

Loss of cell adhesion molecule CHL1 improves homeostatic adaptation and survival in hypoxic stress

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Close homologue of L1 (CHL1) is a transmembrane cell adhesion molecule that is critical for brain development and for the maintenance of neural circuits in adults. Recent studies revealed that CHL1 has diverse roles and is involved in the regulation of recovery after spinal cord injury. CHL1 expression was downregulated in the cerebral cortex, hypothalamus, and brain stem after the induction of acute hypoxia (AH). In the current study, we sought to address the role of CHL1 in regulating homeostasis responses to hypoxia using CHL1-knockout (CHL1^{-/-}) mice. We found that, compared with wild-type littermates, CHL1^{-/-} mice showed a dramatically lower mortality rate and an augmented ventilatory response after they were subjected to AH. Immunofluorescence staining revealed that CHL1 was expressed in the carotid body (CB), the key oxygen sensor in rodents, and CHL1 expression level in the CB as assayed by western blot was decreased after hypoxic exposure. The number of glomus cells and the expression of tyrosine hydroxylase (a marker for glomus cells) in the CB of CHL1^{-/-} mice appeared to be increased compared with CHL1^{+/+} mice. In addition, in the *ex vivo* CB preparation, hypoxia induced a significantly greater afferent nerve discharge in CHL1^{-/-} mice compared with CHL1^{+/+} mice. Furthermore, the arterial blood pressure and plasma catecholamine levels of CHL1^{-/-} mice were also significantly higher than those of CHL1^{+/+} mice. Our findings first demonstrate that CHL1 is a novel intrinsic factor that is involved in CB function and in the ventilatory response to AH.

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Close homologue of L1 (CHL1), a close homolog of L1, is a transmembrane cell adhesion molecule that contains six Ig-like domains and 4–5 fibronectin type III homologous domains in its extracellular region and was first identified in 1996.¹ CHL1 is regionally expressed in the central nervous system, spinal cord, and peripheral nervous system and is critical for neurite outgrowth, neuronal survival, and axonal guidance during development and in the maintenance of neural circuits in adults.^{2,3} In humans, CHL1 (also referred to as CALL) is a candidate gene involved in 3p-syndrome-associated mental impairment.⁴ Recent studies demonstrated that CHL1 is involved in the development of different human cancers and is downregulated/silenced in a majority of primary tumors and upregulated in association with invasive/metastatic growth.⁵ Moreover, CHL1 has been shown to be a novel physiological substrate of beta-site APP cleaving enzyme 1 (BACE1) *in vitro* and *in vivo* and may be involved in the regulation of AD development.⁶

The first CHL1-knockout mice were generated in 2002.⁷ CHL1 deficiency resulted in misguided axonal projections and aberrant axonal connectivity within the central nervous system, and the mice displayed impaired cognitive functions

and sensorimotor co-ordination as well as aberrant emotional reactivity.⁸ Further studies indicated that CHL1 also performs important functions in the lesioned nervous system. The expression of CHL1 was elevated in glia cells in injured optic nerves, and CHL1-knockout mice displayed improved recovery from spinal cord injury owing to a reduction in the glial fibrillary acidic protein (GFAP) expression when compared with wild-type mice.⁹ In our previous work, we found that CHL1 was expressed in neural stem cells and negatively regulated their proliferation and differentiation.¹⁰ In addition, we found that CHL1 protein expression was downregulated in the cerebral cortex, hypothalamus, and brain stem after the induction of acute hypoxia (AH), suggesting that CHL1 might have an important role in the regulation of neuronal responses to hypoxia.¹¹

Oxygen has a central role in cell metabolism and animal physiology. Once oxygen supplies decrease, even temporarily, irreversible cellular damage can result. As part of the mammalian adaptation to hypoxic environments, an important response to hypoxia is to increase breathing and replenish oxygen from the air. The carotid body (CB) is the main arterial chemoreceptor that senses the blood O₂ concentration.

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Abbreviations: CHL1, close homologue of L1; AH, acute hypoxia; AHH, acute hypobaric hypoxia; CB, carotid body; TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein; BACE1, beta-site APP cleaving enzyme 1; HVR, hypoxic ventilatory response

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Upon exposure to AH, neurosecretory type-I glomus cells release transmitters that activate afferent sensory fibers that project to the brain stem to elicit hyperventilation and sympathetic activation. The increased ventilation and sympathoexcitation triggered by hypoxia represent the important homeostasis responses for mammalian adaptation to acute hypoxic environments and survival.^{12,13} Molecules such as hypoxia-inducible factor 1 α , the small membrane-anchoring D subunit of mitochondrial succinate dehydrogenase, heme oxygenase 2, and NOX4 *et al.* have been reported to be involved in the regulation of the CB oxygen-sensing function.^{14,15} However, the precise molecular mechanisms underlying the hypoxic response in the CB remain poorly understood. Experimental studies on the CB are challenging because of the small size of the organ, which precludes elaborate biochemical and molecular biology experiments. To our knowledge, there are no reports of the involvement of cell adhesion molecules in this process.

The current study was designed to test the possible involvement of CHL1 in the regulation of the homeostasis responses to AH. We found that CHL1 is expressed in mouse CB and its deficiency results in hyperplasia of CB. Importantly, CHL1^{-/-} mice showed an augmented ventilatory response and a lower mortality rate when they were subjected to AH. The blood pressure and plasma catecholamine levels of CHL1^{-/-} mice were also higher than those of CHL1^{+/+} mice. The findings suggest that CHL1 might have a role in the chemoreceptor control of the homeostatic adaptation to AH.

Results

Loss of CHL1 increases the survival rate after AH. In our previous study, we found that the expression of CHL1 was

decreased in the cerebral cortex, hypothalamus, and brain stem and increased in the cerebellum after the induction of AH. Thus, we hypothesized that CHL1 may be involved in the regulation of the hypoxic response *in vivo*. To address this possibility, CHL1^{-/-} mice and wild-type littermates were subjected to hypoxia (5% O₂) for 10 min. We found that CHL1^{-/-} mice showed a dramatically decreased mortality rate after they were subjected to AH (survival rate: 62.5% in CHL1^{-/-} versus 12.5% in wild-type) (Figure 1a). The survival time after AH was 343.3 \pm 51.9 s for CHL1^{+/+} mice and 403.2 \pm 35.9 s for CHL1^{-/-} mice ($P = 0.0173$, Figure 1b). To further investigate the above findings, CHL1^{-/-} mice and CHL1^{+/+} littermates generated from breeding CHL1^{+/-} mice were treated with hypobaric hypoxia for 15 min. Again, we observed an increased survival rate in CHL1^{-/-} mice (86% versus 13.4% in wild-type, $P = 0.0019$, Figures 1c and d). Thus, CHL1^{-/-} mice exhibited lower mortality compared with wild-type mice after exposure to AH.

CHL1^{-/-} mice exhibit an augmented ventilatory response to hypoxia. To further understand why the loss of CHL1 results in an increased survival rate after AH, we observed the changes in respiration of mice upon exposure to hypoxia. Six- to eight-week-old unanesthetized male CHL1^{+/+} and CHL1^{-/-} mice were exposed to 10% O₂ (15 min) and 5% O₂ (15 min), and the respiratory rate (RR) and tidal volume (V_T) were continuously monitored using whole-body plethysmography. The ventilation during normoxia (21% O₂) was similar between the CHL1^{-/-} and CHL1^{+/+} groups. When the O₂ content of the inspired air was dropped to 10%, the breathing rate accelerated, whereas V_T remained unaltered in both groups of mice. The peak increase in minute ventilation upon exposure to 10% O₂ was not significantly different between CHL1^{-/-} and

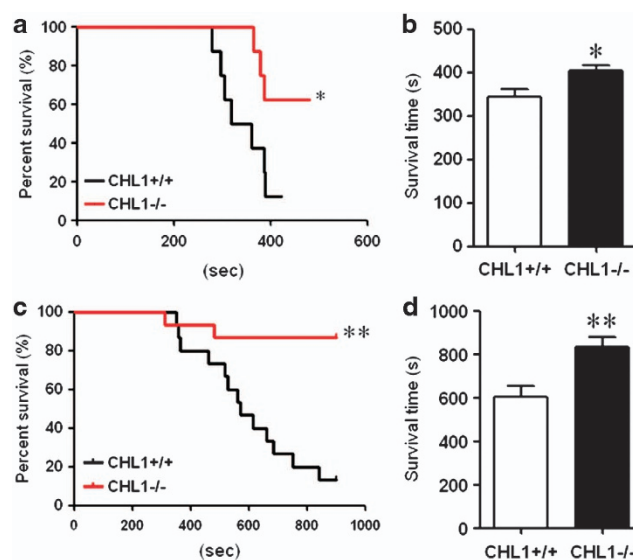


Figure 1 CHL1 deficiency enhances the survival rate and survival time of mice subjected to AH. (a) Kaplan–Meier curves show a significant difference in survival rates between CHL1^{+/+} and CHL1^{-/-} mice subjected to AH. (b) CHL1^{-/-} mice show a significant increase in survival time after AH ($n = 8$, * $P < 0.05$). (c) Kaplan–Meier curves show a significant difference in survival rates between CHL1^{+/+} and CHL1^{-/-} mice subjected to acute hypobaric hypoxia (AHH). (d) CHL1^{-/-} mice show a significant increase in survival time after AHH ($n = 20$, ** $P < 0.01$)

CHL1^{+/+} mice. As the O₂ content in inspired air was further dropped to 5%, the CHL1^{+/+} mice responded with an increase in V_T but with no further increase in breathing rate, whereas the CHL1^{-/-} mice responded with an increase in both V_T and breathing rate. Consequently, the minute ventilation increased to a significantly greater extent in CHL1^{-/-} mice than in the wild-type counterparts (Figures 2a–c). We further analyzed the oxygen saturation level in the mice during normoxia and hypoxia. The blood oxygen saturation levels during normoxia were not significantly different between the two groups. However, oxygen saturation at 10 and 15 min after exposure to hypoxia (5% O₂) was significantly greater in CHL1^{-/-} mice (77.4 ± 10.4 and 70.3 ± 11.2) than in CHL1^{+/+} mice (72.4 ± 11.5 and 64.1 ± 4.4) (see Table1). These results indicate that CHL1 deficiency augments the ventilatory response to AH.

To confirm whether the increased respiratory response in the CHL1^{-/-} mice is specific to hypoxia, we examined the respiratory response to hypercapnia. We monitored respiration in response to hypercapnia (21% O₂ + 5% CO₂ and 21% O₂ + 3% CO₂). Hypercapnia induced a profound increase in ventilation in all groups of animals. However, there were no significant differences in ventilation (RR, V_T, and V_E) between CHL1^{-/-} and CHL1^{+/+} mice when the concentration of CO₂ in the inspired air was increased to 3% and then to 5%

(Figures 2d–f). These results demonstrate that CHL1 deficiency does not affect the ventilatory responses to hypercapnia.

Expression and localization of CHL1 in the CB. The primary site of oxygen sensing is the CB, which can instantaneously sense changes in the composition of the arterial blood, and the afferent signal is relayed to the brain stem to stimulate the respiratory center (and to counteract the direct inhibitory effects of hypoxia on respiratory center) to produce hyperventilation. The CB, a small neural crest-derived paired organ located at the carotid bifurcation, is composed of clusters of neuron-like glomus or type-I cells enveloped by glia-like sustentacular or type-II cells.¹⁶ To investigate the role of CHL1 in this peripheral chemoreceptor, CBs were sectioned and analyzed by immunofluorescence staining. Strong CHL1 immunofluorescence was seen surrounding the immunofluorescence for tyrosine hydroxylase (TH), an established marker of glomus cells (Figure 3A). The staining pattern suggests that CHL1 is primarily localized to type-II cells or afferent terminals. To further study the localization of CHL1, the CBs of adult mice were isolated, enzymatically dissociated, and then cultured as previously described.¹⁶ The cultured CB cells are primarily composed of clusters of neuron-like glomus (TH-positive

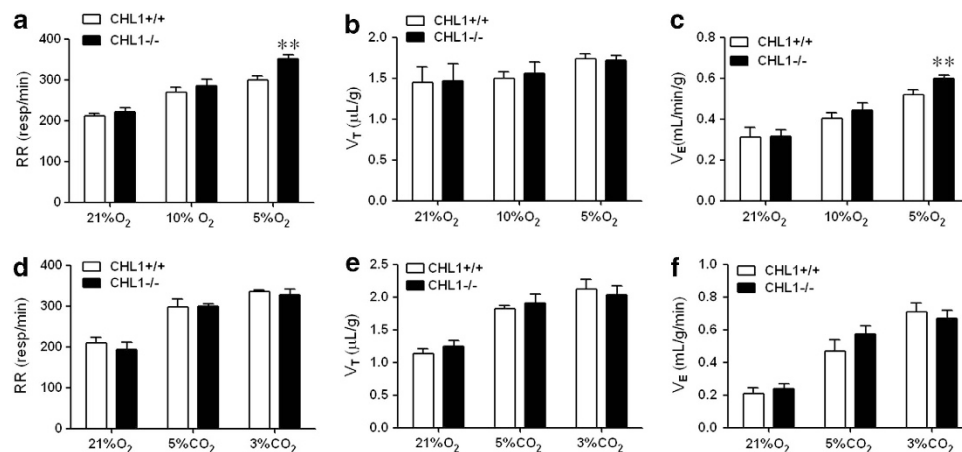


Figure 2 Ventilatory responses to hypoxia and hypercapnia in CHL1^{+/+} and CHL1^{-/-} mice. RR, V_T, and V_E were measured in unanesthetized mice by whole-body plethysmography in normoxia (21% O₂), hypoxia (10% O₂, 5% O₂), and hypercapnia (5% CO₂, 3% CO₂) conditions. The average values of RR, V_T, and V_E in response to 10% O₂, 5% O₂ hypoxia are shown in (a–c) and in response to 5% CO₂, 3% CO₂ hypercapnia are shown in (d–f). Data shown are the mean ± S.E.M. from eight mice in each group. **P < 0.01 compared with the CHL1^{+/+} group in response to hypoxia

Table1 Measurement of physiological parameters before and after acute hypoxia (15 min)

Parameters	Genotype	Normoxia (20% O ₂)	Exposure to hypoxia (5% O ₂)		
			5 min	10 min	15 min
SaO ₂ (%)	Wild-type	96.9 ± 1.8	87.7 ± 14.0	72.4 ± 11.5	64.1 ± 4.4**
	CHL1 ^{-/-}	98.4 ± 0.8	89.2 ± 10.7	77.4 ± 10.4	70.3 ± 11.2**
HR (b.p.m.)	Wild-type	239.4 ± 14.2	250.8 ± 11.9	261.5 ± 13.4	263.9 ± 13.2*
	CHL1 ^{-/-}	216.2 ± 13.7	228.5 ± 14.1	236.8 ± 14.8	245.5 ± 13.2*
BR (b.r.p.m.)	Wild-type	77.2 ± 10.2	84.5 ± 8.3	82.1 ± 7.4	83.3 ± 10.6*
	CHL1 ^{-/-}	73.2 ± 5.7	79.3 ± 10.3	84.1 ± 9.2	86.6 ± 11.2*

Abbreviations: SaO₂, arterial oxygen saturation; HR, heart rate; BR, breath rate

cells) cells and are enveloped by glia-like sustentacular (GFAP and nestin-positive cells) cells (Figure 3B). Strong immunofluorescence for CHL1 was identified in GFAP⁺ sustentacular cells and, moderate CHL1 immunostaining was also observed in TH⁺ glomus cells (Figure 3C).

We then investigated whether the expression of CHL1 would be altered in mice that were subjected to prolonged hypoxia. CHL1 protein levels in the CB were determined by western blot. The levels of CHL1 expression were significantly decreased in mice exposed to hypoxia (8% O₂) for 8 h compared with those exposed to normoxia (Figures 3D and E). These results suggest that CHL1 expression in the chemoreceptive CB is altered in response to hypoxia.

CHL1 deficiency enhances the CB response to hypoxia in mice. The CB senses changes in arterial PO₂ during AH and converts the hypoxic stimulus to afferent nerve activation. Then, the increased sensory discharge is transmitted to the central nervous system, which in turn triggers appropriate cardio-respiratory responses, including a stimulation of breathing and a rise in blood pressure.^{17–19} The hypoxic ventilatory response represents a major

adaptive reflex triggered by the CB. We next examined whether CHL1 deficiency would alter the response of CB to hypoxia. Chemoreceptive afferent nerve activity was recorded in the *ex vivo* CB/sinus nerve preparations taken from CHL1^{-/-} mice and wild-type littermates under normoxia and hypoxia conditions. There were no significant differences in the baseline sinus nerve discharge between CHL1^{-/-} and wild-type littermates under normoxia condition. When the superfusion solution was switched to the hypoxic solution (5% O₂, for 5 min), sinus nerve discharge markedly increased in both groups (Figure 4a). The magnitude of hypoxia-induced afferent response, however, was significantly greater in CHL1^{-/-} than in wild-type preparations. The afferent discharge rate increased from 11.7 ± 2.11 to a peak of 340.32 ± 70.32 spikes/s in CHL1^{-/-} preparations (*n* = 6) as compared with an increase from 10.2 ± 1.72 spikes/s to a peak of 171.82 ± 20.79 spikes/s (*n* = 6) in wild-type mice (*P* < 0.05, Figure 4c). We further investigated the sinus nerve activity in response to 15 min of continuous hypoxia and found that the afferent response in CHL1^{-/-} preparations was also significantly increased compared with wild-type preparations (Figures 4b–d).

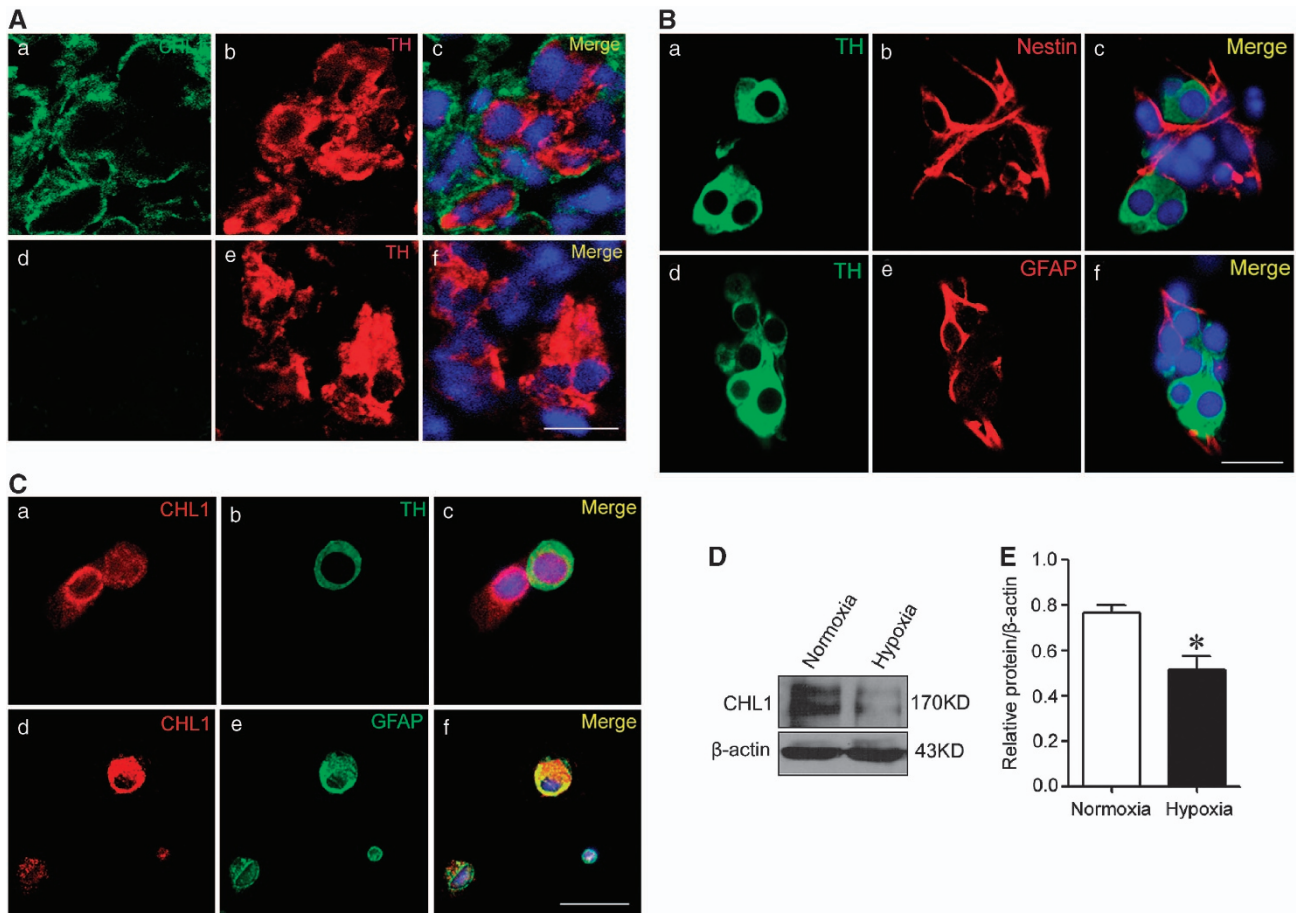


Figure 3 CHL1 localization in the mouse CB. (A) CHL1 expression in CBs. CB sections were stained with antibodies specific against CHL1 (green) and TH, a marker of glomus cells (red). (B) The cultured CB cells are primarily composed of clusters of TH⁺ cells (a–d) and enveloped by GFAP⁺ (b) and nestin⁺ (e) cells. (C) CHL1 expression in cultured glomus cells. CHL1 (red) colocalized with TH⁺ (green) glomus cells (a–c) and GFAP⁺ (green) sustentacular cells (d–f) in cultured CB cells. Scale bar: 20 μm. (D) Immunoblot assays of CHL1 protein from mouse CBs in response to hypoxia. (E) Quantitative analysis of CHL1 protein expression in response to hypoxia. Data are presented as the mean ± S.E.M. from five experiments. **P* < 0.05 compared with control normoxic mice

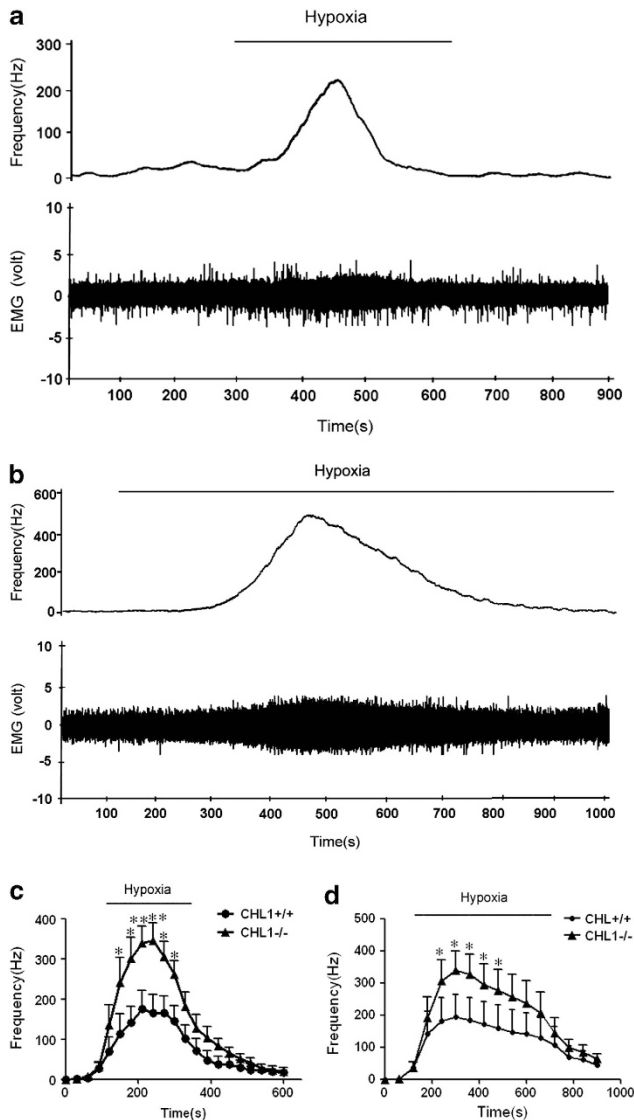


Figure 4 Recording of the afferent nerve responses to hypoxia in the isolated CB–sinus nerve preparation taken from CHL1^{+/+} and CHL1^{-/-} mice. (a) Representative recording of the afferent nerve response to hypoxia in CHL1^{+/+} mice. (b) Representative recording of the afferent nerve response to hypoxia in CHL1^{-/-} mice. (c) Average data showing the sinus nerve discharge of the CB from CHL1^{+/+} and CHL1^{-/-} mice exposed to hypoxia (5 min). (d) Average data showing the sinus nerve discharge of the CB from CHL1^{+/+} and CHL1^{-/-} mice exposed to hypoxia (15 min). Data presented are the mean \pm S.E.M. from eight mice in the CHL1^{+/+} control and the CHL1^{-/-} group. * $P < 0.05$, ** $P < 0.01$ compared with control CBs

CHL1 deficiency leads to hyperplasia of the CB. The glomus cells in the CB are considered to be the primary hypoxia-sensing cells, and a reduction in the oxygen supply activates glomus cells, resulting in membrane depolarization and release of neurotransmitters such as ATP, acetylcholine, and dopamine.^{20–22} Long-term exposure to low-oxygen environments (such as during acclimation to high altitudes) results in a dramatic increase in the number of glomus cells and in CB size.²³ To further understand the potential role of

CHL1 in CB development, a morphological analysis of the CBs from CHL1^{-/-} and CHL1^{+/+} mice was performed. The CBs, nestled at the bifurcation of the internal and external carotid arteries, were removed, fixed in 4% paraformaldehyde, and sectioned for immunohistochemical staining. CHL1^{-/-} exhibited a 1.3-fold-enlarged volume of the CB and an increased number of glomus cells when compared with CHL1^{+/+} mice (Figures 5B and C). However, a difference in the volume of CB between CHL1^{-/-} mice and CHL1^{+/+} mice was not apparent until 14 days after hypoxia (see Supplementary Figure 1). We further studied the expression of TH, a glomus cell-type-specific marker, in the CB using western blot analysis. The level of TH expression in CHL1^{-/-} mice was increased when compared with CHL1^{+/+} mice; however, nestin (a marker of multipotent cells) and GFAP (a marker of sustentacular cells) expressions were not different between these two groups (Figures 5E–G). These results demonstrate that CHL1 negatively regulates the number of glomus cells in the CB and may be involved in the regulation of CB activity.

Blood pressure and catecholamine levels in CHL1^{-/-} mice under hypoxia. In addition to hyperventilation in response to hypoxia, increased CB activity also causes enhanced sympathetic activation and a cardiovascular response.^{14,21} We therefore examined whether there were differences in arterial blood pressure and plasma catecholamine levels between CHL1^{-/-} mice and the wild-type littermates. Arterial blood pressure was determined using the tail-cuff method during plethysmography under normoxic and hypoxic conditions. Mean blood pressure was higher in CHL1^{-/-} mice than in wild-type mice, as were systolic and diastolic pressures (Figures 6a–c). Plasma NE and dopamine levels were also significantly higher in CHL1^{-/-} mice. Unexpectedly, the blood pressure did not alter significantly during hypoxia in either group of mice. However, plasma norepinephrine dropped in CHL1^{-/-} mice ($P = 0.09$) and remained unchanged in wild-type mice. Epinephrine and dopamine levels were similarly increased in CHL1^{-/-} compared with wild-type mice during hypoxia. Thus, it appears that CHL1^{-/-} mice manifested an ongoing hyperactive sympathetic nervous system.

Discussion

The present study reveals a novel physiological role for the cell adhesion molecule CHL1 in the CB. We found that CHL1 is expressed within the CB, the major peripheral chemoreceptor that initiates the homeostasis adaptation to hypoxia. CHL1 deficiency results in hyperplasia of type-I glomus cells, upregulated TH expression, and enhanced carotid sinus nerve activity in response to AH. Concomitantly, CHL1^{-/-} mice manifested increased ventilatory responses and lower mortality following AH. These results reveal a previously unappreciated role of CHL1 in the regulation of the homeostasis responses to hypoxia.

One of the major findings of the current study is that CHL1^{-/-} mice had a greater survival rate during hypoxia. Interestingly, a recent study reported that BACE1^{-/-} mice were more susceptible to seizures upon exposure to AH

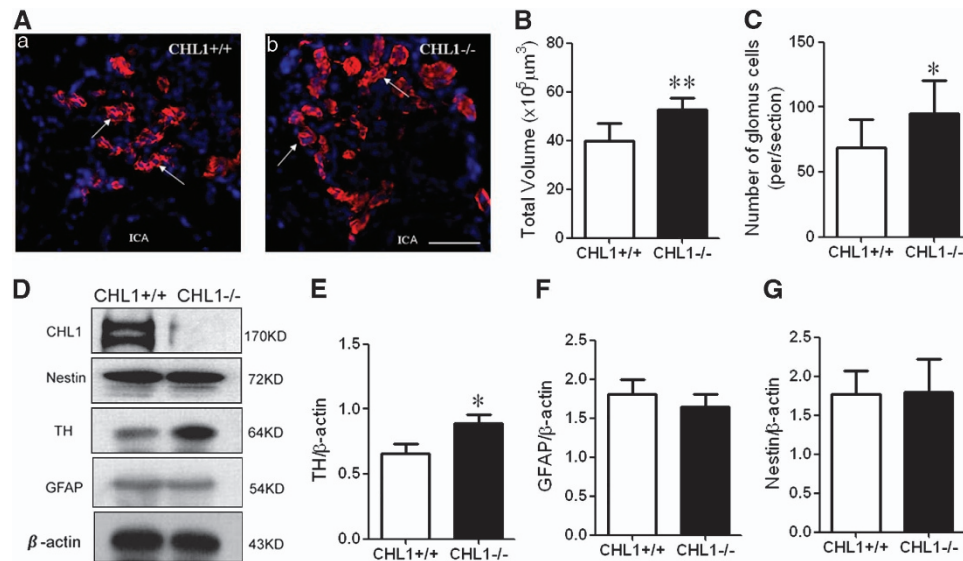


Figure 5 Morphological analysis of the CBs from CHL1^{-/-} mice. **(A)** Representative photomicrographs of CB sections were stained with antibodies specific for TH in CHL1^{-/-} (b) and CHL1^{+/+} (a) mice. **(B)** Statistical analysis of the volume of CBs in CHL1^{+/+} and CHL1^{-/-} mice. CHL1^{-/-} mice exhibit an enlarged CB volume. **(C)** Statistical analysis of the number of glomeruli cells in CHL1^{+/+} and CHL1^{-/-} mice. CHL1^{-/-} mice exhibited an increased volume of glomeruli cells compared with CHL1^{+/+} mice. **(D)** Western blot analysis of the cell-type-specific marker protein in CHL1^{+/+} and CHL1^{-/-} CBs. **(E–G)** The relative intensity of marker expression compared with β -actin. The expression of TH in CHL1^{-/-} mice was increased compared with CHL1^{+/+} controls. Data are presented as the mean \pm S.E.M. from 10 mice in each group. ICA, internal carotid artery. **(E–G)** The relative intensity of marker expression compared with β -actin. Expression of TH in CHL1^{-/-} mice was increased compared with CHL1^{+/+} controls. Data are presented as the means \pm S.E.M. from 10 mice in each group. ICA, internal carotid artery. * $P < 0.05$, ** $P < 0.01$ compared with control CBs

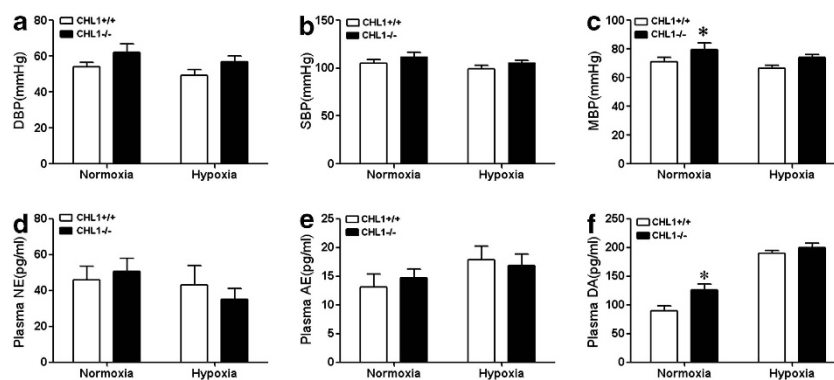


Figure 6 Blood pressure and plasma catecholamine levels in CHL1^{-/-} mice under hypoxia. **(a–c)** Statistical analysis of mean (MBP), systolic (SBP), and diastolic (DBP) blood pressure levels in CHL1^{+/+} and CHL1^{-/-} mice under hypoxia. **(d–e)** Statistical analysis of plasma catecholamine levels in CHL1^{+/+} and CHL1^{-/-} mice under hypoxia. Data are presented as the mean \pm S.E.M. from 10 mice in each group. **(d–f)** Statistical analysis of plasma catecholamine levels in CHL1^{+/+} and CHL1^{-/-} mice under hypoxia. Data are presented as the means \pm S.E.M. from 10 mice in each group. * $P < 0.05$, ** $P < 0.01$ compared with control mice

(5% O₂). In that study, ~60% of BACE1^{-/-} mice died during a 12-min ischemic insult compared with no deaths in the wild-type group. CHL1 was shown to be a physiological substrate of BACE1, and the level of CHL1 expression was elevated in BACE1^{-/-} mice.²⁴ These data together support a novel role for CHL1 in acute hypoxic stress.

Survival of mammals in an acute hypoxic state requires fast respiratory and cardiovascular adjustments to ensure sufficient oxygen supply to tissues, particularly to the brain, which is highly vulnerable to lack of O₂.²⁵ The chemoreceptive CB detects oxygen availability and triggers the adaptive responses to hypoxic conditions. In this regard, a drop in arterial PO₂ leads to an increase in sensory discharge of the

CBs in all mammalian species, and the increased sensory activity is transmitted to the central nervous system, which in turn triggers appropriate autonomic changes, including stimulation of breathing, sympathetic activation, and a rise in blood pressure.²⁶ If the CB dysfunction occurs in patients with ischemia or hypoxia-related illness, then impaired sensitivity to hypoxia and further failure to reduce metabolic oxygen demand in conditions of low O₂ might promote mortality.^{22,23,27} Hence, the CB has a major role sustaining homeostasis in hypoxic conditions.

Previously, it was shown that an enlarged CB with clusters of TH⁺ glomeruli is the typical histological appearance of CBs exposed to hypoxia.^{16,23} Here we show that CHL1

deletion is also sufficient to induce hyperplasia of the CB characterized by increased CB mass, increased number of glomus cells, and upregulated TH expression. The increased number of glomus cells leads to the release of more neurotransmitters, which then induced a greater afferent nerve discharge in CHL1^{-/-} mice compared with the CHL1^{+/+} mice. Consistent with the morphological phenotype, the CB of CHL1-deficient mice had an augmented chemosensory response as determined by the sinus nerve activity. Hypoxia induces release of neurotransmitters from CB chemoreceptor cells that increases the activity of afferent chemosensory fibers.²⁸ The increased ventilation and sympathy-excitation triggered by hypoxia in the CHL1-deficient mice represent the important homeostasis responses for mammalian adaptation to acute hypoxic environments. Concomitantly, the mice manifested augmented respiratory adaptation and greater survival rate following AH. It is tempting to assume that the augmented respiratory adaptation partially underlies the improved survival during hypoxic conditions.

Indeed, the increase in the vital capacity and breath rate is critical for the inspiration of more oxygen to prevent a decrease in blood oxygen saturation (SaO₂) and to maintain normal function of the body during hypoxia. Consistently, the blood oxygen saturation at 10 and 15 min after exposure to hypoxia (5% O₂) was significantly greater in CHL1^{-/-} mice (77.4 ± 10.4 and 70.3 ± 11.2) than in CHL1^{+/+} mice (72.4 ± 11.5 and 64.1 ± 4.4), whereas there were no differences between the two groups during the normoxia condition.

On the other hand, upon exposure to AH, the glomus cell membrane depolarization by inhibition of O₂-sensitive K⁺ channels induces extracellular Ca²⁺ influx and then leads to a reversible release of neurotransmitters that activate afferent nerve fibers.¹² In the current study, we confirmed CHL1 expression in the CB and primary distribution in type II cells as well as afferent terminals, although type I glomus (the oxygen-sensing cells) also appeared to express a lower level of CHL1 through immunofluorescence. Importantly, we found that CHL1 expression in the CB was decreased following 8 h of hypoxia (8% O₂), and hypoxia induced a significantly greater afferent nerve discharge in CHL1^{-/-} mice compared with CHL1^{+/+} mice. One possible reason is that CHL1 may also be involved in the regulation of synaptic vesicle exo- and endocytosis, as recent work has shown a novel role for CHL1 as a co-chaperone in the presynaptic chaperone machinery consisting of Hsc70, CSP, and aSGT.²⁹ The interactions between CHL1-containing chaperones and proteins of the synaptic vesicle fusion machinery are involved in the regulation of synaptic vesicle recycling following prolonged and stressful synapse activity.³⁰ CHL1 deficiency results in enhanced susceptibility of syntaxin 1B, SNAP25, and VAMP2 to degradation, especially under stressful conditions, such as exposure to heat or prolonged synaptic activity.²⁹ Therefore, whether CHL1 interacts with the synaptic chaperone and mediates the synaptic vesicle exocytosis or endocytosis in glomus cells is an interesting question and needs to be further addressed.

In addition, CHL1^{-/-} mice had higher blood pressure and plasma catecholamine levels under normoxic conditions, indicative of a greater sympathetic tone, compared with

wild-type mice. The significant difference in plasma dopamine levels between CHL1^{-/-} and wild-type mice would suggest greater activation of the sympathetic nerve-adrenal gland axis. The lower heart rate seen in CHL1^{-/-} mice might be due to the influence of the arterial baroreceptor-vagal reflex. At present, however, the possibility that an enhanced chemosensory activity in the CB underlies the greater sympathetic tone in CHL1^{-/-} mice has yet to be explored. We also attempted to compare the sympathetic outflow in CHL1^{-/-} and wild-type mice following AH. Intriguingly, plasma NE, epinephrine and dopamine levels and blood pressure were not significantly different between CHL1^{-/-} and wild-type mice following AH (5% O₂ for 15 min). The results might suggest a dynamic change in the sympathetic limb of the arterial chemoreceptor reflexes in CHL1-deficient mice, which awaits further investigation.

However, even a subtle influence on CB function may lead to significant changes in homeostatic adaptation in hypoxic states. Our data provide evidence that the cell adhesion molecule CHL1 is involved in the regulation of respiration during acute hypoxic stress and affects the survival rate. Targeting cell adhesion molecules may be developed as a potential mode of treatment against, or for the prevention of, various hypoxia-related diseases. Future studies will continue to investigate the role of CHL1 in chronic hypoxia-related diseases, such as brain ischemia or heart failure, to confirm whether this protective effect is applicable to hypoxia-related diseases.

Materials and Methods

Animals. The generation of a CHL1-deficient (CHL1^{-/-}) mouse in the C57BL/6 genetic background has been described previously.⁷ Heterozygous mice were mated to generate wild-type (CHL1^{+/+}) and knockout (CHL1^{-/-}) pairs, and littermates were used in all experiments. Genotyping was performed using tail DNA analysis. All experiments were performed on 2-month-old male CHL1^{+/+} and CHL1^{-/-} mice (22–25 g body weight). Mice were maintained at the animal facility with free access to water and food in accordance with institutional guidelines. The Institutional Animal Care and Use Committee (IACUC) of the Academy of Beijing medical Sciences approved all experiments involving mice.

Immunofluorescence. The carotid bifurcation was fixed in 4% paraformaldehyde, and 10 μm serial sections were cut using a Thermo Cryotome. Sections were incubated for 30 min at room temperature in a block solution (10% normal horse serum, 1% BSA, 0.1% TritonX-100 in PBS). Sections were then incubated overnight at 4 °C with the following primary antibodies: mouse anti-rat CHL1 (1:500; R&D Systems, Minneapolis, MN, USA), rabbit anti-rat TH (1:2000; Millipore, Billerica, MA, USA) or mouse anti-rat Nestin (1:500; Millipore) or rabbit anti-rat GFAP (1:1000; DAKO, Produktionsvej, Denmark). The immunoreactivity was visualized with Alexa Fluor 488- or 594-conjugated secondary antibodies (Molecular Probes). Nuclei were counterstained with DAPI-containing mounting medium (Vector Laboratories, Burlingame, CA, USA). Fluorescence was visualized using a Zeiss LSM5 confocal laser-scanning microscope.

Dissociation of glomus cells. CBs were removed from anesthetized young adult mice.^{24–28} They were then dissected and placed on ice-cold Tyrode solution (Ca²⁺ and Mg²⁺ free) and then incubated for 30 min at 37 °C in an enzyme solution containing collagenase and trypsin in a CO₂ incubator. Finally, the cells were mechanically dispersed. Cell density was determined using a hemocytometer (Millipore). For direct staining of dispersed cells they were plated onto an adherent substrate for 1 h, then fixed in 4% PFA, and stained following the same procedures used in the immunofluorescence staining.

CB sensory activity. Sensory activity from CBs *ex vivo* was recorded as described.³¹ Briefly, CBs along with the sinus nerves were harvested from anesthetized mice, placed in a recording chamber, and superfused with oxygenated

Krebs' solution (140 mM NaCl, 2.7 mM KCl, 2.5 mM CaCl_2 , 0.5 mM MgCl_2 , 5.5 mM Hepes, 11 mM D-glucose and 5 mM sucrose; 21% O_2 + 5% CO_2 + 74% N_2). The sinus nerve was carefully dissected and recorded using a suction electrode. Nerve signal was amplified and filtered using the Neurolog system (Digitimer Ltd, Cambridge, UK), A/D converted (Micro1401, Cambridge Electronic Design, Cambridge, UK), and processed using the Spike2 data capture and analysis program (CED, Cambridge, UK). The preparation was allowed to stabilize for about 30 min and was then intermittently challenged with hypoxic solutions (Krebs bubbled with 5% O_2 + 5% CO_2 + 90% N_2). Hypoxic duration was for 5 or 15 min.

Whole-body plethysmography. Ventilation was monitored using a whole-body plethysmograph in anesthetized mice. Baseline ventilation was recorded for 60 min while the mice breathed under normoxic conditions. To observe hypoxia-induced ventilatory responses, the inspired gas was switched to 10% O_2 + 90% N_2 for 15 min and to 5% O_2 + 95% N_2 for 15 min. To determine the ventilatory response to CO_2 , baseline ventilation was determined while mice breathed 21% O_2 + 89% N_2 , and the inspired gas was switched to a hypercapnic gas (21% O_2 + 5% CO_2 + 74% N_2). All recordings were made at an ambient temperature of 22–24 °C. Variables analyzed were RR, V_T , and V_E (minute ventilation).

Measurements of blood pressure and plasma catecholamines. Blood pressures were determined using the tail-cuff method in conscious mice using a noninvasive BP system (Softron Instruments, Beijing, China); mean (MBP), systolic (SBP), and diastolic (DBP) blood pressure levels were measured. Plasma norepinephrine levels were determined by immunoassay. Blood from adult mice was collected into heparinized tubes placed on ice and was then centrifuged at 3000 g for 5 min at 4 °C to separate the plasma. Plasma catecholamine levels were quantified by immunoassay according to the manufacturer's instructions (IBL-Hamburg, Hamburg, Germany).

Western blot analysis. CBs were harvested from anesthetized $\text{CHL1}^{+/+}$ and $\text{CHL1}^{-/-}$ mice, and total protein was extracted. Equal amounts of protein (50–100 μg) were boiled in sample buffer, separated by electrophoresis on 8–12% SDS-PAGE gels, and transferred to a nitrocellulose membrane for 1–3 h. Membranes were blocked in 5% skimmed-milk powder in TBS-T (TBS plus 0.5% Tween-20) or in 3% BSA in TBS-T for 2 h at room temperature, and then incubated overnight at 4 °C with primary antibody. Primary antibodies included CHL1 (1:500; R&D Systems), TH (1:2000; Millipore), Nestin (1:500; Millipore), GFAP (1:1000; DAKO), and β -actin (1:10 000; Sigma, St. Louis, MO, USA). Membranes were treated with goat anti-mouse or -rabbit HRP-conjugated secondary antibodies (1:5000, MBL). Complexes on the membrane were visualized using an enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA).

Hypoxic treatment. I Mice were exposed to a low-oxygen chamber with gas mixture to hypoxic air (5% oxygen, balance with nitrogen) for 10 min. II Mice were exposed to acute hypobaric hypoxia of 10 000 m at a velocity of 50 m/s for 15 min. Humidity in the chamber was maintained at 40–50% and temperature at 22–24 °C. Survival rate and survival time were measured in $\text{CHL1}^{+/+}$ and $\text{CHL1}^{-/-}$ mice in these models within a certain time (10 and 15 min). The maximal survival time is 10 min (600 s) or 15 min (900 s) if the mice still survive to hypoxic exposure. III Mice were exposed to a hypoxia chamber with gas mixture to hypoxic air (8% oxygen, balance with nitrogen) for 6 h; thereafter, CBs were harvested, and total protein was extracted for SDS-PAGE.

Statistical analysis. All experiments were repeated at least three times, and the measurements were recorded by the observers who were blinded to the groups. Data are presented as group mean values with S.E.M. Asterisks identify experimental groups that were significantly different from control groups according to the Student *t*-test or one-way ANOVA. For all analyses, *P*-values <0.05 were considered significant.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

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- Holm J, Hillenbrand R, Steuber V, Bartsch U, Moos M, Lubbert H *et al*. Structural features of a close homologue of L1 (CHL1) in the mouse: a new member of the L1 family of neural recognition molecules. *Eur J Neurosci* 1996; **8**: 1613–1629.
- Rolf B, Lang D, Hillenbrand R, Richter M, Schachner M, Bartsch U. Altered expression of CHL1 by glial cells in response to optic nerve injury and intravitreal application of fibroblast growth factor-2. *J Neurosci Res* 2003; **71**: 835–843.
- Nishimune H, Bernreuther C, Carroll P, Chen S, Schachner M, Henderson CE. Neural adhesion molecules L1 and CHL1 are survival factors for motoneurons. *J Neurosci Res* 2005; **80**: 593–599.
- Angeloni D, Lindor NM, Pack S, Latif F, Wei MH, Lerman MI. CALL gene is haploinsufficient in a 3p- syndrome patient. *Am J Med Genet* 1999; **86**: 482–485.
- Senchenko VN, Krasnov GS, Dmitriev AA, Kudryavtseva AV, Anedchenko EA, Braga EA *et al*. Differential expression of CHL1 gene during development of major human cancers. *PLoS One* 2011; **6**: e15612.
- Kuhn PH, Koroniak K, Hogl S, Colombo A, Zeitschel U, Willem M *et al*. Secretome protein enrichment identifies physiological BACE1 protease substrates in neurons. *EMBO J* 2012; **31**: 3157–3168.
- Montag-Sallaz M, Schachner M, Montag D. Misguided axonal projections, neural cell adhesion molecule 180 mRNA upregulation, and altered behavior in mice deficient for the close homolog of L1. *Mol Cell Biol* 2002; **22**: 7967–7981.
- Pratte M, Rougon G, Schachner M, Jamon M. Mice deficient for the close homologue of the neural adhesion cell L1 (CHL1) display alterations in emotional reactivity and motor coordination. *Behav Brain Res* 2003; **147**: 31–39.
- Jakovcevski I, Wu J, Karl N, Leshchynska I, Sytnyk V, Chen J *et al*. Glial scar expression of CHL1, the close homolog of the adhesion molecule L1, limits recovery after spinal cord injury. *J Neurosci* 2007; **27**: 7222–7233.
- Huang X, Zhu LL, Zhao T, Wu LY, Wu KW, Schachner M *et al*. CHL1 negatively regulates the proliferation and neuronal differentiation of neural progenitor cells through activation of the ERK1/2 MAPK pathway. *Mol Cell Neurosci* 2011; **46**: 296–307.
- Sun J, Huang X, Zhu LL, Wu KW, Fan M. [Influence of acute hypoxia on CHL1 expression in different tissues of mice]. *Zhongguo Ying Yong Sheng Li Xue Za Zhi* 2011; **27**: 280–283.
- Lopez-Barneo J, Ortega-Saenz P, Pardal R, Pascual A, Piruat JI, Duran R *et al*. Oxygen sensing in the carotid body. *Annals NY Acad Sciences* 2009; **1177**: 119–131.
- Nanduri J, Makarenko V, Reddy VD, Yuan G, Pawar A, Wang N *et al*. Epigenetic regulation of hypoxic sensing disrupts cardiorespiratory homeostasis. *Proc Natl Acad Sci USA* 2012; **109**: 2515–2520.
- Ortega-Saenz P, Pascual A, Piruat JI, Lopez-Barneo J. Mechanisms of acute oxygen sensing by the carotid body: lessons from genetically modified animals. *Resp Physiol Neurobiol* 2007; **157**: 140–147.
- Kline DD, Peng YJ, Manalo DJ, Semenza GL, Prabhakar NR. Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1 alpha. *Proc Natl Acad Sci USA* 2002; **99**: 821–826.
- Pardal R, Ortega-Saenz P, Duran R, Lopez-Barneo J. Glia-like stem cells sustain physiologic neurogenesis in the adult mammalian carotid body. *Cell* 2007; **131**: 364–377.
- Abdala AP, McBryde FD, Marina N, Hendy EB, Engelman ZJ, Fudim M *et al*. Hypertension is critically dependent on the carotid body input in the spontaneously hypertensive rat. *J Physiol* 2012; **590**(Pt 17): 4269–4277.
- Dahan A, Nieuwenhuijs D, Teppema L. Plasticity of central chemoreceptors: effect of bilateral carotid body resection on central CO_2 sensitivity. *PLoS Med* 2007; **4**: e239.
- Campanucci VA, Fearon IM, Nurse CA. O_2 -sensing mechanisms in efferent neurons to the rat carotid body. *Adv Exp Med Biol* 2003; **536**: 179–185.

20. Teppema LJ, Dahan A. The ventilatory response to hypoxia in mammals: mechanisms, measurement, and analysis. *Physiol Rev* 2010; **90**: 675–754.
21. Peers C, Wyatt CN, Evans AM. Mechanisms for acute oxygen sensing in the carotid body. *Respir Physiol Neurobiol* 2010; **174**: 292–298.
22. Lopez-Barneo J. Oxygen and glucose sensing by carotid body glomus cells. *Curr Opin Neurobiol* 2003; **13**: 493–499.
23. Semenza GL. Oxygen sensing, homeostasis, and disease. *N Engl J Med* 2011; **365**: 537–547.
24. Koike MA, Lin AJ, Pham J, Nguyen E, Yeh JJ, Rahimian R *et al*. APP knockout mice experience acute mortality as the result of ischemia. *PLoS One* 2012; **7**: e42665.
25. Serra A, Brozoski D, Hedin N, Franciosi R, Forster HV. Mortality after carotid body denervation in rats. *J Appl Physiol* 2001; **91**: 1298–1306.
26. Prabhakar NR. O₂ sensing at the mammalian carotid body: why multiple O₂ sensors and multiple transmitters? *Exp Physiol* 2006; **91**: 17–23.
27. Prabhakar NR, Peng YJ. Peripheral chemoreceptors in health and disease. *J Appl Physiol* 2004; **96**: 359–366.
28. Gonzalez C, Almaraz L, Obeso A, Rigual R. Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiol Rev* 1994; **74**: 829–898.
29. Andreyeva A, Leshchynska I, Knepper M, Betzel C, Redecke L, Sytnyk V *et al*. CHL1 is a selective organizer of the presynaptic machinery chaperoning the SNARE complex. *PLoS One* 2010; **5**: e12018.
30. Leshchynska I, Sytnyk V, Richter M, Andreyeva A, Puchkov D, Schachner M. The adhesion molecule CHL1 regulates uncoating of clathrin-coated synaptic vesicles. *Neuron* 2006; **52**: 1011–1025.
31. Rong W, Gourine AV, Cockayne DA, Xiang Z, Ford AP, Spyer KM *et al*. Pivotal role of nucleotide P2 × 2 receptor subunit of the ATP-gated ion channel mediating ventilatory responses to hypoxia. *J Neurosci* 2003; **23**: 11315–11321.



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