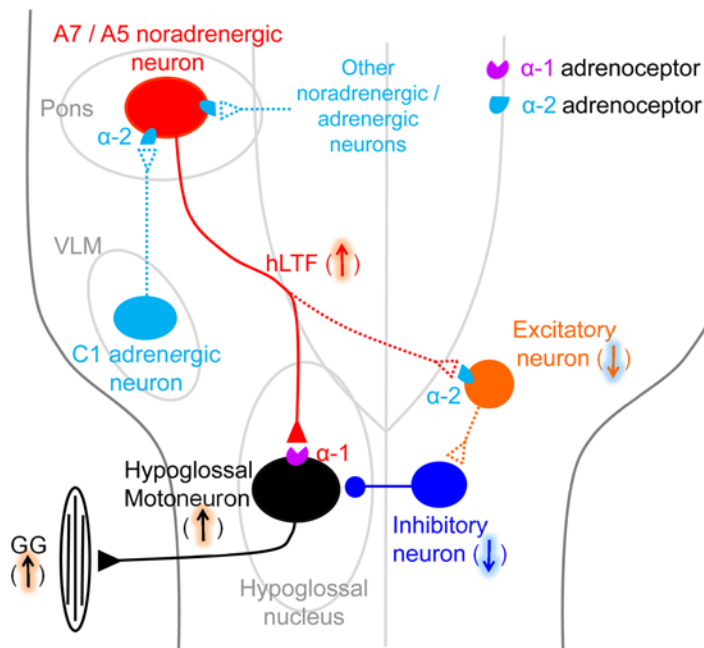
**Figure 7. Activation of  $\alpha_2$ -adrenoceptors on A7 or A5 neurons impaired obstructive apnea-induced hLTF. (A–C)** Tracings of integrated genioglossus electromyogram (JGG EMG) and diaphragm EMG (JDia EMG) in 1 urethane-anesthetized, spontaneously breathing rat. **(A)** Microinjection of the  $\alpha_2$ -adrenoceptor agonist clonidine at bilateral A7 region (~50 nl at 5 mM at each injection site) reduced baseline GG activity (JGG EMG amplitude). See Supplemental Figure 1 for microinjection loci. **(B)** After microinjection of clonidine, episodic obstructive apnea (denoted by dots above the JGG EMG recording) elicited relatively weak reflexive facilitation of GG activity during each apnea episode with no evidence of hLTF (hypoglossal long-term facilitation) afterward. **(C)** After washout of clonidine (2–3 hours after microinjection), baseline GG activity returned to control level. Episodic obstructive apnea elicited much stronger reflexive facilitation of GG activity during each apnea episode, with pronounced long-term facilitation of the inspiratory-phasic component of GG activity afterward. Note that, in **B** and **C**, a tonic component of GG activity (shaded in light blue) was recruited during obstructive apnea, but this tonic component decayed rapidly after each obstructive apnea episode and did not exhibit long-term facilitation afterward. **(D)** Bar graphs (overlaid with individual data points) showing the significant adverse effects ( $P < 0.05$ , 2-way ANOVA with repeated measures) of clonidine injection at bilateral A7 ( $n = 8$ ) or A5 ( $n = 6$ ) region (vs. corresponding control values after washout) on baseline GG activity before episodic obstructive apnea (left panel), as well as the facilitation of GG activity during the first and last apnea episodes (middle panel) and at 5 and 40 minutes after the last apnea episode (right panel). In the middle panel, areas of the bar graphs shaded in light blue indicate the magnitudes of the tonic component that contributed to GG activity; balance of the same bar graphs (not shaded in light blue) indicate the magnitudes of the corresponding inspiratory-phasic component. \* $P < 0.05$  between values as indicated, Tukey post-hoc test.

noradrenergic neurons, including A7/A5 neurons, are inhibited (via increasing  $\alpha_2$ -adrenergic receptor activation) by inputs from extrapontine noradrenergic/adrenergic cell groups that are active during REM sleep. The resultant decreases in noradrenergic input, along with a simultaneous increase in REM sleep-dependent inhibitory input, precipitate a fall in baseline HM activity and corresponding GG muscle tone,

Indeed, certain medullary adrenergic neurons (such as the C1 cell group) are known to be active during REM sleep and exert inhibitory influences on pontine noradrenergic neurons via  $\alpha_2$ -adrenoceptors (44, 45), although recent studies revealed that a subset of C1 neurons may paradoxically also exert stimulation frequency-dependent excitatory influences on pontine noradrenergic neurons (46). Of note, previous studies have shown that HMs may be subject to an  $\alpha_{2C}$ -adrenoceptor (but not  $\alpha_{2A}$ -adrenoceptor) subtype-mediated inhibition (47, 48). However, subsequent studies revealed that this effect is prominent only in neonatal animals (49) and does not involve pre- or postsynaptic inhibition of glutamatergic transmission (50). Thus, the mechanism of action of yohimbine therapy in adult animals probably does not involve the modulation of glutamate transmission of inspiratory drive to HMs through blockade of pre- or postsynaptic  $\alpha_{2C}$ -adrenoceptor subtype. Further work is needed to elucidate how blockade of  $\alpha_2$ -adrenoceptors on A7 and A5 neurons by yohimbine may simultaneously excite and disinhibit HMs during REM sleep.

In light of the above, we propose the following 2-tier mechanism of noradrenergic-dependent pathogenesis of OSA and corresponding mechanism of action of yohimbine therapy for further investigation (Figure 8).

During REM sleep, pontine



**Figure 8. Neural network diagram showing the proposed 2-tier mechanism of noradrenergic-dependent pathogenesis of OSA and corresponding mechanism of action of  $\alpha_2$ -adrenergic blocker therapy.** During REM sleep, pontine A7 and A5 noradrenergic neurons are inhibited by extrapontine noradrenergic/adrenergic inputs, including those possibly from the C1 adrenergic cell group in ventrolateral medulla (VLM), which become active during this state. Resultant disfacilitation (possibly together with concurrently increased inhibition) of the hypoglossal motoneurons and blunting of hypoglossal long-term facilitation (hLTF) precipitate obstructive sleep apnea (OSA). An  $\alpha_2$ -adrenergic blocker such as yohimbine reverses these depressant effects of REM sleep by disinhibiting A7 and A5 neurons, hence restoring the excitatory modulations of central noradrenergic drive on hypoglossal motoneuron activity and hLTF (upward arrows) in defense against OSA. How restoration of central noradrenergic drive by yohimbine may simultaneously gate off (downward arrow) inhibitory influences on hypoglossal motoneurons during REM sleep is unknown. GG, genioglossus muscle.

triggering the onset of OSA in at-risk patients. Additionally, with diminished noradrenergic drive during REM sleep, obstructive apnea-induced hLTF is also greatly attenuated, hence allowing the developing obstructive apnea to persist and recur. This 2-tier mechanism of noradrenergic-dependent depression of the first- and second-line motor defenses against OSA underlies the pathogenesis of full-blown repetitive and unremitting OSA that can be broken only by arousal (Figure 1). To reverse this, administration of an  $\alpha_2$ -adrenergic blocker such as yohimbine effectively disinhibits A7/A5 neurons, hence restoring the noradrenergic-dependent first- and second-line motor defenses against OSA while simultaneously gating off inhibitory inputs to the HMs during REM sleep (Figure 8).

*Clinical aspects of  $\alpha_2$ -adrenergic blocker therapy for OSA.* Currently, yohimbine is the only prescription  $\alpha_2$ -adrenergic blocker (currently available over-the-counter in the USA as a dietary supplement without the need for prescription) that has been tested extensively in acute and long-term clinical studies to verify its relative safety when administered at a clinically recommended dose (51, 52); others, such as atipemazole, are currently for animal use only and are unsafe for humans. Within this safe dose range, yohimbine does not disrupt REM sleep (53–55) or cause weight gain (56). At much higher doses, yohimbine may cause hypertension, tachycardia, anxiety, and agitation, but these adverse effects are easily reversible upon termination of use (52, 56). For comparison, a yohimbine dose of 0.5 mg/kg presently used in rats amounts to a human equivalent dose of 5.6 mg for a 70-kg person (57), in excellent agreement with the standard prescription dose (5.4 mg 3 times a day) for its purported treatment of erectile dysfunction in men (51). Because yohimbine per os has a relatively short elimination half-life of approximately 36 minutes in humans (58), an extended-release yohimbine formulation taken before bedtime is desirable for effective repurposed treatment of OSA throughout sleep. For risk/benefit analysis, the off-target profile of yohimbine as a prototypic  $\alpha_2$ -adrenergic blocker is comparable to those of traditional  $\beta_1$ -adrenergic blockers widely used for the treatment of hypertension and heart diseases (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/jci.insight.91456DS1). Future drug designs with improved selectivity for  $\alpha_2$ -autoreceptor subtypes expressed on central noradrenergic neurons (26), together with a combination drug therapy approach to control off-target effects (59), would further limit potential adverse effects of  $\alpha_2$ -adrenergic blocker therapy for OSA. On the other hand, although the sites of action of yohimbine therapy have been verified by the present study to include A7 and A5 noradrenergic neurons, possible therapeutic contributions of other yohimbine-sensitive receptors (in addition to  $\alpha_2$ -adrenoceptors) expressed on these neurons cannot be ruled out (Supplemental Table 1).

Although the promise of  $\alpha_2$ -adrenergic blockade as a sufficient monotherapy for OSA in its own right has been hitherto unrecognized, anecdotal evidence does exist in the literature that unwittingly implicates the potential benefits of yohimbine therapy for OSA patients in a clinical setting. In a previous

randomized double-blind controlled clinical study, the antidepressant mirtazapine (a mixed antagonist for serotonin 5HT<sub>2</sub>/5HT<sub>3</sub> receptors, histamine H<sub>1</sub> receptor, and  $\alpha_{2A}/\alpha_{2C}$  adrenoceptors; see Supplemental Table 1) was proposed to be efficacious for adult patients with OSA mainly through its antagonism of 5HT<sub>2</sub>/5HT<sub>3</sub> receptors, an effect that was thought to promote serotonin release in the brain (16). However, a subsequent trial failed to verify the suggested benefits of mirtazapine, especially at higher doses (17). The discrepancy may be explained in part by the inverse dose-dependent effects of mirtazapine on GG activity, indicating possible within-drug interaction (60). Because serotonin therapy has been shown to be largely ineffective for patients with OSA and in animal models (8–10), any beneficial effects of low-dose mirtazapine (16, 60) in retrospect are likely mediated by its antagonism of  $\alpha_2$ -adrenergic receptors. At higher doses, the resultant increase in serotonin release may actually suppress excitatory inputs to HMs via presynaptic inhibition (reviewed in ref. 10), thus negating any beneficial effects of mirtazapine as a nonselective  $\alpha_2$ -adrenergic blocker. This, plus the fact that mirtazapine is notoriously associated with sedation and weight gain and is a much weaker antagonist for  $\alpha_{2A}/\alpha_{2C}$  adrenergic receptors than is yohimbine (Supplemental Table 1), makes it a nonideal drug candidate for OSA. In contrast, yohimbine, when administered at the clinically recommended dose, was reported in an early (and much neglected) preliminary study to be surprisingly effective in treating snoring, a key symptom of OSA (61). These early clinical data, while inconclusive and incomplete, do add to the present proposition that an  $\alpha_2$ -adrenergic blocker such as yohimbine may be a promising drug candidate for the treatment of OSA.

*Limitations of the study.* To the extent that the present study has been aimed at primarily verifying the viability and sites of action of yohimbine therapy as a novel drug treatment of OSA, many underlying mechanistic details as discussed above remain to be clarified in the future (Figure 8). Another limitation of the present study is the necessary use of urethane anesthesia in order to avoid arousal during obstructive apnea in tracheostomized rats, which is typical of such studies (24). Unlike other anesthetics, urethane anesthesia retains the sleep-like alterations in brain state, GG activity, breathing pattern, and chemosensitivity similar to those seen during natural sleep in unanesthetized rats (29, 62, 63). Our finding that yohimbine had similar beneficial effects under both cholinergic-induced REM-like sleep and spontaneous REM sleep in urethane-anesthetized rats (Figure 3) further substantiated the suggested robustness of yohimbine therapy in potentially mitigating obstructive apnea — particularly those occurring predominantly during REM sleep, a common yet often under-diagnosed form of OSA (64). Given that hypotonia of GG muscle is generally less severe in non-REM sleep than in REM sleep (36, 37), yohimbine therapy should be beneficial also during non-REM sleep, although further studies are needed to confirm this. Ultimate proof in future clinical trials of the efficacy and long-term safety of yohimbine or other  $\alpha_2$ -adrenoceptor subtype-specific antagonists in mitigating OSA in all sleep states would open a long-sought-after pharmacotherapy alternative to current standards of care for this widespread disease.

## Methods

*Animal preparation and electrophysiology.* Experiments were performed on 45 male adult Sprague-Dawley rats (300–400 g, Charles River Laboratories) under urethane anesthesia (initial dose 1.5 g/kg, i.p.; supplemental dose 1/10 of initial dose, i.p. or i.v. when necessary) and sublingual tracheal cannulation with a Y-shaped tracheal cannula. Femoral vein and artery were cannulated for i.v. infusion and monitoring blood pressure, respectively. Rectal temperature was maintained at 36.5°C–37.5°C with a thermostatic heating pad. In all experiments, both vagus nerves were kept intact because repetitive interruption of vagal lung volume feedback was necessary for the induction of hLTF (24).

GG or diaphragm EMG was recorded by inserting a pair of isolated silver wires (outer diameter [OD] 0.127 mm) into the muscle. The wire tips were exposed for 1 mm and separated by approximately 5 mm once inserted into the muscle. In some rats, the medial branch of hypoglossal nerve (which innervates the GG and other tongue protrusion muscles) and phrenic nerve (which innervates the diaphragm) were isolated and severed. After the rat was mounted onto the stereotaxic frame, the central end of the nerve was exposed from the back of the neck (dorsal approach) and mounted onto parallel bipolar wire electrodes for recording. These rats were artificially ventilated with oxygen-enriched (40% O<sub>2</sub>) medical air and paralyzed with pancuronium bromide. The nerve discharge or EMG signals were amplified (CyberAmp 380, Axon Instruments), integrated (time constant 0.1 s, MA821 RSP Moving Averager, CWE Inc.), and sampled at 10 KHz into a Dell PC with LabView software (National Instruments). The recordings were stabilized for at least 1 hour before any data collection and experimental manipulations were performed.

*Brain microinjections.* For brain microinjections, an occipital craniotomy was performed to expose the brain surface. A glass micropipette (tip OD 10–20  $\mu\text{m}$ ) filled with chemical solution was inserted to the target structure according to its stereotaxic coordinates. Injections (25–50 nl per injection) were performed by applying pressure pulses to the micropipette and confirmed by the movement of the intrapipette solution meniscus. All chemicals were purchased from Sigma-Aldrich and dissolved in ACSF at concentration of 10 mM. Stereotaxic coordinates of the A7 region: 2.3–2.6 mm from midline, 0–0.5 mm rostral from interaural level, and 8.0–8.5 mm from lambda surface; of the A5 region: 0–0.5 mm caudal from interaural level, 2.2–2.7 mm from midline, and 9.5–10 mm from lambda surface; and of the dorsomedial pons: 1–1.5 mm from midline, 0.2–0.7 mm rostral from interaural level, and 7.5–8.5 mm from lambda surface.

*Induction of obstructive apnea.* Recurrent obstructive apneas during spontaneous breathing in urethane-anesthetized rats were simulated as described in ref. 24 by applying 10–12 episodes of end-expiratory airway occlusion to prevent lung inflation (for 10–12 sec) at 1 episode per minute. During mechanical ventilation in urethane-anesthetized and pancuronium-paralyzed rats, recurrent obstructive apneas were simulated by periodically stopping the ventilator at end-expiration to prevent lung inflation in a similar manner.

*Cholinergic-induced REM sleep-like state.* A REM sleep-like state was induced by microinjection of the cholinergic agonist carbachol at unilateral dorsomedial pons (65) and was detected by the decrease of hypoglossal/GG EMG activity and the appearance of hippocampal  $\theta$  discharge. The latter was recorded by using a parallel bipolar wire electrode inserted into the hippocampal CA1 region at the following stereotaxic coordinates: 3.7 mm caudal from Bregma, 2.2 mm lateral from midline, and depth of 2.4 mm from brain surface.

*Tyrosine hydroxylase immunohistology.* To confirm that the microinjections of clonidine and yohimbine covered the A7 and A5 groups of noradrenergic neurons, the loci of microinjections were marked by microinjections of fluorescent microspheres (RetroBeads, Lumafuor Inc.) at the end of the experiment. The animal was killed by urethane overdose and perfused with PBS, followed by paraformaldehyde solution. The brain was removed, postfixed, and cut into 40- $\mu\text{m}$  coronal sections on a freezing microtome. Sections of rostral pons were processed for immunofluorescent visualization of tyrosine hydroxylase. Briefly, the sections were incubated in a rabbit polyclonal anti-tyrosine hydroxylase antibody (EMD Millipore, catalog AB152, RRID: AB\_390204) at 1:500 dilution in PBS containing 5% normal goat serum for 48 hours at 4°C. Then the sections were rinsed (3 times for 15 min in PBS) and incubated in Alexa Fluor-488-labeled goat anti-rabbit IgG (Invitrogen, catalog A11008) at 1:1,000 dilution for 3 hours at room temperature and thoroughly rinsed.

*Dopamine  $\beta$ -hydroxylase and c-Fos immunohistology.* To detect changes in c-Fos expression in pontine noradrenergic neurons following episodic obstructive apnea (2 experimental series separated by 10 min in each rat), brainstem sections from rats in the experimental group and in the control group (not exposed to episodic obstructive apnea) were incubated in a mixture of goat polyclonal anti-dopamine  $\beta$ -hydroxylase (Santa Cruz Biotechnology Inc., catalog sc-7487, RRID: AB\_2230289) and rabbit polyclonal anti-c-Fos (EMD Millipore, catalog ABE457) antibodies at 1:500 dilution in PBS containing 5% normal donkey serum for 48 hours at 4°C. Then, the sections were rinsed (3 times for 15 min) and incubated in a mixture of Alexa Fluor-488-labeled donkey anti-rabbit IgG and Alexa Fluor-594-labeled donkey anti-goat IgG (Invitrogen, catalogs A21206 and A11058, respectively) at 1:500 dilution for 3 hours at room temperature and thoroughly rinsed.

*Fluorescent microscopy.* Brainstem sections processed for immunostaining as described above were mounted onto slides, dried and coverslipped, and observed under fluorescent microscope (Zeiss fluorescent Axio microscope, Zeiss). Photos were captured with AxioCam (Zeiss) and analyzed with AV Rel 4.8.2 software (Zeiss).

*Optogenetics methods.* Experiments were performed on 6 homozygous TH-cre transgenic Sprague-Dawley rats (TH-cre<sup>tm1sage</sup>, TGRA8400, male, 275–300 g, Horizon Discovery) that expressed Cre recombinase in catecholamine neurons under the control of the endogenous tyrosine hydroxylase (TH) promoter. Surgeries were performed under pentobarbital anesthesia (60 mg/kg, i.p.) and sterile condition. The first 3 rats were microinjected a Cre-dependent short-term herpes simplex virus (HSV) vector encoding ChR2-EYFP (HSV-LS1L-hChR2(H134R)-EYFP; purchased from MIT Viral Core Facility) at unilateral A7 ( $n = 2$ ) or A5 ( $n = 1$ ). The dosage was 2  $\mu\text{l}$  at viral concentration  $\geq 3 \times 10^8$  transducing units/ml. This vector used nonleaky lox-stop-lox cassette that was induced robustly in the presence of Cre to drive the expression of ChR2-EYFP for a short period of 3–7 days. To cross-check the data with the short-term HSV vector, the other 3 rats

were microinjected a traditional Cre-dependent AAV vector encoding ChR2-EYFP (AAV9-hEF1a-DIO-hChR2(H134R)-EYFP; packaged by Virovek) at unilateral A7 (65). The dosage was 2  $\mu$ l at viral concentration  $2.22 \times 10^{13}$  transducing units/ml. After the injection, rats were maintained under standard postsurgical care until the ChR2-EYFP transduction was complete (4–5 days for rats microinjected with HSV vector and 4 weeks for rats microinjected with AAV vector) before being subjected to photostimulation experiments.

**Photostimulation.** In rats that were preinjected with viral vectors at A7 or A5, an  $\varnothing$ 200  $\mu$ m optical fiber was inserted stereotaxically into the A7 or A5 regions. The optical fiber was connected to a 473-nm laser light source (IKE-PS-500, Ikecool) to deliver photostimulation. For episodic photostimulation, 10 square-wave light pulses (each pulse lasting 15 sec) were delivered at 1 pulse per minute for 10 minutes.

**Statistics.** The amplitudes of the integrated GG EMG (or hypoglossal nerve discharge) and integrated diaphragm EMG (or phrenic nerve discharge) recordings were measured and normalized to the control baseline values. All values are expressed as means  $\pm$  SEM. One- or 2-way ANOVA with repeated measures followed by Tukey post-hoc analysis (or 2-tailed Student's *t* test where appropriate) was used to test for statistical significance at the 95% CI.

**Study approval.** All experimental protocols were reviewed and approved by the MIT Committee on Animal Care (Cambridge, Massachusetts, USA) in accordance with published guidelines.

## Author contributions

GS deigned the research studies, conducted the experiments, acquired and analyzed data, and wrote the manuscript. CSP initiated and designed the research studies, analyzed data, and wrote the manuscript.

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