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ORIGINAL ARTICLE

A latent serotonin-1A receptor-gated spinal afferent pathway inhibiting breathing

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Abstract Spinal afferents such as nociceptive afferents and group III–IV muscle afferents are known to exert an acute excitatory effect on breathing when activated. Here, we report the surprising existence of latent spinal afferents which exerted tonic inhibitory influence on breathing subliminally in anesthetized rats, an effect which was reversed upon activation of serotonin-1A receptors (5-HT_{1A}Rs) in lumbar spinal cord, lesion of pontine lateral parabrachial nucleus or suppression of the adjacent Kölliker-Fuse nucleus with NMDA receptor blockade. Small-interfering RNA knockdown of 5-HT_{1A}Rs in lumbar spinal cord unequivocally localized the site of 5-HT_{1A}R-mediated gating of these respiratory-inhibiting interoceptive afferents to relay neurons in the spinal superficial dorsal horn at the lumbar level and not cervical spinal or supraspinal levels. Our results reveal a novel somatosensory/viscerosensory mechanism which exerts tonic inhibitory influence on homeostatic regulation of breathing independent from the classical chemoreflex excitatory pathways, and suggest a hitherto unrecognized therapeutic target in spinal dorsal horn for 5-HT_{1A}R-based treatment of a variety of respiratory abnormalities.

Keywords 5-HT_{1A}R · Spinal cord · Respiration · Pontine · siRNA

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Introduction

Serotonin or 5-hydroxytryptamine (5-HT) is a ubiquitous monoamine neurotransmitter which plays an important role in many physiological and behavioral functions. Among the family of 5-HT receptors, the 1A receptor subtype (5-HT_{1A}R) is most pervasive in the central nervous system (Fiorino et al. 2014) and has been widely considered as a therapeutic target for various brain disorders including anxiety and depression, schizophrenia, Parkinson's disease and cognitive dysfunction (Lacivita et al. 2012; Celada et al. 2013). There is increasing evidence that 5-HT_{1A}R activation may be beneficial in reversing a variety of respiratory abnormalities such as irregular apneas in sudden infant death syndrome (Audero et al. 2008; Duncan et al. 2010) and Rett syndrome (Abdala et al. 2010), apneustic disturbances following brain injuries (Lalley et al. 1994; Wilken et al. 1997; El-Khatib et al. 2003), and respiratory depression caused by spinal cord injury (Teng et al. 2003; Choi et al. 2005) or opioids (Sahibzada et al. 2000; Zhuang et al. 2012).

In contrast to 5-HT_{1A}R's well-known actions in brain circuits, 5-HT_{1A}Rs are also heavily localized in the superficial laminae (I and II) of spinal cord dorsal horn especially at the lumbar and sacral levels (Marlier et al. 1991; Thor et al. 1993; Manzke et al. 2009). Neurons in these dorsal horn regions receive monosynaptic inputs from small-diameter (Aδ and C) primary afferent fibers which convey diverse sensory information from the periphery such as pain, temperature, itch, sensual touch, as well as muscular and visceral sensations and vasomotor activity (Craig 2002, 2003, 2015; Todd 2010; Jankowski et al. 2013). Transmission of these interoceptive afferent signals is gated by serotonergic inhibition of spinal superficial dorsal horn neurons through the activation of

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pre- and/or postsynaptic 5-HT_{1A}Rs (Yoshimura and Furue 2006; Lu and Perl 2007; Jeong et al. 2012). Lamina I neurons receiving these interoceptive inputs send ascending projections to the thalamus en route to the somatosensory cortex to modulate affective arousal (Craig et al. 1994; Craig 2002, 2015). These spinothalamic projections also have extensive collaterals that terminate predominantly in the lateral parabrachial nucleus (LPBN) (Cechetto et al. 1985; Hylden et al. 1989; Feil and Herbert 1995; Gauriau and Bernard 2002; Nakamura and Morrison 2008; Kayalioglu 2009; Nakamura and Morrison 2010) and adjacent Kölliker-Fuse nucleus (KFN) in the parabrachial complex in dorsolateral pons (Cechetto et al. 1985; Feil and Herbert 1995), both of which are known to play an important role in modulating breathing (Song et al. 2006, 2015; Song and Poon 2009a, b; Dutschmann and Dick 2012). Neurons in the LPBN have been shown to mediate the excitatory effect of cutaneous nociception on inspiratory motor activity in anesthetized rats (Jiang et al. 2004).

Because somatosensory/viscerosensory receptors are generally presumed to be quiescent until provoked, the functional significance of 5-HT_{1A}R-dependent gating of interoceptive inputs in spinal dorsal horn has been largely ignored in the literature except as a possible pharmacological target for the development of new therapies for pain relief (Colpaert 2006). Most previous studies have ascribed the complex excitatory-inhibitory effects of systemic 5-HT_{1A}R agonists on breathing exclusively to their actions in brainstem respiratory-related networks (Lalley et al. 1994; Wilken et al. 1997; Sahibzada et al. 2000; El-Khatib et al. 2003; Teng et al. 2003; Choi et al. 2005; Abdala et al. 2010; Zhuang et al. 2012; Corcoran et al. 2014). However, conflicting evidences reported in the literature have led to the hypothesis that the excitatory effect of 5-HT_{1A}R activation on breathing may be attributed in part to disfacilitation of a latent 5-HT_{1A}R-gated spinal afferent pathway which normally exerts a paradoxical tonic inhibitory effect on breathing even in the quiescent state without explicit sensory provocations (Poon 2009). Here, we employed small-interfering RNA (siRNA) technology to unequivocally verify the respiratory-inhibiting effect of a hitherto unrecognized 5-HT_{1A}R-gated spinal afferent pathway and to localize the site of action to 5-HT_{1A}Rs in lumbar spinal cord dorsal horn. Our results uncovered a latent somatosensory/viscerosensory mechanism which exerted tonic inhibitory influence on homeostatic regulation of breathing subliminally in the quiescent state independent from the classical chemoreflex excitatory pathways (Mulkey et al. 2004; Kumar and Prabhakar 2012).

Results

Tonic respiratory inhibition by latent 5-HT_{1A}R-gated spinal afferents

First, we tested whether respiration was influenced by 5-HT_{1A}R modulation of spinal afferents that were persistently active in the quiescent state unprovoked (i.e., without any acute somatosensory or viscerosensory stimuli being applied) in anesthetized rats. To minimize possible interference of phrenic motor neurons directly or indirectly through local spinal 5-HT_{1A}R-dependent reflex in cervical spinal cord (Zimmer and Goshgarian 2006), we kept all spinal administrations of 5-HT_{1A}R agonist and antagonist at the lumbar level where 5-HT_{1A}R expression is reportedly the highest especially in the dorsal horn and rarely in the ventral horn (Marlier et al. 1991; Thor et al. 1993). Post-experimental Sky Blue injection (2 %, 20 µl at the same location of drug injection with 20 min diffusion time) showed that the Sky Blue staining of spinal cord was mainly within the lumbar level and no staining could be visually observed beyond lumbar level.

Intrathecal injection of the 5-HT_{1A}R agonist 8-OH-DPAT [(*R*)-(+)-8-Hydroxy-DPAT, 0.5 µg/µl, 20 µl] into the rat spinal cord subarachnoid space at lumbar level caused pronounced respiratory augmentation (Fig. 1a). At ~3 min after injection, inspiratory amplitude (as measured by integrated phrenic activity, $\int$phr) was increased by 57.8 ± 7.9 % (mean ± SE; $p < 0.001$ two-tailed paired *t* test, $n = 11$) and respiratory frequency by 26.6 ± 10.1 % ($p < 0.05$), whereas inspiratory and expiratory durations were decreased by 14.1 ± 7.1 % ($p < 0.05$) and 19.2 ± 3.6 % (both $p < 0.001$), respectively (Fig. 1b). As a result, inspiratory drive (inspiratory amplitude/inspiratory duration) and neural ventilatory output (inspiratory amplitude × respiratory frequency) increased markedly by 102.0 ± 14.1 and 103.0 ± 23.0 %, respectively (Fig. 1c). These respiratory augmentation effects remained stable for >30 min after lumbar-intrathecal 8-OH-DPAT injection while the animal remained anesthetized and unprovoked. Conversely, lumbar-intrathecal injection of the 5-HT_{1A}R antagonist WAY-100635 (2 µg/µl, 20 µl) caused respiratory depression (Fig. 1b). At ~8 min after injection, inspiratory amplitude was reduced by 21.2 ± 2.5 % and inspiratory duration lengthened by 10.4 ± 2.9 % (both $p < 0.001$, $n = 7$) although changes in expiratory duration and respiratory frequency were not statistically significant (both $p > 0.1$, Fig. 1b). Consequently, inspiratory drive and neural ventilatory output showed significant decreases by 27.3 ± 2.9 and 24.8 ± 4.2 %, respectively (both $p < 0.001$, Fig. 1c). The effects of lumbar-intrathecal 8-OH-DPAT and WAY-100635 were without explicit provocation of any somatosensory/viscerosensory stimuli.

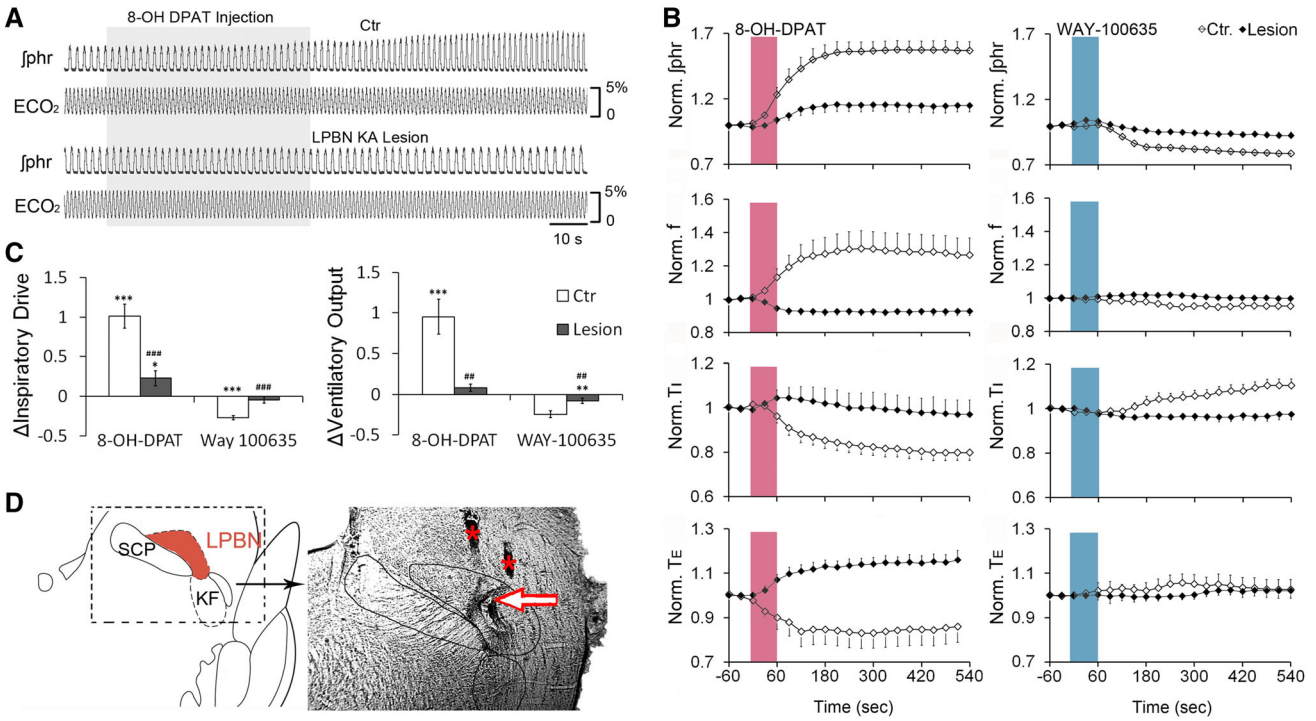

Fig. 1 A 5-HT_{1A}R-gated spinal-LPBN pathway mediating somatosensory/viscerosensory modulation of respiration in anesthetized rats without sensory provocations. **a** Phrenic motor activity at constant end-tidal PCO_2 (ECO_2) before and after intrathecal administration of the 5-HT_{1A}R agonist 8-OH-DPAT at lumbar level in control rat (*upper*) and LPBN kainic acid-lesioned rat (*lower*). **b** Normalized group data showing the effects of lumbar-intrathecal administrations of 8-OH-DPAT and the 5-HT_{1A}R antagonist WAY-100635 on respiratory pattern before and after LPBN lesion. *Amp* amplitude of $\int$ phr, *f* respiratory frequency, T_E expiratory duration, T_I inspiratory duration. **c** Corresponding effects on inspiratory drive (Amp/T_I) and neural ventilatory output (Amp × *f*). **d** Histological section showing the center (*red arrow*) of kainic acid microinjection in LPBN in one representative animal. *Red asterisks* indicate artifacts (which are of no consequence to the study). *KF* Kölliker-Fuse nucleus, *LPBN* lateral parabrachial nucleus, *SCP* superior cerebellar peduncle

Table 1 Effects of systemic 5-HT_{1A}R agonist followed by systemic or intrathecal antagonist on respiration

	8-OH-DPAT i.v. ($n = 8$) (%)	WAY-100635 i.v. ($n = 3$) (%)	WAY-100635 intrathecal ($n = 5$) (%)
Norm. $\int$ phr	45.5 ± 6.0*	−5 ± 11	9.2 ± 3.5*
Norm. T_I	−14.8 ± 2.3*	−7.5 ± 8.3	−7.9 ± 6.9
Norm. T_E	14.5 ± 4.8*	10.9 ± 4.0	10.0 ± 7.5

Antagonists were applied at 5 min post-intravenous agonist injection. All values are percentage changes over pre-injection baseline levels

* $p < 0.05$ vs. pre-injection baseline

As shown in Table 1, intravenous administration of 8-OH-DPAT also elicited an augmentation of inspiratory amplitude (by 45.5 ± 6.0 %) and shortening of inspiratory duration (by −14.8 ± 2.3 %). However, these effects were accompanied by a prolongation of expiratory duration (by 14.5 ± 4.8 %) unlike lumbar-intrathecal 8-OH-DPAT, which induced a shortening of expiratory duration (Fig. 1b). The effects of intravenous 8-OH-DPAT on respiration were attenuated or abolished by subsequent WAY-100635 administration (5-min post-intravenous agonist injection), either intravenously or intrathecally at the lumbar level (Table 1). Thus, the respiratory effects of intravenous 8-OH-DPAT were ascribable largely (but not totally) to activation of spinal 5-HT_{1A}Rs.

Lateral parabrachial nucleus mediation of spinal 5-HT_{1A}R disinhibition of respiration

Next, we sought to identify the brainstem sites potentially mediating the observed lumbar-spinal 5-HT_{1A}R

Table 2 Baseline respiratory patterns before and after pontine microinjections

	Before	After
LPBN KA ($n = 9$)		
Amp	5.42 ± 0.44	6.01 ± 0.56
f (min^{-1})	40.2 ± 2.2	45.2 ± 4.2
T_E (s)	1.03 ± 0.08	0.88 ± 0.1
T_I (s)	0.52 ± 0.03	0.52 ± 0.07
KFN AP5 ($n = 6$)		
Amp	8.6 ± 0.3	6.5 ± 0.5*
f (min^{-1})	36.4 ± 2.6	28.7 ± 3.0*
T_E (s)	1.13 ± 0.11	1.08 ± 0.1
T_I (s)	0.60 ± 0.06	0.85 ± 0.07*

Data are mean ± SE of 1-min recordings

KA kainic acid

* $p < 0.05$ (two-tailed paired t test)

disinhibition of respiration. In animals subjected to unilateral lesion of LPBN with kainic acid microinjection (Fig. 1d), no significant changes in respiratory parameters were observed 30 min after lesion (Table 2), as previously reported (Song and Poon 2009a). However, LPBN lesion reversed the expiratory duration and respiratory frequency responses to lumbar-intrathecal injection of 8-OH-DPAT (Fig. 1a, b), increasing (instead of decreasing) expiratory duration by 15.9 ± 4.0 % and decreasing (instead of increasing) respiratory frequency by 9.0 ± 2.6 % at peak ($p < 0.01$ and $p < 0.05$, respectively, $n = 8$). After LPBN lesion, lumbar-intrathecal 8-OH-DPAT injection caused much smaller (albeit significant) increase in inspiratory amplitude (22.7 ± 9.3 %, $p < 0.05$) while the shortening of inspiratory duration was all but abolished ($p > 0.1$). Similarly, the effects of WAY-100635 on inspiratory amplitude and inspiratory duration were significantly reduced after LPBN lesion (both $p < 0.05$, $n = 7$; Fig. 1b). Consequently, the responses in inspiratory drive and neural ventilatory output to 8-OH-DPAT and WAY-100635 were markedly suppressed after unilateral LPBN lesion (all $p < 0.001$; Fig. 1c), indicating that integrity of the LPBN was required in mediating the effects of intrathecal 8-OH-DPAT and WAY-100635 on respiration.

siRNA knockdown of lumbar-spinal dorsal horn 5-HT$_{1A}$Rs

Pharmacological modulation of spinal 5-HT$_{1A}$R activity suffers from potential confounds such as limited selectivity and durability of the injected agonist and antagonist as well as their possible diffusion from the lumbar spinal cord to respiratory-related circuits in the cervical spinal cord or supraspinal structures. To circumvent these potential drawbacks, small-interfering RNA (siRNA; Invitrogen^{TM}) with high target specificity for 5HT$_{1A}$R and minimum in vivo immune response or its scrambled negative control was injected to rat spinal cord between L4 and L5 under pentobarbital anesthesia. Previous studies have shown that in vivo siRNA knock-down of receptor or protein expressions typically peaks at 7–14 days post-injection, and disappears after 21 days post-injection (Song et al. 2003; Tan et al. 2005). Accordingly, in this study all tests were performed on the 14th day post-injection.

Fourteen days post-injection, baseline respiration in 5-HT$_{1A}$R knockdown rats was depressed (i.e., more inhibited) compared with negative controls mainly due to a significant decrease in respiratory frequency secondary to a prolongation of expiratory duration (both $p < 0.05$, Fig. 2a). As in normal animals, scrambled negative control rats showed pronounced increases in inspiratory amplitude and respiratory frequency with significant shortening of expiratory and inspiratory durations in response to lumbar-intrathecal 8-OH-DPAT injection (all $p < 0.01$ at ~8 min, $n = 8$; Fig. 2b). Corresponding responses were greatly attenuated or abolished in 5-HT$_{1A}$R knockdown rats compared with negative controls (Fig. 2b, $p < 0.01$, $n = 8$).

Immunohistological examination post-experiment revealed that the level of 5-HT$_{1A}$R expression in lumbar spinal cord was markedly reduced (by >60 %) in 5-HT$_{1A}$R knockdown rats ($n = 8$) compared with negative controls ($n = 8$)—the latter showing the highest density of 5-HT$_{1A}$R immunopositive staining in the superficial layers (laminae I and II) of dorsal horn and much lower in the ventral horn (Fig. 2c). 5-HT$_{1A}$R expression at the cervical level was not affected by lumbar siRNA injection (Fig. 2c). These data confirm that the respiratory disinhibition effects of lumbar-intrathecal 8-OH-DPAT injection were mediated by activation of 5-HT$_{1A}$Rs localized primarily in the superficial layers of lumbar-spinal dorsal horn independent of cervical spinal and supraspinal 5-HT$_{1A}$Rs.

Distinct spinal/supraspinal 5-HT$_{1A}$R modulation of respiration

Previous studies have shown that systemic 5-HT$_{1A}$R agonists can reverse the apneustic disturbances (i.e., pronounced prolongation of inspiratory duration) resulting from a variety of respiratory defects in patients and animal models (Wilken et al. 1997), presumably via 5-HT$_{1A}$R-mediated enhancement of glycinergic inhibition in brainstem respiratory networks (Manzke et al. 2009, 2010). Consistent with this view, we found that microinjection of the specific NMDA (*N*-Methyl-D-aspartate) receptor antagonist AP5 (20 mM) into the KFN unilaterally induced an apneustic pattern (Fig. 3a; Table 2) (Song et al. 2015) that was completely reversed by intravenous administration of 8-OH-DPAT (20 μg/kg)

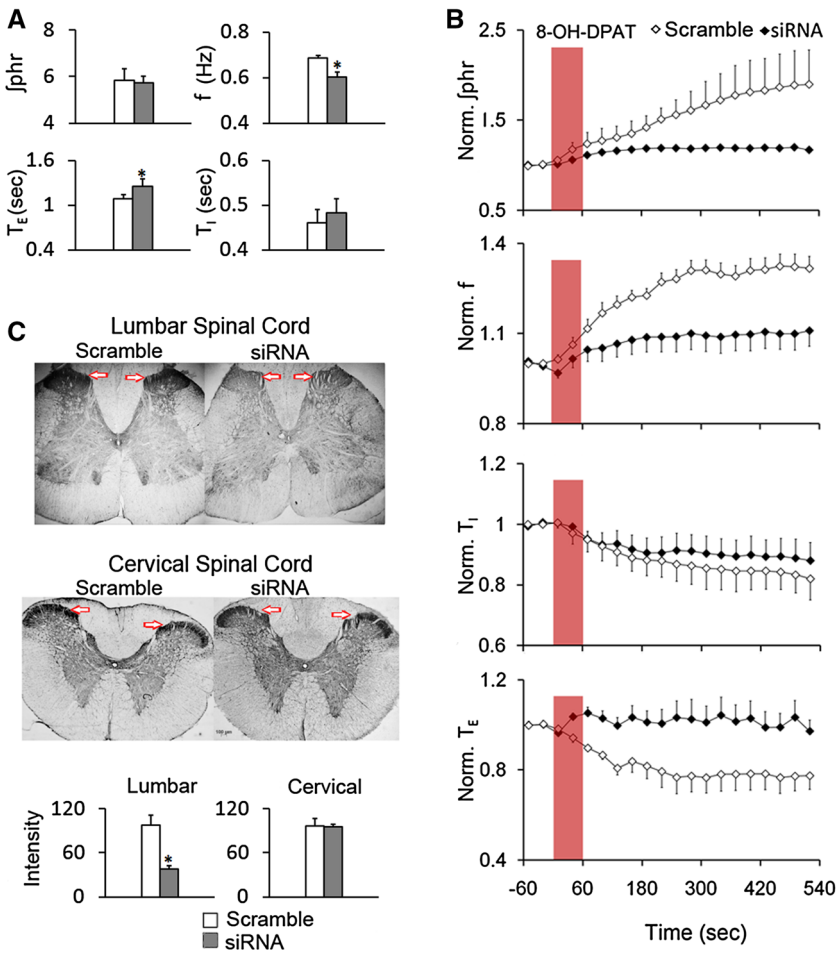

Fig. 2 Effects of siRNA knockdown of 5-HT_{1A}Rs in lumbar spinal cord on respiration. **a** Baseline respiratory patterns of 5-HT_{1A}R knockdown rats vs. scrambled negative controls. **b** Normalized group data showing the respiratory responses to lumbar-intrathecal 8-OH-DPAT injection in 5-HT_{1A}R knockdown rats vs. scrambled negative controls. (Note that in the *top panel* (Norm. $\int$phr) the *error bars* for the siRNA group are too small to be visible). **c** 5-HT_{1A}R expression in lumbar and cervical spinal cord of 5-HT_{1A}R knockdown rat vs. scrambled negative control 14 days post-siRNA injection at the lumbar spinal cord. *Red arrows* indicate 5-HT_{1A}R expression in superficial layers of dorsal horns. *Bar graphs at bottom* show relative intensity of 5-HT_{1A}R expression (in pixels/sq. in) in the dorsal horn of lumbar vs. cervical spinal cords of 5-HT_{1A}R knockdown rats vs. scrambled negative controls

($n = 3, p < 0.05$; Fig. 3a, c). In contrast, lumbar-intrathecal injection of 8-OH-DPAT had no effect on the apneustic pattern ($p > 0.1$, Fig. 3b). These data confirm that the anti-apneustic effect of 8-OH-DPAT was mediated predominantly by activation of 5-HT_{1A}Rs in supraspinal respiratory networks rather than spinal dorsal horn.

Kölliker-Fuse nucleus mediation of spinal 5-HT_{1A}R disinhibition of respiration

After microinjection of AP5 into KFN, 8-OH-DPAT whether administered intrathecally or intravenously no longer elicited a disinhibition of respiration (Fig. 3b, c). These data indicate that the KFN also contributed importantly to the lumbar-spinal 5-HT_{1A}R modulation of respiration independently from the LPBN.

Discussion

The present results provide the first direct experimental evidence revealing a latent (i.e., persistently active in the quiescent state without explicit sensory provocation) 5-HT_{1A}R-gated spinal afferent pathway (or collection of pathways) which exerts profound tonic inhibitory influence on respiration, as conjectured previously (Poon 2009). Our results with lumbar-intrathecal 8-OH-DPAT injection after siRNA knockdown of 5-HT_{1A}R unequivocally localized the site of action of 8-OH-DPAT to (pre- and/or postsynaptic) 5-HT_{1A}Rs in the superficial layers of spinal dorsal horn at the lumbar level and not cervical level or higher. Hence, potential confounds associated with the activation of presynaptic or postsynaptic 5-HT_{1A}Rs in cervical spinal cord or brainstem respiratory-related circuits were eliminated, although possible contribution of other receptor subtypes cannot be excluded. Because only a very low dosage of 8-OH-DPAT or WAY-100635 was injected intrathecally at the lumbar level, any resultant changes in blood pressure and their effects on breathing should be minimal. This latent 5-HT_{1A}R-gated respiratory-inhibiting spinal afferent pathway signifies a novel somatosensory/viscerosensory mechanism which contributes importantly to the homeostatic regulation of breathing in the quiescent state independent of the classical chemoreflex excitatory pathways (Mulkey et al. 2004; Kumar and Prabhakar 2012).

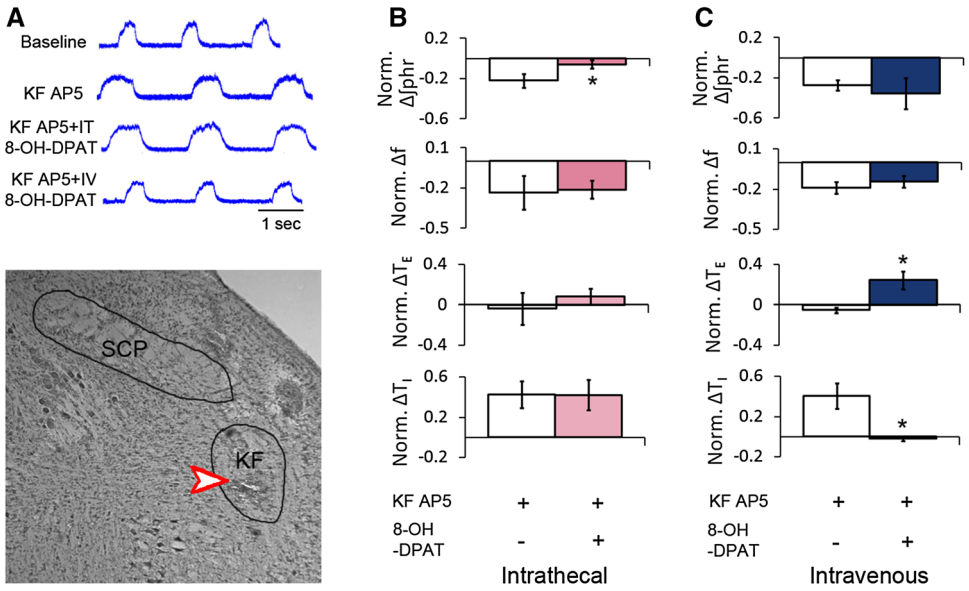

Fig. 3 A 5-HT$_{1A}$R-gated spinal-KFN pathway mediating somatosensory/viscerosensory modulation of respiration in anesthetized rats without sensory provocations. **a** *Top panel*: examples of phrenic motor activity in control rat and after KFN AP5 injection, lumbar-intrathecal injection and intravenous injection 8-OH-DPAT. *Bottom panel*: *red arrowhead* indicates the center of AP5 microinjection in KFN. **b** Normalized group data showing the effects of lumbar-intrathecal 8-OH-DPAT injection on respiratory pattern after KFN AP5 microinjection. **c** Corresponding effects of intravenous 8-OH-DPAT injection

Suppression of lumbar-spinal 5-HT$_{1A}$Rs with chronic siRNA knockdown or acute antagonism with WAY-100635 both resulted in respiratory depression. However, the respiratory depression in 5-HT$_{1A}$R knockdown rats was manifested as a decrease in respiratory frequency secondary to a prolongation of expiratory duration instead of a decrease in inspiratory amplitude and prolongation of inspiratory duration that were seen after lumbar-intrathecal WAY-100635 injection in normal rats. Possible causes for such subtle discrepancy include the limited specificity of WAY-100635 and limited potency of siRNA, or that the siRNA-treated rats adapted their respiratory pattern in compensation for the effects of the 5-HT$_{1A}$R knockdown over the 14 days incubation period.

It is presently unclear which specific somatosensory/viscerosensory modalities underlie this latent inhibitory effect on respiration. Certain modalities such as pain afferents (Jiang et al. 2004) and small-fiber (group III–IV) muscle mechanosensitive or metabosensitive afferents (Poon and Song 2015) may be ruled out in this regard because of their known excitatory (instead of inhibitory) effects on breathing. Similarly, large-fiber (group I and II) proprioceptive afferents from limb muscles and joints [mainly via the spinal dorsal column (Kayalioglu 2009; Craig 2015) instead of dorsal horn] may also be ruled out because of their reported excitatory entrainment effects on breathing (Potts et al. 2005; Giraudin et al. 2012). A spinoparabrachial thermoregulatory pathway has been previously proposed as a possible candidate that potentially contributes to a spinal 5-HT$_{1A}$R-gated respiratory inhibition (Poon 2009). However, although cutaneous warm and cold stimuli have been shown to elicit a powerful defensive autonomic response via this spinoparabrachial pathway (Nakamura and Morrison 2008, 2010), there is currently no evidence that these cutaneous thermosensory inputs exert a tonic inhibitory effect on breathing particularly in the quiescent state unprovoked. Indeed, cutaneous temperature sensors such as the family of transient receptor potential (TRP) channels are known to also serve as nociceptors (Clapham 2003), the activation of which has been shown to stimulate instead of inhibit breathing (Jiang et al. 2004). Similar considerations may also be applicable to spinoparabrachial mediation of bone nociception (Williams and Ivanusic 2008). Besides these somatosensory modalities, little is currently known about a diverse array of spinal afferents mediating viscerosensory information and their effects on control of breathing (Bernard et al. 1994; Cervero and Laird 1999). Further studies are needed to pinpoint the specific somatosensory/viscerosensory mechanisms underlying the latent spinal 5-HT$_{1A}$R-gated respiratory-inhibiting effect revealed by the present study.

In the rat, neurons in the superficial dorsal horn of lumbar spinal segments project primarily to the dorsal subdivision of LPBN and to a lesser extent the central subdivision (Feil and Herbert 1995). Although the loci of our kainic acid microinjections were localized mainly to

the central subdivision of LPBN (as verified by post-experimental histological examination Fig. 1d), the dosage of injection (50 nl at a concentration of 1 μg/μl) was such that a major part of the LPBN (including its dorsal subdivision) located within 0.5–1.0 mm around the center of injection would have been destroyed (Mizusawa et al. 1995; Song and Poon 2009a, b). Hence, the respiratory effects of LPBN lesion elicited by lumbar-intrathecal injection of 8-OH-DPAT or WAY-100635 are ascribable to the interruption of a 5-HT_{1A}R-gated lumbar spinoparabrachial pathway, as previously conjectured (Poon 2009). Interestingly, blockade of NMDA receptors at KFN was similarly effective as LPBN lesion in reversing the respiratory-disinhibiting effect of 8-OH-DPAT. In the rat, KFN does not receive projections from lumbar superficial dorsal horn neurons but instead receives projections from those at the cervical levels (Feil and Herbert 1995). However, KFN receives projections from LPBN (Yoshida et al. 1997) that are targets of the 5-HT_{1A}R-gated lumbar spinoparabrachial pathway mediating the latent respiratory-inhibiting afferents. Blockade of NMDA receptors at KFN may therefore indirectly disrupt the respiratory-inhibiting effect of the lumbar spinoparabrachial pathway by suppressing certain NMDA receptor-dependent neurons [such as post-inspiratory driver neurons (Song et al. 2015)] in KFN that are the downstream relaying neurons of LPBN.

LPBN neurons may be either excited or inhibited by somatosensory/viscerosensory stimuli (Bernard et al. 1994). Future studies should verify whether this 5-HT_{1A}R-gated lumbar spinoparabrachial respiratory-inhibiting pathway is glutamatergic (impinging on a respiratory-inhibiting network in LPBN-KFN) or GABAergic/glycinergic (impinging on a respiratory-exciting network). In addition, somatic afferents (e.g., those from skeletal muscles) may also be relayed by spinal lamina I neurons directly to the medullary respiratory circuits (Wilson et al. 2002). Whether this pathway is also subject to 5-HT_{1A}R-gating remains to be studied.

Our results support the notion that the anti-apneustic effect of systemic 5-HT_{1A}R activation is mediated by the action of 5-HT_{1A}R in supraspinal rather than spinal respiratory networks. In the present study, apneusis-like breathing was induced in vagotomized rats by NMDA receptor blockade at KFN, a procedure which has been shown to suppress post-inspiratory neural activity and abolish the post-inspiratory phase of the respiratory rhythm (Song et al. 2015). Our results suggest that activation of supraspinal 5-HT_{1A}Rs reversed the apneusis and restored normal inspiratory–expiratory phase transition by activating brainstem respiratory-related circuits that are independent from post-inspiratory activity and spinoparabrachial afferent activity.

In conclusion, we have identified a latent 5-HT_{1A}R-gated spinal afferent pathway (or collection of afferent pathways) which exerted powerful tonic inhibition on respiration subliminally without any explicit sensory provocations. Activation of 5-HT_{1A}Rs in spinal dorsal horn at the lumbar level or suppression of the neural circuits in LPBN or KFN in dorsolateral pons shut down this latent pathway thereby eliciting pronounced respiratory disinhibition. The remarkable potency of this latent 5-HT_{1A}R-gated spinoparabrachial afferent pathway suggests that homeostatic regulation of breathing may be continually modulated subliminally by certain tonic somatosensory and/or viscerosensory respiratory-inhibiting inputs independent from the classical chemoreflex excitatory pathways. Further studies are needed to investigate whether these latent somatosensory/viscerosensory respiratory-inhibiting inputs are also active in the unanesthetized state and exert tonic modulation of affective arousal via the spinothalamic pathway in behaving animals. These findings are of potential translational significance in light of the increasing proposed use of 5-HT_{1A}R-based therapeutics for a wide range of neurological and respiratory disorders and chronic pain.

Materials and methods

Animal preparation

Experiments were performed on 35 Sprague–Dawley male rats (330–380 g Charles River Laboratories, Wilmington, MA). Experimental protocols were as reviewed and approved by the M.I.T. Committee on Animal Care in accordance with published guidelines. Rats were anesthetized with urethane at an initial dose of 1.5 g/kg (i.p.). Supplemental dose (1/10 of the initial dose) was given when a noxious stimulus (clamp at hind paw) caused withdrawal response, changes in respiratory rhythm and heart rate. Other animal procedures were as previously described (Song and Poon 2009a). Rats were paralyzed with pancuronium bromide (Hospira Inc. Lake Forest, IL; initial dose 0.5 mg, i.v., supplemented every hour at 0.1 mg, i.v.) and ventilated with hyperoxic medical air (O_2 enriched to 40 %) by using a CWE AVS-1 ventilator (CWE, Ardmore, PA). A respiratory gas analyzer (CWE Gemini) was used to monitor end-tidal O_2 and CO_2 levels. Throughout the experiment the end-tidal CO_2 level was maintained at 5.0 ± 0.2 % (38 ± 1.5 mmHg), which was the CO_2-recruitment threshold (Boden et al. 1998) plus 1.0 %. Body temperature was kept at 36.5 ± 0.2 °C with a temperature controller (CWE, TC-831). During the experiment, the depth of anesthesia was checked every 15 min. Whenever a noxious stimulus (clamping the hind

paw) caused changes in pupil size, respiration and heart rate or elicited a withdrawal reflex, a supplementary dose of urethane (1/10 original dosage) was given intravenously to maintain adequate anesthesia. The right phrenic nerve and both vagus nerves were isolated and severed at the cervical level from ventral approach. The head of the rat was then fixed in a stereotaxic frame (KOPF 1430, David Kopf Instruments, Tujunga, CA) in a tilted position (with Bregma 1.5 mm higher than Lambda) with the dorsolateral pons being readily accessible from a vertical dorsal approach.

Electrophysiological recording

To record phrenic discharge, the isolated right phrenic nerve was exposed from dorsal approach and mounted on a parallel bipolar platinum wire electrode. The raw phrenic discharge signal (Phr) was amplified (CyberAmp 380, Axon Instruments, Union City) and sampled (at 10 kHz) into a Dell PC with LabView (National Instruments, Austin, TX). In most experiments, the phrenic discharge was integrated with an analog Paynter filter (time constant 15 ms).

Intrathecal injection

Procedures for intrathecal injection were as described in (Yaksh and Rudy 1976). Briefly, the atlanto-occipital membrane was exposed by retracting the superficial neck muscles and removing overlying soft tissue. Then a small puncture was made in the membrane. A thin sterilized polyethylene tube (PE-10) filled with ACSF was inserted into the spinal subarachnoid space through this small puncture and advanced caudally. Once the tube was inserted to a pre-determined depth (lumbar spinal cord at vertebral level T9, 60 mm from atlanto-occipital foramen), the neck muscles and skin were sutured. At the end of the experiment, the animal's spinal cord was exposed to check the actual location of tube. If the spinal cord was damaged by the tube, or the tube was misplaced to a wrong spinal segment, related data were abandoned. Injections of 8-OH-DPAT or WAY-100635 (Sigma-Aldrich, St. Louis, MO; dissolved in ACSF at 0.5 and 2 μg/μl, respectively) were performed using a Hamilton microsyringe at a speed of 1 μl/3 s. The total volume of injection for each drug was 20 μl. ACSF was used for control injection. To verify the extent of diffusion of the injection, at the end of the experiment 20 μl of a Sky Blue solution (2 %, dissolved in ACSF) was injected to the same spinal cord segment. 20 min after injection of the Sky Blue solution, the animal was euthanized with urethane overdose (2 g/kg, i.v.) and the spinal cord immediately removed to examine the Sky Blue staining of spinal cord.

Pontine microinjection

Chemical lesion of the LPBN was made unilaterally using a glass micropipette (tip diameter 15–30 μm) filled with kainic acid solution (Sigma-Aldrich; 1 μg/μl in ACSF). Stereotaxic coordinates of the LPBN were −0.12 mm (caudal) to 0.24 mm (rostral) from interaural level, 2.3–2.6 mm lateral from midline, and 7–7.5 mm below lambda surface (Paxinos and Watson 2007). Microinjection was made at loci where electrical stimulation caused increase of respiratory frequency and inspiratory amplitude (Lara et al. 1994). A total volume of 50 nl was injected by applying multiple pressure pulses to the micropipette using a BH2 microinjector (Harvard Apparatus, Holliston, MA).

AP5 (Sigma-Aldrich; 20 mM in ACSF) was injected bilaterally into KFN with the same method as described above for kainic acid microinjection. The stereotaxic coordinates of the KFN were 2.4–2.8 mm lateral from midline, −0.12 mm (caudal) to 0.36 mm (rostral) from interaural level, vertical depth at 7.4–8 mm from lambda surface (Paxinos and Watson 2007). Injection was made at loci where electrical stimulation caused inspiratory inhibition (Song et al. 2015, BSF).

For all microinjections, a small lesion was made at the injection site at the end of the experiment by passing anodal D.C. current (100 μA for 60 s) through the injection pipette. The rat was then euthanized with urethane overdose (2 g/kg, i.v.) and perfused with 4 % paraformaldehyde. The brainstem was removed and cut into 100-μm sections for histological examination of the injection loci (Fig. 1). Animals with inadvertent misplaced microinjections (outside of LPBN or KFN) were excluded from analysis.

siRNA knockdown of spinal 5-HT_{1A}Rs and immunohistochemistry

5-HT_{1A}R Stealth™ in vivo siRNA 5′-ACGUGACCUUCAGCUACCAAGUGAU and its scrambled negative control (Medium GC Duplex, Cat. No. 12935-300, Invitrogen™) were dissolved in 5 % glucose solution with RNase-free water at a concentration of 1 μg/μl as stock solution. One microgram of siRNA was mixed with 0.18 μl of polyethyleneimine (PEI, Fermentas). Totally 5 μg siRNA in 20 μl was intrathecally delivered by bolus injection after a puncture was made by a 27G needle between vertebral levels T9 and T10 (corresponding to spinal cord L4 and L5 segments) under pentobarbital anesthesia. Because siRNA effect typically peaks at 7th to 14th day post-injection (Song et al. 2003; Tan et al. 2005), rats were studied 14 days post-injection under urethane anesthesia as described above for normal rats (not injected with siRNA or scrambled control).

Immunomicroscopy

After the siRNA knockdown experiment, rats were euthanized with urethane overdose (2 g/kg, i.v.) and transcardially perfused with PBS followed by 4 % paraformaldehyde (dissolved in PBS). 1 cm of cervical spinal cord was taken out and post-fixed in 4 % paraformaldehyde. 40-μm frozen sections were cut with frozen microtome and the sections were incubated in primary rabbit anti-5-HT_{1A} antibody (EMD Millipore Cat# AB15350 RRID:AB_805421) at 1:500 dilution for 48 h at 4 °C, rinsed, incubated in biotinated secondary antibody and visualized with standard ABC-DAB method. All spinal sections were processed together under identical conditions. Quantitative analysis of immunohistochemistry positive staining intensity was done with National Institutes of Health ImageJ image processing and analysis software.

Data analyses

The amplitude of integrated phrenic discharge ($\int$phr), inspiratory and expiratory durations (T_I, T_E) and respiratory frequency (f) were measured for each respiratory cycle. All measured values were normalized against corresponding pre-test baseline values in control or post-injection conditions, and averaged every 10 s and expressed as mean ± SE. Student t test and ANOVA (one-way or two-way ANOVA with repeated measures followed by Tukey post hoc analysis) were used to determine statistical significance. A confidence level of 95 % was used.

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