**TASK1 and TASK3 in orexin neuron of lateral hypothalamus contribute to respiratory chemoreflex by projecting to nucleus tractus solitarius**

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**Abstract**

TWIK-related acid-sensitive potassium channels (TASKs)-like current was recorded in orexin neurons in the lateral hypothalamus (LH), which are essential in respiratory chemoreflex. However, the specific mechanism responsible for the pH-sensitivity remains elusive. Thus, we hypothesized that TASKs contribute to respiratory chemoreflex. In the present study, we found that TASK1 and TASK3 were expressed in orexin neurons. Blocking TASKs or microinjecting acid artificial cerebrospinal fluid (ACSF) in the LH stimulated breathing. In contrast, alkaline ACSF inhibited breathing, which was attenuated by blocking TASK1. Damage of orexin neurons attenuated the stimulatory effect on respiration caused by microinjection of acid ACSF (at a pH of 6.5) or TASKs antagonists. The orexinA-positive fiber and orexin type 1 receptor (OX1R) neurons were located in the nucleus tractus solitarius (NTS). The exciting effect of acidosis in the LH on respiration was inhibited by blocking OX1R of the NTS. Taken together, we conclude that orexin neurons sense the extracellular pH change through TASKs and regulate respiration by projecting to the NTS.

**KEYWORDS**

chemoreflex, lateral hypothalamus, nucleus tractus solitarius, orexin, TASK1, TASK3

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Abbreviations: ACSF, acid artificial cerebrospinal fluid; ASIC1, acid-sensing ion channel 1; iPND, the integrating value of phrenic nerve discharge; LC, locus coeruleus; LH, lateral hypothalamus; NTS, nucleus tractus solitarius; OX1R, orexin type 1 receptor; RTN, retrotapezoid nucleus; TASKs, TWIK-related acid-sensitive potassium channels; VLM, ventrolateral medulla.

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Background K⁺ channels, characterized by a leak K⁺ currents, play a critical role in regulating the resting membrane potential.¹ They are formed by the two-pore domain potassium (K2P) channel subunits also called TWIK-related subunits.² In neurons, many endogenous neurochemicals and physicochemical factors allows K2P channels to control the membrane potential, and thereby regulate the electrical excitability. Among K2P channels, TWIK-related acid-sensitive potassium channels (TASKs) including three subunits (TASK1, TASK2, and TASK3) are sensitive to pH change in the physiological condition.³ Homo- or heterodimers of TASK1 and TASK3 channels are widely distributed in nervous system.⁴ They are modulated by relatively small deviations of extracellular pH in the physiological environment. Extracellular acidification inhibits TASKs activity and enhances neurons excitability, while alkalinization decreases the excitability of TASKs-positive neurons. The pH sensitivities are used to differentiate TASK1 from TASK3.⁵ The pK value of TASK1 ranges from pH 7.3 to 7.5 and that of TASK3 ranges from pH 6.5 to 6.7.⁶ Our previous study demonstrated that TASK1 and TASK3 are coexpressed with acid-sensing ion channel 1 (ASIC1) in ventrolateral medulla (VLM) and contribute to the central regulation of breathing by coordinating with each other to perceive local pH change.⁶ However, the role of TASKs in the respiratory chemoreflex remains to be explored.

It has been reported that chemoreceptors exist in many brain areas including raphe, retrotrapezoid nucleus (RTN), nucleus tractus solitarius (NTS), locus coeruleus (LC), and lateral hypothalamus (LH).⁶ Chemoreceptors sense changes of H⁺ concentration in cerebrospinal fluid (CSF) and local extracellular fluid to regulate the respiratory acid-base homeostasis. The physiological stimulation of the central chemoreceptor is H⁺ in the CSF and local extracellular fluid. It was reported that orexin neurons in the LH were pH-sensitive and contribute to in regulation of breathing.⁷ Central respiratory chemoreception is established early in development as central chemoreceptors appear to be present within days after birth.⁸,⁹ It has been reported that elevated PaCO₂ increases the fetal breathing. Therefore, the change of TASKs expression in the LH from neonate to adult may reflect the development of respiratory chemoreception in rats.

Orexins are neuropeptides synthesized from neurons localized in the LH and perifornical region.¹⁰ Orexin neurons in the LH directly project to respiratory centers in the brainstem such as NTS, which express orexin receptors. Injection of orexins into these respiratory centers stimulates breathing, which indicates that orexins were related to the regulation of breathing.¹¹,¹² Knockout of the orexin gene in mice reduces CO₂-induced increases in breathing by 50%.¹³ Our previous study also demonstrated that acidification of LH stimulates breathing via activation of ASIC1a on orexin neurons.⁷ Accumulated evidences indicate that TASK1 and TASK3 take an important part in chemoreception and respiratory regulation. However, the distribution of TASKs 1 and 3 in the LH and the function of them in the chemoreception of orexin neurons have not been addressed. In our present study, we found TASKs (1 and 3) expressed in the LH of rats. We then investigated the role of TASKs (1 and 3) in chemoreception of the LH. Our data showed that TASKs (1 and 3) were colocalized with orexin neurons in the LH. Microinjection of different TASKs blockers, including a nonselective antagonist bupivacaine (BUP) and a specific TASK3 antagonist ruthenium red (RR) into the LH facilitated phrenic nerve discharge (PND). In addition, microinjection of ACSF at a pH of 7.0, 6.5, and 6.0 increased integrated PND (iPND), inspiratory time (Ti), and respiratory drive. In contrast, the alkaline ACSF decreased iPND, Ti, and respiratory drive, and this effect was attenuated by a specific TASK1 antagonist AEA. Damage of orexin neurons blocked the exciting effect caused by acidification or TASKs antagonists on respiration. Inhibiting OX1R in the NTS blunted the stimulatory effect of respiration induced by acidifying LH. Our results indicated that TASK1 and TASK3 comprised chemoreceptor of orexin neurons that regulate respiratory activity by projecting to the NTS. This investigation will help to establish a new understanding of the pH-sensing mechanism of chemosensitive neurons in the LH.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Neonatal, 4-week-old and 8-week-old male Sprague Dawley (SD) rats were obtained from Shanghai Jiesijie Experimental Animal Co. Ltd. (Shanghai, China). All animals were kept in a room under a 12-h light-dark cycle, an ambient temperature maintained at a temperature of 23°C, and a relative humidity of 50% ± 10%. Food and water were given freely. The animal experiments were conducted in strict accordance with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Experimental Research, Shanghai Medical College, Fudan University.

### 2.2 | Quantitative real-time PCR

The total RNA was extracted from hypothalamus of neonatal, 4-week-old and 8-week-old SD rats using TRIZol Reagent (Invitrogen Corporation, USA). First-strand cDNA was synthesized and amplified from 0.5 µg of total RNA using ReverTra Ace qPCR RT Kit (Toyobo, Japan). Then, the mRNA levels of TASK1 and TASK3 were analyzed using SYBR Green Real-time PCR Master Mix (Toyobo, Japan) in a total volume of
20 μL according to the manufacturer’s protocol. The target gene and their primer sequences are shown in Table 1. The PCR amplification consisted of 40 cycles of denaturation (94°C, 15 seconds), annealing (60°C, 30 seconds), and extension (72°C, 30 seconds). The relative quantification of gene expression was normalized to GAPDH using $2^{-\Delta\Delta C_t}$ method.

### 2.3 Western blot

The hypothalamus of neonatal, 4-week-old and 8-week-old SD rats was dissected. Tissues were placed in liquid nitrogen immediately and kept at −80°C for further use. The samples were homogenized in RIPA lysis buffer containing phosphatase inhibitor (Roche, UK) and then, centrifuged at 12 000 $\times$ g at 4°C for 15 minutes to obtain supernatants. Protein concentration was measured by protein quantitative analysis kit (Beyotime Institute of Biotechnology, China). Total protein (20 μg) were loaded in 10% SDS-polyacrylamide gels and transferred to a PVDF membrane, the membranes were incubated with rabbit anti-TASK1 antibody, rabbit anti-TASK3 antibody (Alomone Lab, Israel), or mouse anti-GAPDH (Beyotime Institute of Biotechnology, China) with a 1:1000 dilution at 4°C overnight. The next day, membranes were incubated with anti-rabbit IgG or anti-mouse IgG (Beyotime Institute of Biotechnology, China) for 1 hours at room temperature after washing with TBST and probed by the addition of enhanced Chemiluminescent Substrate (ECL). The optical density of each blot was normalized to that of GAPDH, which represented as relative optical density.

### 2.4 Immunohistochemistry

Adult Sprague Dawley rats were anesthetized with urethane (1 g·kg$^{-1}$) and perfused through the left ventricle with normal saline followed by 4% paraformaldehyde. After perfusion, the hypothalamus and medulla were dissected, transferred to graded sucrose solution (20% and 30%) until sinking, and cut into coronal sections at 25 μm with Leica freezing microtome. Slices were washed with 0.01 M of PBS and blocked in 1% BSA for 1 hours at room temperature. After blocking, hypothalamus slices were incubated with primary antibody TASK1 or TASK3 (Alomone Lab, Israel, 1:100); medulla slides were incubated with primary antibody orexinA (Alomone Lab, Israel, 1:100) and the controls were in 0.01 M of PBS without primary antibody overnight. Then, the reaction was detected by avidin-biotin-HRP complex (ABC) immunohistochemical kit (Boshide, China). Slices were dried in the drying oven and mounted with coverslips after dehydration and transparency, then observed and taken photographs under microscope.

### 2.5 Double immunofluorescence technique

Slices were washed with 0.01 M of PBS and blocked with 5% mixture serum of donkey and goat for 1 hours at room temperature. After blocking, slices were incubated with primary antibody TASK1 or TASK3, orexinA diluted in 0.01 M of PBS (1:100) overnight. After washed, slices were incubated with fluorescent dyes-conjugated second antibodies (donkey-anti-rabbit-FITC-conjugated antibody and donkey anti-goat-CY3-conjugated antibody, 1:100, Beyotime Institute of Biotechnology, China) for 1 hours in the dark. Then, the slices were mounted in antifading medium (Beyotime Institute of Biotechnology, China), and fluorescence was detected by Zeiss LSM confocal laser system.

### 2.6 Drug application

Nonselective TASK antagonist BUP (200 μM), specific TASK3 antagonist RR (10 μM), and OX1R antagonist SB408124 (10 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA) were freshly prepared in ACSF immediately before administration. A specific TASK1 antagonist AEA (Sigma-Aldrich, St. Louis, MO, USA) was prepared in ethanol. The ACSF solutions were prepared at different pH (8.0, 7.4, 7.0, 6.5, and 6.0). ACSF containing (mM): NaCl 130, NaHCO3 26, KCl 5, CaCl2 2.6, MgSO4 1.2, NaH2PO4 1.6, glucose 11, and sucrose 10 at pH 7.4 and ethanol served as the vehicle and volume controls.

### 2.7 Phrenic nerve discharge recording

The detail method was reported in our previous publication. PND was recorded with platinum bipolar electrodes, which were amplified (filters set at 5.0 kHz) using a Polygraph System (NIHON KOKDEN) and digitized using a SMUP system (SMUP-E, Shanghai Medical College, Fudan University). The experiments were started after stabilization of the phrenic activity (about 30 minutes). The integrating value of phrenic nerve discharge (iPND) was obtained by a

| Gene name | Forward (5′-3′) | Reverse (5′-3′) |
|-----------|----------------|----------------|
| TASK1     | CGGCTTCCGCAACGTTAT | TTGTACCAGGGACGAGCA |
| TASK3     | GAGCTCAGGGAACACCTACTT | GTGTTGCACTCAGGAGGGA |
| GAPDH     | GAGAATGGGAAGCTGGTCATCAAC | ACTCCACGACATACCTGACCCAG |

**TABLE 1** PCR primer sequence
moving average of the phrenic signal. Inspiratory time (Ti) was averaged over 30 seconds. The respiratory drive was reflected by the value of iPND/Ti.

2.8 | Microinjection

Rats were held in stereotaxic frames with heads inclined forward at 45° to the level of dorsal surface of brain stem after anesthetization. A stainless steel was used to unilateral microinjection into the LH (2.6 mm posterior, 1.6 mm lateral, and 7.0 mm dorsal from bregma), the NTS (1.7 mm anterior, 2.1 mm lateral from obex, and 1.5 mm dorsal from brain surface) at a volume of 0.1 μL or the lateral ventricles (LV) (0.9 mm posterior, 1.5 mm lateral, 3.5 mm dorsal from bregma) at a volume of 0.3 μL. At the end of the experiment, 2% pontamine sky blue was microinjected into the same injection point. Brains were removed, fixed in 10% formalin solution. After 48 hours brain stem was coronally sectioned (30 μm) and stained with neutral red to determine the injection site.

2.9 | LH lesion with orexin-SAP

Rats were bilaterally microinjected with orexin-SAP or blank-SAP (0.43 ng/nL, 400 nL, Advanced Targeting Systems, San Diego, CA, USA) into the LH. The agents were delivered via a sterilized glass micropipette with a tip diameter of 20 μm, coupled to a pressure injector (Picospritzer; General Valve, Fairfield, NJ). After injection, the pipette was left in place for 5 minutes and then, withdrawn slowly. The incision was then closed by suture. Rats were administrated with antibiotic by intraperitoneal injection and allowed 14 days for lesions of orexin neurons to develop and for recovery. Then, the phrenic response was examined after microinjecting ACSF with different pH and TASKs inhibitors.

2.10 | Statistical analysis

Data are expressed as the mean ± SD. The significance of differences among the groups was evaluated using one-way or two-way ANOVA, with Tukey’s post hoc tests where appropriate. A value of \( P < .05 \) was considered statistically significant.

3 | RESULTS

3.1 | The expression of TASK1 and TASK3 in the hypothalamus of rats at different ages

The protein levels of TASK1 and TASK3 were measured by western blot (Figure 1A,B). Bands of TASK1 and TASK3 in the hypothalamus of neonatal, 4-week-old and 8-week-old rats were shown in Figure 1A. Our data showed that the protein levels of TASK1 and TASK3 of hypothalamus in 4-week-old and 8-week-old rats were decreased compared to neonatal (Figure 1B). In contrast, the RNA levels of TASK1 and TASK3 were increased as maturing (Figure 1C). These results suggest that TASK1 and TASK3 were expressed in the hypothalamus and the changes of protein levels change were the opposite to that of the mRNA levels.

3.2 | Distribution of TASK1 and TASK3 in the LH and dorsal hypothalamus area (DA) of adult rats

Both TASK1 and TASK3 were expressed in the hypothalamus. They were mainly distributed in the LH and DA with different expression patterns. TASK1- and TASK3-positive cells in the LH were more than that of the DA (Figure 2).

3.3 | The effects of TASK1 and TASK3 antagonists on respiration

To explore the effect of TASKs in the LH on the respiratory regulation, the BUP (nonselective TASK antagonist that can block both TASK1 and TASK3), RR (selective TASK3 inhibitor), and AEA (selective TASK1 inhibitor) was applied. We microinjected 0.1μL of BUP (200 μM), RR (10 μM), or AEA (100 μM) into LH to observe the consequent changes of PND and iPND. The injection spot was confirmed by histological staining (Figure 3A,B). Microinjection of BUP or RR triggered significant changes in iPND, Ti, and respiratory drive (iPND/Ti), while administration of AEA had no effect on respiratory activity (Figure 3C-E). The iPND value was increased by approximately 30%, while Ti was shortened after microinjection of BUP. Thus, the respiratory drive was also increased (Figure 3C). Additionally, RR but not AEA stimulates respiratory activity (Figure 3D,E). These results indicated that TASKs especially TASK3 are involved in maintaining resting membrane potential of neurons in the LH. Inhibition of TASKs leads to cell depolarization by decreasing K+ efflux, thus stimulating respiration.

It is known that TASKs are sensitive to changes in extracellular proton. Our previous study proved that inhibition of TASKs in the VLM by extracellular acidification excited brainstem respiratory neurons, while, activation of TASKs by alkalinization inhibited. Therefore, we microinjected ACSF at acidic pH (7.0, 6.5, and 6.0) into the LH and observed the change of respiration. Microinjection of acidic ACSF caused significant increases in iPND, Ti, and respiratory drive (Figure 3F). ACSF at a pH 6.5 and 6.0 seemed
to cause the most significant increase. TASK1 and TASK3 exhibit different pH sensitivity. TASK1 is activated strongly by pH over 7.3, whereas, TASK3 is nearly fully activated at pH 7.3, and additional alkalization has little effect on TASK3 currents. Thus, we select AEA to explore the effect of TASK1 on respiration after local alkalization. We microinjected alkaline ACSF (pH 8.0) and found that alkaline ACSF triggers decrease of iPND, Ti, and respiratory drive, which were inhibited by AEA (Figure 3G). The result indicated that TASK1 contributed to alkalization-induced inhibition of breathing.

### 3.4 Co-localization of TASK1 and TASK3 with orexinA in the LH of adult rats

Accumulated evidence indicated that orexin neurons take an important part in the respiratory chemoreflex. First, the co-expression of TASK1 and TASK3 with neurofilament was detected by double immunofluorescence. Our data showed that TASK1 and TASK3 were localized with neurons of LH. In addition, they were co-expressed in the LH (Figure 4A-I). To detect whether TASK1 and TASK3 are localized in orexin neurons, TASK1, TASK3, and orexinA cells were examined.

Our data demonstrated both TASK1 and TASK3 were localized in orexinA neurons (Figure 4J-O). Coronal diagram of rat brain was shown in Figure 4P, the red area represents the immune reactive domain.

### 3.5 Damage of orexin neurons in the LH blunted the effect of acidification, BUP, and RR on respiration

In order to investigate whether TASKs (1 and 3) in orexin neurons are involved in central regulation of respiration, we lesioned orexin neurons by administrating orexin-SAP in the bilateral LH, and observed the PND after microinjection of different drugs into the LH (Figure 5A). As results showed that 2 weeks after orexin-SAP treatment, there was a significant loss of the body weight (Figure 5C) and about 80% of orexin neurons were vanished (Figure 5B). To confirm the specific lesion effect of orexin-SAP in orexin neurons, we employed Nissl staining and immunofluorescence staining of neurofilament-H (NF-H, the neuron marker) and ionized calcium-binding adapter molecule 1 (Iba1, the microglia marker). The results indicated that microinjection of orexin-SAP leads to loss of neurons but not microglia in the LH.
FIGURE 2 Distribution of TASK1 and TASK3 in the hypothalamus of rats. A, B, TASK1-positive neurons in the dorsal hypothalamus area (DA) (a, b, c) and in the lateral hypothalamus (LH) (d, e, f). C, D, TASK3-positive neurons in the DA (g, h, i) and the LH (j, k, l). E, 1% BSA controls and the peptides absorbed antibody control. F, Group data show the numbers of TASK1- and TASK3-positive cells per visual field under microscope (200×) in the LH and DA. *P < .05, **P < .01, n = 6. Scale bar: 200 μm (a, d, g, j, E); 80 μm (b, e, h, k); and 20 μm (c, f, i, l)
WANG et al. (Supplemental Figure SI). In addition, after lesion of orexin neurons, microinjection of BUP, RR, or acidic ACSF into the LH no longer stimulated breathing (Figure 5D-G).

3.6 | Orexin neurons in the LH regulated respiration by innervating to the NTS

Though several lines of evidence demonstrated the role of orexin neurons of the LH in the control of breathing, very little is known regarding the nervous signaling pathway. Our study found that orexin type 1 receptor (OX1R) was existed in neuron of NTS and orexinA-positive nerve fiber was innervated into the NTS (Figure 6A). Moreover, administration of orexinA (10, 100, 1000 μg/mL, and 0.3 μL) into the LV stimulated respiration (Figure 6B-D). To explore the role of LH-NTS pathway in the respiratory chemoreflex, we blocked OX1R of NTS by injection SB408124 (10 μg/mL and 0.1 μL), and observed that the effect of acidizing the LH on respiration. Our results indicated that the effect of acidosis in the LH was inhibited after blocking OX1R in NTS. But blocking OX1R alone had no effect on respiration (Figure 6E-G).
further proved that orexin neuron in the LH regulate respiration by innervating NTS in medulla. Together, these findings indicate that TASKs (1 and 3) expressed in orexin neurons of the LH may participate in regulation of breathing during local acidification or alkalization under physiological condition.

4 | DISCUSSION

The central chemoreflex is essential for maintaining circulatory acid-base homeostasis by adjusting the activity of breathing. Accumulated evidence demonstrated that the LH is chemosensitive region and participates in respiratory regulation. Extracellular acidification activates ASIC1a on orexin neurons of the LH exert an excitation on respiration. In addition, TASKs are also found to be sensitive to extracellular change of pH. In contrast to the ASICs, TASKs can be inhibited by extracellular acidification and activated by alkalization. However, the expression of TASK1 and TASK3 in the hypothalamus and their exact function in central chemoreception are still under debate. In the present study, we found TASK1 and TASK3 were localized in the orexin neurons in the LH; blocking TASK3 in the LH stimulated breathing; blocking TASK1 in the LH neutralize inhibitory effect of alkalization on respiration; lesion of orexin neurons attenuated the effect of TASKs inhibitors BUP, RR, and acidification on breathing; blocking OX1R in the NTS blunted stimulated effect of respiration induced by acidifying the LH. These results indicated that TASK1 and TASK3 in the orexin neurons contribute to chemoreception of the LH.

The different developmental patterns of ventilatory response to CO₂ may relate to the development after birth. It is reported that the developmental changes in the ventilatory response to hypercapnia could be partly due to changes of either the responsiveness or the number of chemosensitive neurons. In the present study, we found that the expression of TASK1 and TASK3 in the hypothalamus varies as maturation. The mRNA level of TASK1 and TASK3 in the hypothalamus of rats was increased significantly from 0 to 4 and 8 weeks. In contrast, the protein level of them was decreased as maturing (Figure 1). The different expression pattern of mRNA and protein may due to posttranslational modifications of TASK1 and TASK3 during growth. Moreover, TASK1 and TASK3 are inhibited by pH reduction and O₂ deprivation in mouse brain and are protective in ischemic/hypoxia injury. Thus, the expression changes of TASK1 and TASK3 in the hypothalamus as maturation may reflect the development of respiratory chemoreception.

TASKs have been proposed to contribute to both peripheral and central chemical regulation of breathing by CO₂/H⁺. It was reported that TASK1 and TASK3 are widely expressed in several central areas related to regulation of respiration, such as the nucleus of pre-Bötzing, RTN, the NTS, locus coeruleus (LC), and VLM. The
TASKs provide acidosis- and hypoxia-inhibited potassium outward currents, which regulate cellular resting membrane potential and excitability.¹⁸ TASKs current was recorded in central chemoreceptive neurons such as in the nucleus of pre-Bötzinger and RTN and peripheral chemoreceptor, glomus cells in carotid body.¹⁹⁻²¹ However, it was recorded that...
the ventilatory response to CO₂ was completely retained in TASK1, TASK3, or TASK1/3 double knockout mice. Thus, some researchers concluded that TASK1 and TASK3 do not contribute to central respiratory chemosensitivity. It is well known that activation of TASKs mediates outward current and leads to hyperpolarization of cell membrane. In this context, knockout TASKs should increase but not ablated ventilatory response to CO₂. Our results confirmed this reasoning. In the present study, we uncovered the distribution of TASK1 and TASK3 in the LH of adult rats.

Figure 6 Orexin neurons in the LH regulated respiration by innervating to the NTS. A, OX1R-positive neuron and orexinA-positive nerve fiber in the NTS. B, C, Microinjection of orexinA (10, 100, 1000 μg/mL, and 0.3 μL) into the lateral ventricle (LV) stimulated respiration. D, Group data from C. Different doses of orexinA increase Ti, iPND, and respiratory drive. *P < .05, **P < .01, ***P < .001 compared with ACSF, *P < .05, n = 5. E, Microinjection of SB408124 (10 μg/mL and 0.1 μL) into the NTS to block OX1R, and then microinjection of acidic ACSF (pH6.5) immediately, normal ACSF (pH7.4) serves as control. Plot of the injection sites projected on Bregma 2.56 and 11.6 mm section: the blue dots were normal ACSF (pH7.4), the yellow squares were acidic ACSF (pH6.5), and the yellow triangles were SB408124 inside the aimed nucleus. And, the red pentagons represent the ones outside of the aimed nucleus. F, The effect of acidosis in the LH is inhibited by blocking OX1R of NTS. Blocking OX1R of NTS alone had no effect on respiration. The blue and red arrows in ① ② ③ ④ were time points that microinjection into the NTS and LH. ① injection of normal ACSF into the NTS and LH; ② injection of normal ACSF into the NTS and acidic ACSF (pH6.5) into the LH; ③ injection of SB408124 into the NTS and acidic ACSF (pH6.5) into the LH; ④ injection of SB408124 into the NTS and normal ACSF into the LH; G, Group data from F. *P < .05, n = 6.
not stimulate breathing, but neutralized the inhibition effect of alkalized ACSF (Figure 3E, G). The reasons leading to this phenomenon may be that TASK-1 nearly closes at physiological pH (pH 7.3-7.4), whereas, TASK3 fully opens at pH over 7. Additional alkalization has little effect on TASK-3 currents.23 Thus, we conclude that TASK3 takes part in maintaining the resting potential of chemosensitive neuron and TASK1 contributes to the alkali sensitivity of chemoreceptor. An important issue we should take into account is that general anesthesia has effect on ventilation in normal condition and many anesthetics are known to decrease ventilation and metabolic rate.24 But in this study, we observed phrenic nerve discharge after stimulating local brain area with different drugs, which should be done under anesthesia by stereotaxic technique. In addition, microinjecting to the specific points in brain area can reflect respiratory activities regulated by respiration-related neuron of central chemoreceptors in response to the different stimulation rather than that of peripheral chemoreceptors. As we know, many nervous system disorders such as Parkinson’s, epilepsy are associated with local microenvironment change in brain areas, studying central regulation of respiration will help us to have a better understanding of the effect of microenvironment change in local brain area on respiratory activity. However, more evidences are needed to clarify the exact roles of TASKs in the central chemoreception.

The LH is important in the regulation of breathing. Electrolytic lesions or inhibition of the LH by barbiturates in cat reduced respiration.25 It was shown that microdialysis of CO2 into the LH increases ventilation, which suggested that the LH is a central chemosensitive site.26 It was found that microinjection of acidic ACSF into the LH increases respiration (Figure 3F). We also observed that microinjection of solutions with pH 6.0 and 6.5 had almost the same response. We think that it may be caused by the different pH sensitivity of TASK1 and TASK3. At the pH of 6.5 TASK1 is fully inhibited and about half of TASK3 is nearly inhibited, thus further decreasing the pH could not trigger stronger response. Another reason for this, we considered is that other pH-sensitive channels such as ASIC1 is also almost activated at pH of 6.5, which is the saturation state. However, more research should be done to explore this phenomenon. In contrast, alkali ACSF inhibited respiration, which confirmed the property of chemoreception in LH (Figure 3G). Lines of evidences indicated that the chemosensitivity of LH was attributed to orexin neurons. The orexin neurons are activated by CO2/pH in vitro and in vivo.27,28 The prepro-orexin knockout mice have a 50% decrease in the ventilatory CO2 response, an effect that is reversible by administration of orexin.29 In the next study, we confirmed that the expression of TASK1 and TASK3 in orexin neurons of the LH by morphology experiment (Figure 4). Our data showed that TASK1 and TASK3 were expressed in orexin neurons. As the distribution of the neurons is restricted to the bilateral LH, specific lesions at this site will remove the endogenous resource of orexins. Thus, we used a specific neurotoxin orexin-SAP to lesion the neurons in the bilateral LH and found that about 80% orexin-positive neurons were lost. This is consistent with the previous study that more than 76% of orexin neurons are found to undergo apoptosis 12 days after orexin-SAP administration.30 Besides, they also reported that melanin-concentrating hormone (MCH) neurons and adenosine deaminase-containing (ADA) neurons in the LH were found to be partly decreased at the same time after orexin-SAP injection, seems that this toxin does not only target orexin neurons. However, there is no convincing evidence demonstrating MCH and ADA neurons sense to local extracellular pH change. What’s more, another research demonstrated that only high concentration of the orexin-SAP (450 ng) cause a significant loss of NeuN neurons in the DMH or VMH.31 In our previous and present studies, only 172 ng of orexin-SAP was used, which lead to loss of Nissl bodies in the LH after treatment.7,32 We also observed that there was slight decrease of neurons in LH. However, we failed to see a decrease of microglial in LH, indicating this concentration of orexin-SAP caused little non-specific damage in LH (Supplemental Figure SI). Therefore, the result that lesion of orexin neurons blunted the active effect of BUP, RR, and acidification on respiration (Figure 5) suggested that enhanced phrenic nerve discharge was mainly due to the increased orexin activity. Though we demonstrated that orexin neurons are essential in the regulation of breathing, the orexin-SAP also affects other excitatory neurotransmitters expressing on orexin neurons such as, dynorphin, neurotensin, and glutamate, and the lesion is likely changing the effects of these neurotransmitters on breathing. As orexins act on two specific GPCRs, OX1 and OX2 receptors, in target cells.33 Next, OX1R antagonist SB408124 was applied to investigate whether the orexins was involved in this regulation pattern. The neuronal circuit mechanism involved in such a physiological function remains to be elucidated. Our present works and some other previous researches all unrecovered that orexin fibers present throughout the medulla with most dense in the NTS and its receptors OX1R is expressed in the NTS (Figure 6A).34-36 Moreover, a paper implicated that orexinA can depolarize NTS neurons.37 The orexin neuron/NTS circuit has been reported to take important part in cardiovascular function and energy balance.38,39 It was evident that NTS neurons have great impact on the regulation of respiratory activities.40 Thus, we investigated the orexin neuron/NTS circuit in the respiratory chemoreflex by blocking OX1R in the NTS. Our observation indicated that the stimulation effect of respiration induced by acidifying the LH was reduced.
It is also reported that inhibition of orexin receptors (OXRs) also reduced CO₂-induced ventilation. Our present study showed that supplement of exogenous orexinA by microinjection into the LV stimulated respiration. Thus, we conclude that pH decrease may stimulate orexin neuron to release orexin that act on OXRs to stimulate breathing. However, the chemosensory mechanism of orexin neurons remains elusive and controversial. In contrast, another work found that knockout of TASK1 and TASK3 channels did not abolish pH responses of orexin neurons in mice. Since activation of TASKs limits neuronal excitability as discussed above, the conclusion that TASK1/3 subunits are not essential for orexin cell responses to pH should be debated. What we should mention is that TASK is not the only acid-sensing channel. In our previous studies, we have explored that acid sensing ion channels (ASICs) are also expressed in the orexin neuron and contributes to regulation of breathing. More efforts are needed to illustrate whether TASKs and ASICs interacted with each other to elicit chemosensitivity of orexin neuron synergistically.

**CONCLUSIONS**

Taken together, TASK1 and TASK3 channels contribute to chemosensitivity of orexin neurons; extracellular acidification blocks TASKs and increase the excitability of orexin neurons, thereby might release orexinA to the NTS and stimulate breathing (Figure 7). Extracellular pH is a critical signal in the central regulation of breathing. The present study focused on the roles of TASKs and orexin neurons in chemoreflex. Further study will be needed to clarify the complicated mechanism involving in the central chemoreception.
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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
L. Shen and N. Song participated in the research design and revised the manuscript. X. Wang performed most experiments, statistical analysis, and paper writing. J. Chen performed immunohistochemistry staining and pathological analysis. D. Zhu contributed to technical assistance. R. Guan and X. Zhao assisted with the design of the study.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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