The role of Olfr78 in the breathing circuit of mice

ARISING FROM A. J. Chang, F. E. Ortega, J. Riegler, D. V. Madison & M. A. Krasnow *Nature* **527**, 240–244 (2015); https://doi.org/10.1038/nature15721

The carotid body is essential for the adaptation of mammals to environmental or pathological conditions that result in hypoxaemia. In response to hypoxia, neuron-like oxygen-sensitive glomus cells in the carotid body release neurotransmitters that rapidly activate afferent sensory fibres that stimulate the respiratory centre and induce hyperventilation[1], although the mechanisms by which glomus cells detect changes in blood oxygen tension remain unclear[2,3]. Single dissociated glomus cells can respond robustly to hypoxia when superfused with standard, lactate-free hypoxic solutions; it was therefore surprising that Chang et al.[4] claimed that lactate activation of the odorant receptor Olfr78, which is abundantly expressed in the carotid body, is required for oxygen regulation of breathing. We are unable to replicate these findings and show that *Olfr78*$^{-/-}$ mice have a normal ventilatory response to hypoxia and that the physiological responses of single glomus cells to hypoxia and lactate are indistinguishable between wild-type and *Olfr78*$^{-/-}$ mice. There is a Reply to this Comment by Chang, A. J. et al. *Nature* **561**, https://doi.org/10.1038/s41586-018-0547-7 (2018).

In addition to being expressed in the main olfactory epithelium, the odorant receptor Olfr78 (also known as MOL2.3 and MOR18-2) is ectopically expressed in the carotid body[4,5] and other mouse tissues, such as medulla oblongata[6], various sympathetic and parasympathetic ganglia[7], and the kidney[8] and colon[9]. This odorant receptor confers responsiveness to short-chain fatty acids[8,9]. Three independent sets of plethysmographic experiments[3,10,11] were performed in Cambridge (UK) with mice carrying an *Olfr78* null allele[12] that were bred and maintained in Frankfurt (Germany), and are henceforth referred to as 'FRA' mice. These experiments revealed a similar ventilatory response to hypoxia in *Olfr78*$^{-/-}$ mice compared with wild-type *Olfr78*$^{+/+}$ or heterozygous *Olfr78*$^{+/-}$ mice (Extended Data Fig. 1a, b). The first set of experiments was carried out before the publication of the previous study[4], and was followed by two sets of experiments performed by the same investigator without knowledge of the genotype. These preliminary findings led us to design an independent plethysmographic study with wild-type and *Olfr78*$^{-/-}$ FRA mice in Seville (Spain), where these whole-animal experiments were followed by analyses of single glomus cell responsiveness to hypoxia in in vitro preparations[1,3]. Wild-type and *Olfr78*$^{-/-}$ littermates were bred and genotyped in Frankfurt, and shipped to Seville without providing information about the genotype; the coat colour was not indicative of the genotype. The genotypes were confirmed in Seville by PCR of genomic DNA (Extended Data Fig. 1c, d) after completion of the plethysmographic and cellular (amperometric) analyses. A second group of FRA mice, of which the genotype was disclosed in advance, was used to study the effect of hypoxia or lactate on cytosolic Ca^{2+} levels in single dissociated glomus cells from wild-type and *Olfr78*$^{-/-}$ mice.

Exposure of FRA mice to hypoxia (10% O_2) or hypercapnia (5% CO_2) elicited characteristic increases in breathing frequency, which did not significantly differ between *Olfr78*$^{-/-}$ and wild-type mice (Fig. 1a, b and Extended Data Fig. 2a–d). In parallel experiments performed independently at Duke University (United States), normal ventilatory responses to hypoxia were observed in homozygous mice carrying a different *Olfr78*-null allele, which had been generated by Lexicon Genetics (Extended Data Fig. 2e, 'LEX' mice).

It cannot be excluded formally that genetic drift, suppressor mutations or other genetic or epigenetic changes might have occurred in the FRA mouse colony. Therefore, in 2017 a new cryorecovery was ordered and the resulting heterozygous mice were shipped directly to Seville and Duke Universities, without passing through the Frankfurt animal facility. These colonies (referred to as 'JAX' mice) were expanded locally and studied after they reached adulthood. Plethysmographic analyses performed in the two laboratories showed similar ventilatory responses to hypoxia and hypercapnia in wild-type and *Olfr78*$^{-/-}$ JAX mice (Fig. 1c, d and Extended Data Fig. 2f–h). Our results contrast markedly with the previous study[4], which reported a complete abolition of the ventilatory response to hypoxia in *Olfr78*$^{-/-}$ JAX mice. Their mice carried the same *Olfr78*-null allele[12], and had also been obtained from The Jackson Laboratory, without passing through Frankfurt.

To investigate the oxygen-sensing properties of Olfr78-deficient glomus cells, tissue slices or enzymatically dissociated cells were obtained from the carotid bodies that were dissected out from wild-type and

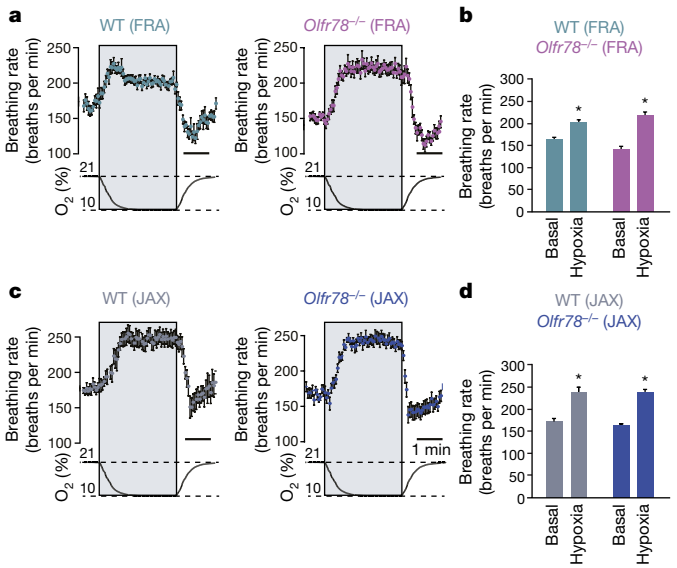

Fig. 1 | Ventilatory responses to hypoxia of wild-type and *Olfr78*$^{-/-}$ mice. a, Plethysmographic recordings (breathing frequency as a measure of time) of the ventilatory response to hypoxia (10% O_2) performed on wild-type (WT; $n = 10$) and *Olfr78*$^{-/-}$ ($n = 10$) FRA mice. Each data point represents the mean ± s.e.m. of the values for the group of 10 mice. Oxygen (percentage O_2) tensions are indicated at the bottom. **b**, Breathing frequency in normoxia (21% O_2) and during exposure to hypoxia (10% O_2) in *Olfr78*$^{-/-}$ FRA mice ($n = 10$) compared to their wild-type littermates ($n = 10$). **c**, **d**, Similar experiments as in **a**, **b** performed with wild-type mice ($n = 10$) and *Olfr78*$^{-/-}$ mice ($n = 10$) from the JAX colony. Data are mean ± s.e.m. Statistically significant differences compared to basal values are indicated; $^*P < 0.001$.

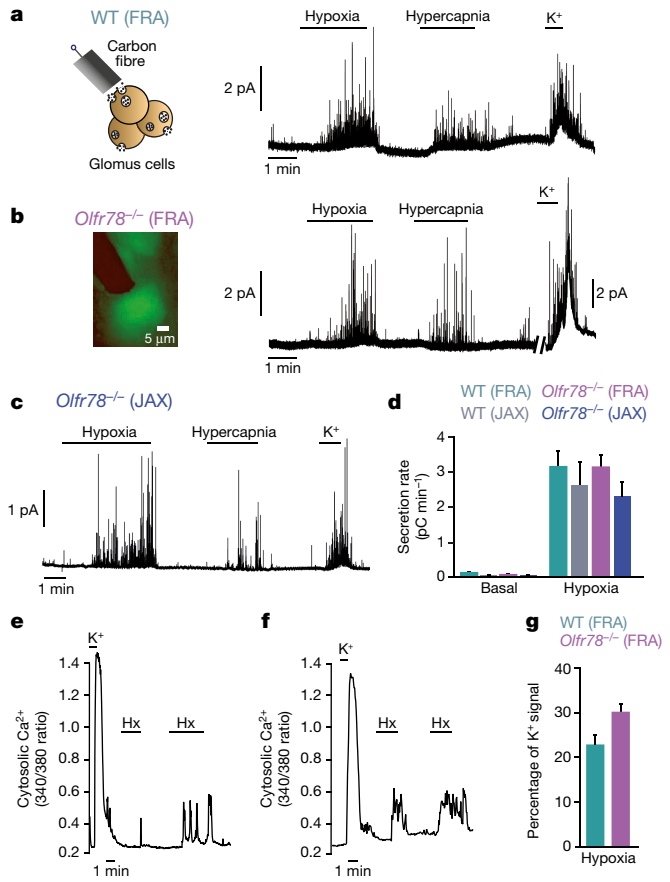

Fig. 2 | Physiological responses to acute hypoxia in carotid body slices and single dissociated glomus cells from wild-type and *Olfr78*$^{-/-}$ mice. **a**–**c**, Amperometric recordings showing glomus cell secretory responses to hypoxia ($p_{O_2} \approx 15$ mm Hg), hypercapnia (5% CO_2) and 40 mM KCl in carotid body slices from wild-type (**a**) and *Olfr78*$^{-/-}$ (**b**) mice of the FRA colony, as well as *Olfr78*$^{-/-}$ JAX mice (**c**). **a**, The drawing on the left illustrates the detection of exocytotic dopamine release by amperometry using a polarized carbon-fibre electrode. **b**, The microphotograph shows the carbon-fibre electrode and the intrinsic GFP fluorescence of glomus cells from *Olfr78*$^{-/-}$ mice. **d**, Secretion rate (picocoulombs per min) in basal conditions (normoxia, $p_{O_2} \approx 145$ mm Hg) and in response to hypoxia ($p_{O_2} \approx 15$ mm Hg) in carotid body slices from wild-type FRA mice (green; $n = 23$ cells, 9 mice) and wild-type JAX mice (grey; $n = 12$ cells, 7 mice) as well as *Olfr78*$^{-/-}$ FRA mice (purple; $n = 19$ cells, 7 mice) and *Olfr78*$^{-/-}$ JAX mice (blue; $n = 11$ cells, 6 mice). **e**, **f**, Increases in cytosolic Ca^{2+} levels elicited by hypoxia (Hx; approximately 15 mm Hg) in single, dissociated, Fura-2-loaded glomus cells from wild-type (**e**) and *Olfr78*$^{-/-}$ (**f**) FRA mice. **g**, Amplitude of changes in cytosolic Ca^{2+} levels induced by hypoxia in glomus cells from wild-type FRA mice (green; $n = 17$ cells, 5 mice) and *Olfr78*$^{-/-}$ FRA mice (purple; $n = 25$ cells, 5 mice) relative to the signal obtained with 40 mM K^+. Data are expressed as mean ± s.e.m.

Olfr78$^{-/-}$ mice. It is well-established that glomus cells, which can be activated by hypoxia, hypercapnia and other stimuli, are responsible for the chemosensory properties of the carotid body[1]. Cells in slices or dissociated glomus cells exhibit increases in exocytotic dopamine release or cytosolic [Ca^{2+}] as an inverse function of oxygen tension (p_{O_2}), which parallel the relationship between ventilation and p_{O_2} observed in the entire animal[1,13,14]. We fixed slices of the carotid body after studying the cellular responses to hypoxia, and determined co-expression of GFP (contained in the gene-targeted *Olfr78* allele[4,10]; Extended Data Fig. 1c) and tyroxine hydroxylase (TH), a characteristic marker of glomus cells (Extended Data Fig. 3a). In the initial in vitro experiments, glomus cells from wild-type and *Olfr78*$^{-/-}$ FRA mice showed similar secretory responses to hypoxia, hypercapnia and high K^+, as measured by amperometry using a polarized carbon fibre electrode, placed near the cells to oxidize the dopamine molecules that were released[3,14] (Fig. 2a, b). A normal responsiveness to hypoxia and the other stimuli was confirmed in glomus cells from *Olfr78*$^{-/-}$ JAX mice (Fig. 2c). The secretion rate (a measure of the number of dopamine molecules released per unit of time)[1,3] during exposure to hypoxia were the same in glomus cells from wild-type and *Olfr78*$^{-/-}$ mice of the FRA and JAX colonies (Fig. 2d). In addition, single dissociated glomus cells of wild-type and *Olfr78*$^{-/-}$ FRA mice, loaded with Fura-2[3,14], showed no difference in their increases of cytosolic Ca^{2+} levels to hypoxia (Fig. 2e–g). Therefore, our results at the cellular level are consistent with our results at the whole-animal level. We confirmed by quantitative PCR the absence of *Olfr78* mRNA in superior cervical ganglion tissue obtained from *Olfr78*$^{-/-}$ mice. The mRNA levels normalized to wild-type mice were: *Olfr78*$^{+/+}$, 1 ± 0.18, $n = 4$; *Olfr78*$^{+/-}$, 0.52 ± 0.10, $n = 5$; *Olfr78*$^{-/-}$, 0.0 ± 0.0, $n = 5$.

We thus cannot confirm the main conclusion reached in the previous study[4]. Genetic drift in a cohort of mice used by Chang et al. could have caused a loss of sensitivity of the carotid body to hypoxia in a way that is unrelated to the *Olfr78* mutation. Another possibility is that environmental factors, diet or co-existing microbiome that is specific to the animal facility in Stanford at the time of the experiments could have modulated the glomus cell function to become dependent on Olfr78. However, we obtained highly consistent data using several sources of mice bred or maintained in animal facilities in four countries (Spain, Germany, the United Kingdom and the United States).

The absolute requirement of lactate for carotid body activation during hypoxia suggested by Chang et al.[4] is incompatible with abundant data demonstrating that acute responsiveness to hypoxia is a cell-autonomous phenomenon, maintained in single dissociated glomus cells perfused with lactate-free solutions[1,3,13,14] (Fig. 2e–g). Unfortunately, Chang et al. did not support their conclusion with recordings of single cells. We have also observed that lactate at relatively high concentrations (around 5 mM or higher) can activate glomus cells from wild-type as well as *Olfr78*$^{-/-}$ FRA mice (Extended Data Fig. 3b–d). It seems, therefore, that Olfr78 is not required to confer lactate responsiveness to glomus cells. Two recent studies reported that lactate is a poor agonist for Olfr78[5,15], using the same heterologous expression system that was used in Chang et al.[4].

In summary, we conclude that Olfr78 is not required for the ventilatory response to hypoxia in mice, and is not required for activation of carotid body cells by lactate.

Data availability

Data are available upon request from the corresponding author.

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Author contributions All authors participated in the design of the experiments. H.T.-T., P.O.-S., D.M., M.O. and T.Z. performed the experiments and analysed data. R.S.J., J.L.-B., H.M. and P.M. supervised the study and wrote the manuscript.

Competing interests Declared none.

Additional information
Extended data accompanies this Comment.
Supplementary information accompanies this Comment.
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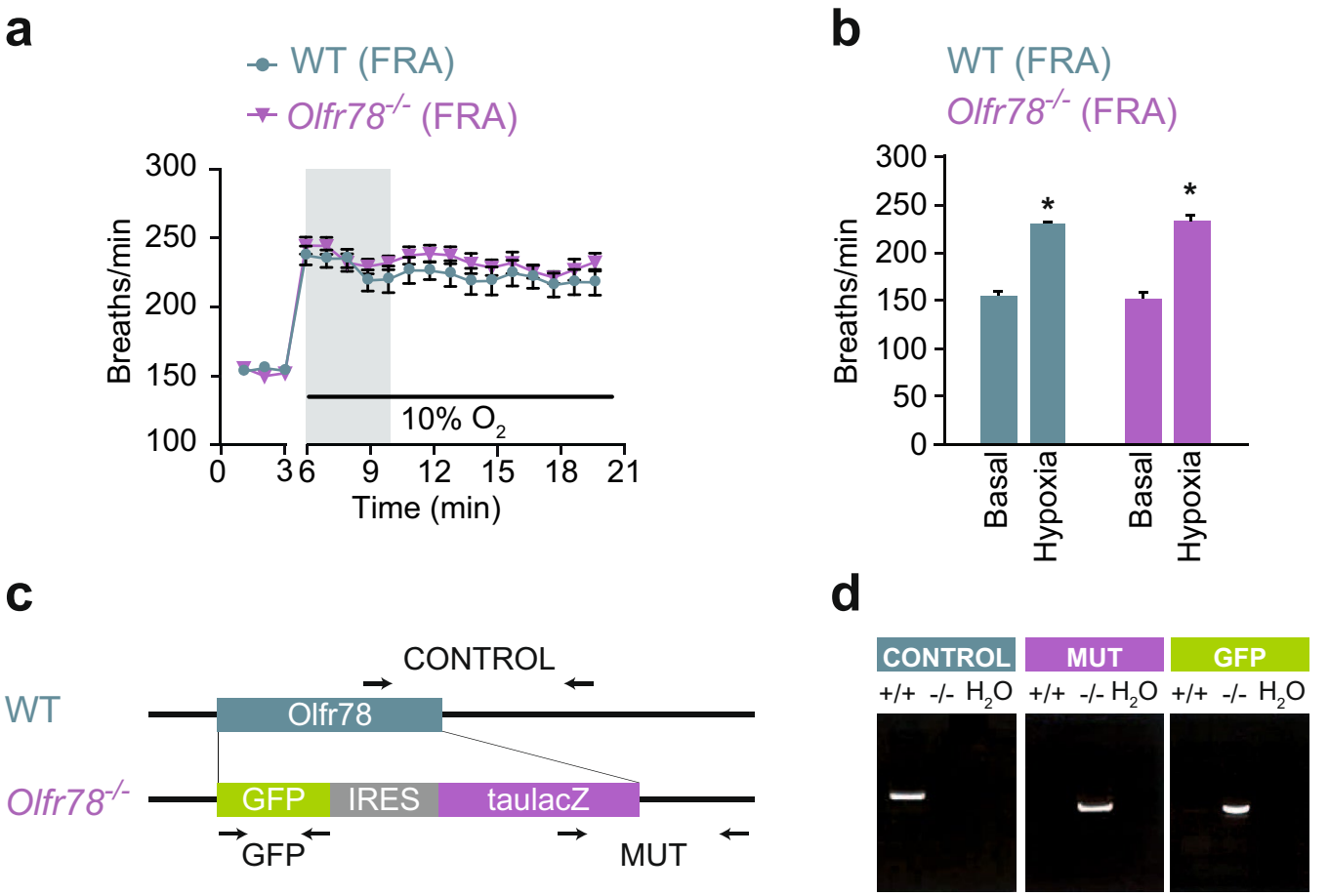
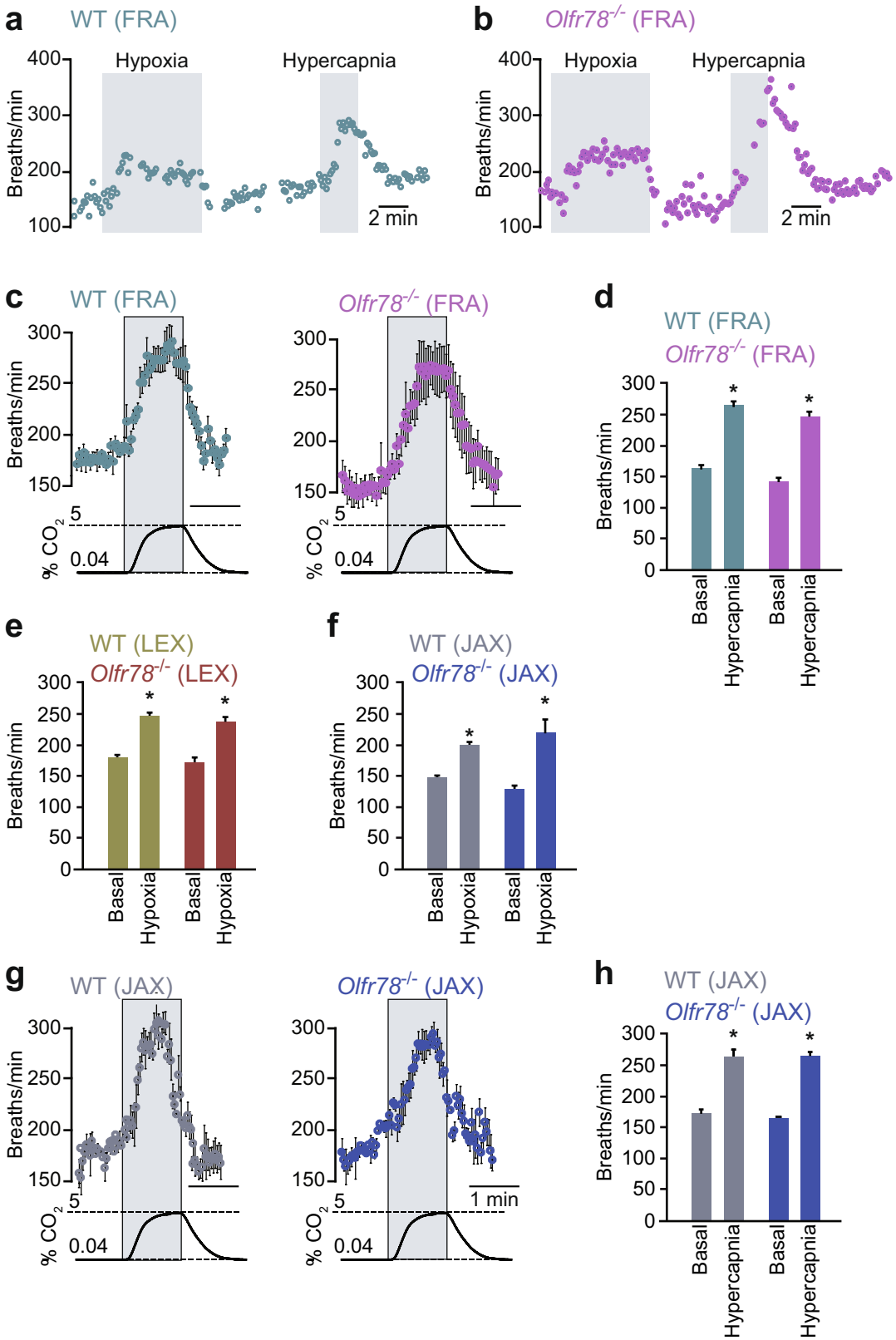
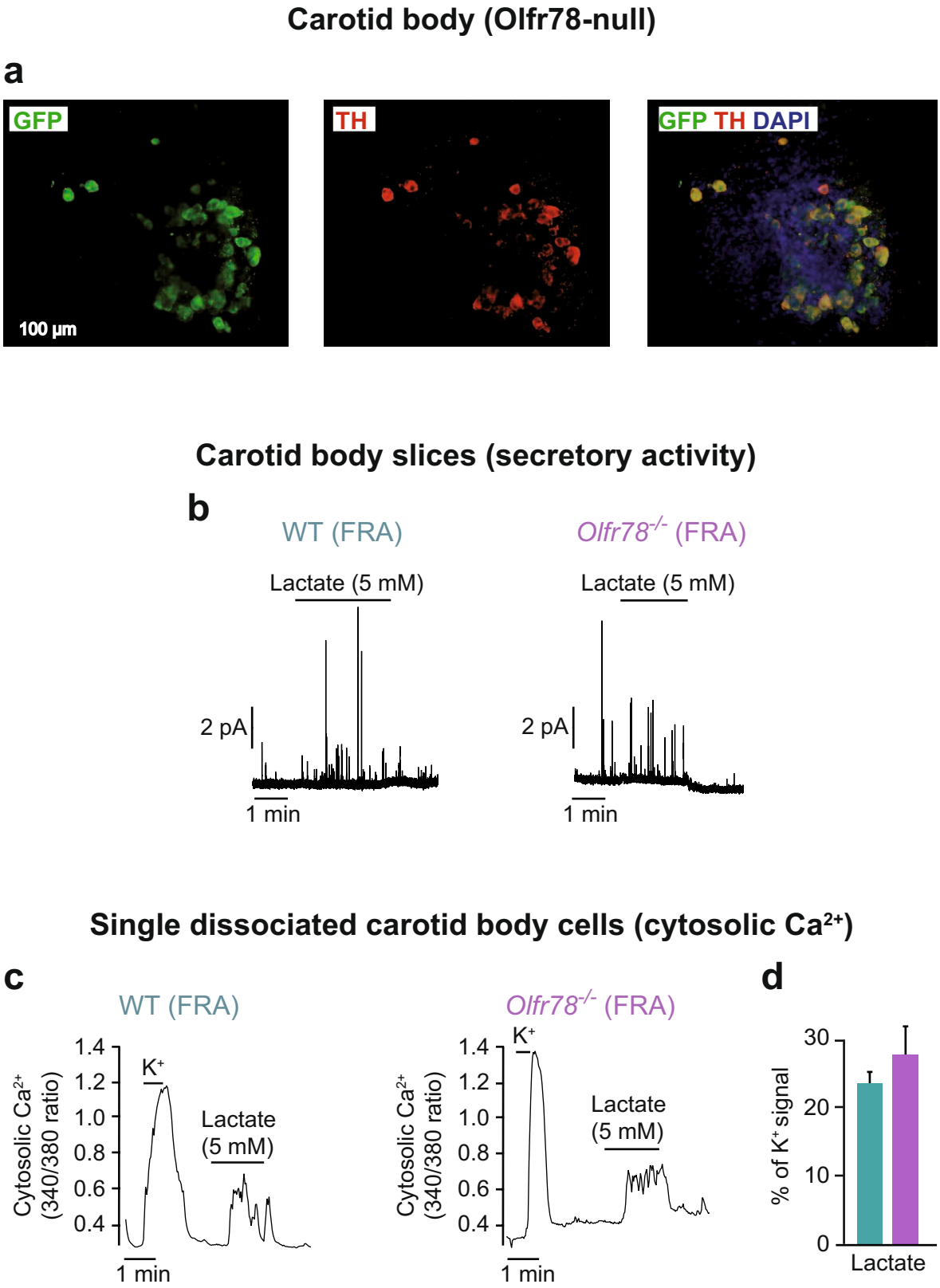


Extended Data Fig. 1 | Ventilatory responses to hypoxia and genotyping in wild-type and *Olfr78*$^{-/-}$ mice. a, Plethysmographic recordings (breathing frequency as a measure of time) of the ventilatory response to hypoxia (10% O_2). Each data point represents the mean ± s.e.m. of the values for control mice ($n = 10$; 7 wild-type and 3 heterozygous mice pooled together) and *Olfr78*$^{-/-}$ mice ($n = 9$) of the FRA colony. The grey-shaded area indicates the first five consecutive measurements after the transition to hypoxia used for quantification (see Supplementary Information). **b**, Breathing frequency in normoxia (basal) and during exposure to hypoxia (10% O_2) of control FRA mice ($n = 10$, 7 wild-type and 3 heterozygous mice) and *Olfr78*$^{-/-}$ FRA mice ($n = 9$). Data are mean ± s.e.m. Statistically significant differences compared to basal values are indicated; $^*P < 0.001$. **c**, Schematic of the wild-type and gene-targeted *Olfr78* alleles. Olfr78, intronless coding region of *Olfr78*; GFP, green-fluorescent protein; IRES, internal ribosome entry site; taulacZ, fusion of bovine tau with β-galactosidase. The arrows indicate the position and orientation of PCR primers used for genotyping. **d**, Genotyping of genomic tail DNA of wild-type (*Olfr78*$^{+/+}$) and homozygous (*Olfr78*$^{-/-}$) mice by PCR. The PCR primer pair 'CONTROL' amplifies the wild-type *Olfr78* allele; the PCR primer pair 'GFP' amplifies internal *GFP* sequences; and the PCR primer pair 'MUT' amplifies the gene-targeted *Olfr78* allele.



Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Ventilatory responses to hypoxia and hypercapnia in wild-type and *Olfr78*$^{-/-}$ mice. a, **b**, Representative examples of plethysmographic recordings (breathing frequency) during exposure to hypoxia (10% O_2) and hypercapnia (5% CO_2) in a wild-type FRA mouse and in a *Olfr78*$^{-/-}$ FRA mouse. **c**, Plethysmographic recordings (breathing frequency as a measure of time) of the ventilatory response to hypercapnia (5% CO_2) performed on wild-type ($n = 10$) and *Olfr78*$^{-/-}$ ($n = 10$) FRA mice. Each data point represents the mean ± s.e.m. of the values for the group of 10 mice. CO_2 (percentage CO_2) tensions are indicated at the bottom. **d**, Breathing frequency during exposure to hypercapnia (5% CO_2) in *Olfr78*$^{-/-}$ FRA mice ($n = 10$) compared to their wild-type littermates ($n = 10$). **e**, Breathing frequency during exposure to hypoxia (10% O_2) in *Olfr78*$^{-/-}$ LEX mice compared to wild-type LEX mice ($n = 10$ for each genotype, 7 pairs in a C57BL/6 background, 3 pairs in a C57BL/6:129S5 mixed background, 9 out of 10 pairs are sex-matched littermates). **f**, Breathing frequency during exposure to hypoxia (10% O_2) in *Olfr78*$^{-/-}$ JAX mice ($n = 6$) compared to their wild-type littermates ($n = 5$), in experiments carried out at Duke University. **g**, Plethysmographic recordings (breathing frequency as a measure of time) of the ventilatory response to hypercapnia (5% CO_2) performed on wild-type ($n = 7$) and *Olfr78*$^{-/-}$ ($n = 7$) JAX mice. Each data point represents the mean ± s.e.m. CO_2 (percentage CO_2) tensions are indicated at the bottom. **h**, Breathing frequency during exposure to hypercapnia (5% CO_2) in *Olfr78*$^{-/-}$ JAX mice ($n = 7$) compared to their wild-type littermates ($n = 7$). Data are mean ± s.e.m. Statistically significant differences compared to basal values are indicated; $^*P < 0.001$.



Extended Data Fig. 3 | See next page for caption.

Extended Data Fig. 3 | Changes in cellular parameters elicited by lactate in carotid body glomus cells from wild-type and *Olfr78*$^{-/-}$ FRA mice. **a**, Immunohistochemical detection of GFP and tyrosine hydroxylase (TH) in a carotid body slice from an *Olfr78*$^{-/-}$ FRA mouse. **b**, Representative examples of quantal dopamine secretion from glomus cells in carotid body slices of wild-type FRA mice (left) and *Olfr78*$^{-/-}$ FRA mice (right) in response to external application of L-lactate (sodium lactate, 5 mM). **c**, Representative examples of increase in cytosolic Ca^{2+} levels elicited by L-lactate (5 mM) in single dissociated glomus cells from wild-type FRA mice (left) and *Olfr78*$^{-/-}$ FRA mice (right). **d**, Amplitude of changes in cytosolic Ca^{2+} levels induced by lactate in glomus cells from wild-type FRA mice (green, $n = 16$ cells, 5 mice) and *Olfr78*$^{-/-}$ FRA mice (purple, $n = 14$ cells, 5 mice) relative to the signal obtained with 40 mM K^{+}. Data are mean ± s.e.m.

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Software and code

Policy information about availability of computer code

Data collection	All the commercial software used for data collection is clearly described in the Extended Data File. Methods.
Data analysis	Sigmaplot 12 and Igor Pro 4.08 carbon were used for routine data analysis.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	We defined the number of independent measurements necessary to ascertain if two parameters have similar or different values based on the experimental evidence and the previous experience in our laboratory. The analysis of the hypoxic ventilatory response (the most critical variable analyzed in our study) was done with 10 mice of the different strains used.
Data exclusions	Not applicable
Replication	Our study on the hypoxic ventilatory response is based on data replicated in three independent laboratories. The data obtained from in vitro preparations were systematically and clearly replicated in several independent experiments.
Randomization	Recordings from wild-type and Olfr78 knockout animals were done in paralel to allow day to day comparison of the parameters measured.
Blinding	The most critical experiments were blinded (see Extended Data File. Methods).

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☐	☒ Unique biological materials
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials	Please see Extended Data File. Methods.

Antibodies

Antibodies used	Primary antibodies: tyrosine hydroxylase antibody (TH) (cat. NB300-109) Novus; green fluorescence protein (GFP) (cat. GFP1020) AvesLab (see Extended data file. Methods). Secondary antibodies: Alexa 568 (cat. A11011) Invitrogen; Alexa 488 (cat. 103-545-155) Jackson ImmunoResearch Laboratories
Validation	Mouse TH primary antibody has previously been used in our laboratory (see Fernández-Agüera et al., Cell Metab 22: 825-837, 2015). GFP antibody has previously been validated by Rojas et al., Neuron 74:151-176, 2012.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	All the laboratory animals used are clearly indicated and explained in the Extended Data File. Methods.
Wild animals	The study did not involve wild animals

Field-collected samples	The study did not involve samples collected from the field

Chang et al. reply

REPLYING TO H. Torres-Torrelo et al. *Nature* **561**, https://doi.org/10.1038/s41586-018-0545-9 (2018)

In the accompanying Comment, Torres-Torrelo et al.[1], and others previously[2], confirmed the following key findings of our paper[3]: *Olfr78* is highly and selectively expressed in carotid body glomus cells; Olfr78 can be activated by lactate, as measured in *Olfr78*-transfected cultured cells; and lactate activates glomus cells at low-millimolar concentrations, within the physiological concentration range of lactate and the range that we measured for lactate activation (half-maximum effective concentration (EC_{50}) of around 4 mM) of *Olfr78*-transfected cultured cells.

The major difference that can be compared is in the observed behavioural response of $Olfr78^{-/-}$ mutant mice to acute hypoxia (hypoxic ventilatory response). The $Olfr78^{tm1Mom}$ mutants used in our original study were cryorecovered by JAX in 2010 from sperm in a mixed 129P2/OlaHsd × C57BL/6J background[3,4], but a marked decline in breeding in heterozygous intercrossed mice within three years of obtaining the animals prevented us from retesting the same cohort now. To address differences between the results presented by Torres-Torrelo et al.[1] and our previous study[3], we instead tested female progeny of newly cryorecovered $Olfr78^{+/-}$ mutant mice from JAX as well as $Olfr78^{+/-}$ mutant mice from the Frankfurt colony using behavioural protocols similar to our original study and the protocol used by Torres-Torrelo et al.[1]. Surprisingly, we did not observe a consistent defect in the hypoxic ventilatory response of either current strain under these conditions. Although we cannot exclude an effect due to differences in our current versus previous test conditions (the animal facility and equipment used in our original experiments in 2011 are no longer available), it seems more likely that the original cohort had an unusual genetic background that rendered a defect in this response more apparent in $Olfr78^{-/-}$ mutants. Because the carotid body response to hypoxia is still partially intact in $Olfr78^{-/-}$ mutants, including a fully functional response to acid[3] that contributes to carotid body oxygen sensing[5], the behavioural response to hypoxia is likely to be highly sensitive to differences in assay conditions and genetic background[6], as observed for mutants in the acid-sensing pathway[5,7]. We proposed that glomus cells separately sense both the extracellular lactate and hydrogen ions from lactic acid produced during acute hypoxia[3]; it may be necessary to disrupt both pathways simultaneously and acutely to observe a robust effect in behavioural assays.

Although we did not find a consistent defect in the hypoxic ventilatory response in the current *Olfr78* mutant strains, curiously we did observe a greater variance in their ventilatory frequency in hypoxia compared to wild-type littermates. Therefore, in addition to potential functional redundancy between Olfr78 and acid-sensing pathways, there may be a variable compensatory response induced by *Olfr78* loss. This could involve either of these carotid body pathways, or the peripheral and central adaptation mechanisms that restore a hypoxic ventilatory response following bilateral carotid body denervation[8,9].

The results of Torres-Torrelo et al.[1] from carotid body slices and isolated cells cannot be directly compared to our results derived from monitoring the integrated sensory output of the intact carotid body by recordings of the carotid sinus nerve. There is growing evidence that sensory signalling involves communication between glomus cells and other carotid body cells[10], so the effect of Olfr78 on signalling may be apparent only in the intact organ. Furthermore, Torres-Torrelo et al.[1] measured release of dopamine, which is not an excitatory transmitter in the rodent carotid body[11], and calcium responses of the glomus cells. Olfr78 may regulate a signalling step in glomus cells that is downstream or parallel to these responses, so the observations of Torres-Torrelo et al.[1] are not incompatible with our nerve recordings. A similar pair of seemingly contradictory observations was previously described for acid-sensing mutants, in which the carotid sinus nerve response to hypoxia was partially defective in mutants but the dopamine response was intact[5,12]. It will be important to determine not only how Olfr78 activity in glomus cells is modulated by lactate produced under hypoxia, but also which signalling step(s) the activated receptor regulates.

We thank Torres-Torrelo et al.[1] for their experiments showing that the *Olfr78* mutant phenotype is more complex than our original experiments revealed, and for motivating us and others to carefully define the relationship of Olfr78 to other pathways implicated in oxygen sensing in the carotid body and the physiological and genetic compensatory mechanisms that can influence them and other aspects of the hypoxic ventilatory response.

Three of the listed authors (N.S.K., H.H. and A.D.) contributed only to the work contained in this Reply.

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