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Genetic variation in HIF-2α attenuates ventilatory sensitivity and carotid body growth in chronic hypoxia in high-altitude deer mice

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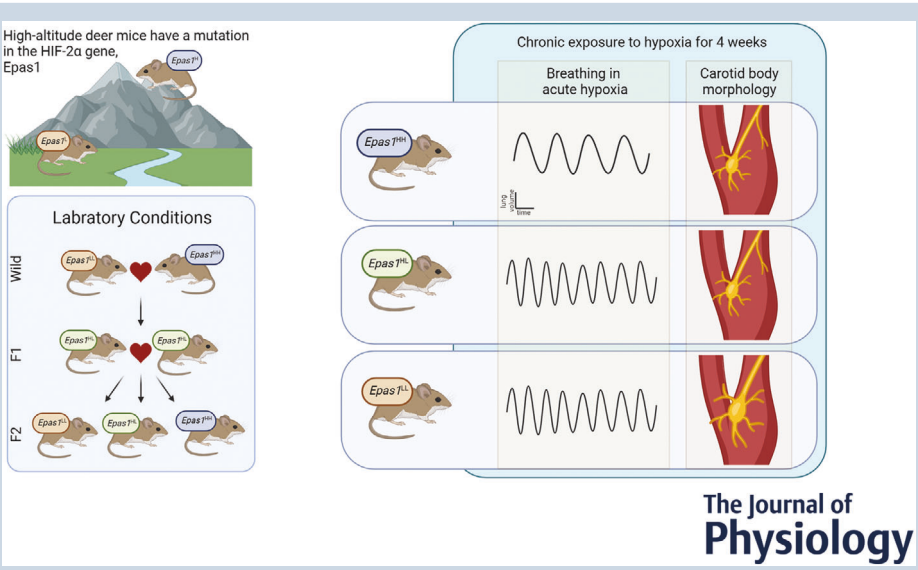

Abstract The gene encoding HIF-2α, *Epas1*, has experienced a history of natural selection in many high-altitude taxa, but the functional role of mutations in this gene is still poorly understood. We investigated the influence of the high-altitude variant of *Epas1* in North American deer mice (*Peromyscus maniculatus*) on the control of breathing and carotid body growth during chronic hypoxia. We created hybrids between high- and low-altitude populations of deer mice to disrupt

Catherine Ivy received her BScH in Zoology at the University of Guelph and her PhD in Biology at McMaster University. Her doctoral thesis investigated breathing responses to hypoxia in high-altitude deer mice and the role of high-altitude genetic variants on the control of breathing and the carotid body. Her doctoral work not only focused on adult breathing responses, but also the ontogeny of breathing in high and low-altitude populations of deer mice. Catherine is now a postdoctoral fellow at Western University where she is investigating the control of breathing in migratory songbirds and shorebirds.

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linkages between genetic loci so that the physiological effects of *Epas1* alleles (*Epas1*^{H} and *Epas1*^{L}, respectively) could be examined on an admixed genomic background. In general, chronic hypoxia (4 weeks at 12 kPa O_2) enhanced ventilatory chemosensitivity (assessed as the acute ventilatory response to hypoxia), increased total ventilation and arterial O_2 saturation during progressive poikilocapnic hypoxia, and increased haematocrit and blood haemoglobin content across genotypes. However, the effects of chronic hypoxia on ventilatory chemosensitivity were attenuated in mice that were homozygous for the high-altitude *Epas1* allele (*Epas1*$^{H/H}$). Carotid body growth and glomus cell hyperplasia, which was strongly induced in *Epas1*$^{L/L}$ mice in chronic hypoxia, was not observed in *Epas1*$^{H/H}$ mice. *Epas1* genotype also modulated the effects of chronic hypoxia on metabolism and body temperature depression in hypoxia, but had no effects on haematological traits. These findings confirm the important role of HIF-2α in modulating ventilatory sensitivity and carotid body growth in chronic hypoxia, and show that genetic variation in *Epas1* is responsible for evolved changes in the control of breathing and metabolism in high-altitude deer mice.

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Abstract figure legend We investigated the role of genetic variants in *Epas1* in high-altitude deer mice on the control of breathing. In the lab, hybrids between high- and low-altitude populations of deer mice were created to disrupt linkages between genetic loci so that the physiological effects of *Epas1* alleles (*Epas1*^{H} and *Epas1*^{L}, respectively) could be examined on an admixed genomic background. The high-altitude variant was associated with reduced ventilatory chemosensitivity and carotid body growth after 4 weeks of chronic hypoxia compared to mice homozygous for the low-altitude allele (*Epas1*^{LL}). These results help us better understand the genetic basis for the unique physiological phenotype of high-altitude natives.

Key points

- High-altitude natives of many species have experienced natural selection on the gene encoding HIF-2α, *Epas1*, including high-altitude populations of deer mice.
- HIF-2α regulates ventilation and carotid body growth in hypoxia, and so the genetic variants in *Epas1* in high-altitude natives may underlie evolved changes in control of breathing.
- Deer mice from controlled crosses between high- and low-altitude populations were used to examine the effects of *Epas1* genotype on an admixed genomic background.
- The high-altitude variant was associated with reduced ventilatory chemosensitivity and carotid body growth in chronic hypoxia, but had no effects on haematology.
- The results help us better understand the genetic basis for the unique physiological phenotype of high-altitude natives.

Introduction

The hypoxic chemoreflex is an important physiological response to environmental hypoxia. Reductions in the partial pressure of O_2 (P_{O_2}) in arterial blood initiate the hypoxic chemoreflex by stimulating the O_2-sensitive type I (glomus) cells in the carotid bodies. Increases in afferent activity from the carotid bodies lead to increases in ventilation termed the hypoxic ventilatory response (Ivy & Scott, 2015; Powell et al., 1998). It also activates the sympathetic nervous system, which can result in α-adrenoreceptor-mediated vasoconstriction that tends to increase vascular resistance in some peripheral tissues, and preferentially redistribute blood flow towards hypoxia-sensitive organs (i.e. brain and heart) (Hainsworth & Drinkhill, 2007; Ivy & Scott, 2015). Days to weeks of hypoxia exposure leads to further increases in ventilation (ventilatory acclimatization to hypoxia, VAH), resulting from increases in O_2 chemosensitivity of the carotid bodies and increases in central gain of afferent signals transmitted to the brain stem (Pamenter et al., 2014; Reid & Powell, 2005), and also leads to growth and neovascularization of the carotid bodies (Kusakabe et al., 1993; Pardal et al., 2007; Powell et al., 1998; Wang et al., 2008). Sympathetic activation can also persist in chronic hypoxia (Bernardi et al., 1998; Calbet, 2003; Hainsworth & Drinkhill, 2007). Although

the hypoxic chemoreflex is critical to survival during acute exposure to severe hypoxia (Slotkin et al., 1988), the value of maintaining or amplifying the hypoxic chemoreflex in chronic hypoxia is less clear. Some associated responses are probably beneficial, particularly the increase in ventilation that can improve pulmonary O_2 uptake (albeit at a metabolic cost and placing greater reliance on mechanisms responsible for maintaining acid–base and water homeostasis). Other associated responses may be counterproductive, such as chronic sympathetic activation, because α-adrenoreceptor stimulation can oppose local vasodilatory factors and act to increase vascular resistance, impede O_2 supply to some peripheral tissues, and increase blood pressure (Bernardi et al., 1998; Calbet, 2003; Hainsworth & Drinkhill, 2007).

Recent evidence suggests that hypoxia-inducible factor (HIF) signalling plays a role in the changes in the hypoxic chemoreflex that occur in response to chronic hypoxia. HIFs act as key transcription factors responsible for co-ordinating many diverse cellular and systemic responses to hypoxia (Prabhakar & Semenza, 2012). HIF-α subunits (of which there are three: HIF-1α, HIF-2α and HIF-3α) are targeted for degradation in the presence of O_2, primarily through O_2-dependent hydroxylation by prolyl hydroxylases. This process is impeded in hypoxia, such that HIF-α accumulates, dimerizes with the HIF-β subunit, and the resulting HIF transcription factor can drive the expression of hypoxia responsive genes (Prabhakar & Semenza, 2012). VAH and carotid body hyperplasia during chronic hypoxia are strongly attenuated by pharmacological inhibition of HIF-2α (Cheng et al., 2020) and by acute inactivation of HIF-2α using Cre-Lox recombination either generally (Hodson et al., 2016) or only in glomus cells and other cells expressing tyrosine hydroxylase (Fielding et al., 2018). However, heterozygous knockout of HIF-2α and HIF-1α have yielded different conclusions in some other studies (Kline et al., 2002; Peng et al., 2011). Nevertheless, the balance of evidence suggests that HIF-2α mediated signalling is critical for the changes in the hypoxic chemoreflex that occur during chronic hypoxia, raising the important question of how variation in HIF-2α signalling between populations or species may affect these changes.

Recent research suggests that the HIF pathway has been a frequent target of selection in high-altitude natives. The gene encoding HIF-2α (*Epas1*) in particular has experienced a history of natural selection in human populations from the Qinghai–Tibet Plateau and in many animals native to high altitude (Ai et al., 2014; Beall et al., 2010; Buroker et al., 2012; Gou et al., 2014; Graham & McCracken, 2019; Li et al., 2013, 2014; Petousi et al., 2014; Qu et al., 2013; Schweizer et al., 2019; Simonson et al., 2012; Song et al., 2016; Yi et al., 2010). Studies have revealed that high-altitude *Epas1* variants appear to be associated with lower haemoglobin concentrations in Tibetans (Beall et al., 2010) and with altered cardiovascular function in hypoxia in high-altitude deer mice (Schweizer et al., 2019). Given the probable role of HIF-2α in VAH and carotid body hyperplasia, selection on *Epas1* could also contribute to some of the evolved differences in breathing and the hypoxic chemoreflex that have been observed in high-altitude natives (Beall et al., 1997; Ivy & Scott, 2018). In one particular study of the effects of *Epas1* genotype within Tibetan humans, there was no clear influence of *Epas1* genotype on ventilatory and cardiovascular responses to acute hypoxia, although this study investigated adults who lived at sea level for 4 years and did not control for developmental environment (Petousi et al., 2014). It remains to be determined whether the genetic variants in *Epas1* that exist in high-altitude natives affect breathing and the hypoxic chemoreflex in chronic hypoxia.

Deer mice (*Peromyscus maniculatus*) are an emerging model species for studying high-altitude adaptation. Deer mice are broadly distributed across North America and can be found from sea level to over 4300 m a.s.l. in the Rocky Mountains (Hock, 1964; Natarajan et al., 2015; Snyder et al., 1982). There is evidence for directional selection for a high aerobic capacity ($\dot{V}_{O_2max}$) for thermogenesis in deer mice at high altitude (Hayes & O'Connor, 1999), which has led to evolved increases in $\dot{V}_{O_2max}$ (Cheviron et al., 2012, 2013, 2014; Lui et al., 2015; Tate et al., 2020) along with evolved changes in haemoglobin (Hb)-O_2 affinity and various other traits that influence O_2 supply and utilization (Lau et al., 2017; Lui et al., 2015; Mahalingam et al., 2017, 2020; Snyder et al., 1982; Storz et al., 2009, 2010; Tate et al., 2020; West, Ivy et al., 2021; West, Wearing et al., 2021). In particular, high-altitude deer mice do not appear to exhibit VAH or carotid body hyperplasia in chronic hypoxia, in contrast to the robust VAH and carotid body growth exhibited by lowlanders (Ivy & Scott, 2017a, 2018). Recent evidence also suggests that there has been a history of spatially varying selection on *Epas1* in high-altitude deer mice, which has resulted in high frequencies of a unique high-altitude variant containing a non-synonymous DNA substitution ($^{C}2288^{T}$) in the 14th exon that leads to a polarity-altering amino acid change (threonine to methionine) at site 755 of the protein sequence ($^{Thr}755^{Met}$) (Schweizer et al., 2019). This mutation disrupts the interaction of HIF-2α with the transcriptional coactivator CREB-binding protein, and thus reduces HIF-2α transcriptional activity (Song et al., 2021). In the present study, we aimed to investigate the influence of this high-altitude variant of *Epas1* on VAH and carotid body hypertrophy in chronic hypoxia. This was achieved by creating hybrids between high- and low-altitude mice using a F_2 intercross breeding design, which helps disrupt the linkages between loci that result from population genetic structure, so that the effects of *Epas1* gene variants on breathing

and carotid body morphology could be evaluated on an admixed genetic background.

Methods

Ethical approval

All animal procedures were approved by the McMaster University Animal Research Ethics Board and the University of Montana Institutional Care and Use Committee, and followed principles set out by the Canadian Council on Animal Care, as well as *The Journal of Physiology* (Grundy, 2015). Mice were provided with unlimited access to water and standard mouse chow, and were maintained under standard holding conditions comprising a 12:12 h light/dark photocycle at ∼23°C. Death occurred via an overdose of anaesthetic (inhalation of >5% isoflurane vapour) followed by decapitation to confirm death.

Deer mice and intercross breeding design

Wild adult deer mice were live-trapped at high altitude on the summit of Mount Evans (Clear Creek County, CO, USA at 39′35′18′N, 105′38′38′W; 4350 m a.s.l.) (*Peromyscus maniculatus rufinus*) and at low altitude on the Great Plains (Nine Mile Prairie, Lancaster County, NE, USA at 40′52′12′N, 96′48′20.3′W; 430 m a.s.l.) (*Peromyscus maniculatus nebrascensis*) and then transported to the University of Montana (978 m a.s.l.). Wild mice from Mount Evans that were homozygous for the derived *Epas1*^{H} allele (H, high-altitude variant) with the non-synonymous substitution in the 14th exon ($^{C}2288^{T}$) were crossed with mice from Lincoln that were homozygous for the ancestral *Epas1*^{L} allele (L, low-altitude variant) at the same position (for genotyping protocols, see below). First-generation hybrid progeny (F_1) from two families (one from crossing a highland male and a lowland female, and one from crossing a highland female and a lowland male) were raised to maturity, and intercrossed in full-sibling matings to produce six families of second-generation progeny (F_2) (two and four families descended from each wild breeding pair, respectively). F_2 intercrossed mice included those that were homozygous (*Epas1*$^{H/H}$ or *Epas1*$^{L/L}$) or heterozygous (*Epas1*$^{H/L}$) for the alternative *Epas1* alleles on an otherwise admixed genomic background. Mice were held under normal atmospheric conditions at the University of Montana until they were shipped to McMaster University (50 m a.s.l.) for use in the experiments described below.

Wild mice and F_2 mice were genotyped using an ear clip sample as described previously (Schweizer et al., 2019). DNA was extracted using a DNeasy kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. PCR amplification of *Epas1* was then conducted with custom exonic primers (5′-GCACGCCTTCCAAGACAC-3′ and 5′-GGTGGCAGGTGTCTCAGT-3′) designed from the *Peromyscus maniculatus bairdii* genome (NCBI Accession GFC_000 500354.1) under the conditions: 94°C for 2 min; 30 cycles of 94°C for 45 s, 58°C for 1 min, 72°C for 1 min; then 72°C for 10 min. To improve amplification specificity for some samples, we used modified primers and PCR conditions (5′- AGGGCAGAGATGTAAACAGC-3′ and 5′-GAATGTGGTGCCGTCTGATG-3′): 94°C for 2 min; 35 cycles of 94°C for 30 s, 62°C for 30 s, 68°C for 1 min; then 68°C for 10 min.

Chronic exposure

Experiments on the role of *Epas1* genotype on responses to chronic hypoxia were carried out at least 5 weeks after transport from the University of Montana, using 35 F_2 mice that were raised in normobaric normoxia under standard holding conditions, and were 1.5–2 years old at the time of experiments. It is important to note that *Peromyscus* mice can live well beyond this age in captivity, with a maximum lifespan potential of ∼8 years, more than double that of *Mus* (Csiszar et al., 2007). Mice were then subjected to measurements of acute hypoxia responses (described below), after which a subset of mice were killed and then sampled for carotid body morphology and haematology (n = 5 *Epas1*$^{H/H}$, 3 female, 2 male; n = 6 *Epas1*$^{H/L}$, 3 female, 3 male; n = 6 *Epas1*$^{L/L}$, 3 female, 3 male). The remaining subset of mice were non-terminally sampled for haematology (using ∼30 μL of blood collected from the tail vein) and then subjected to 4 weeks of hypobaric hypoxia simulating the hypoxic conditions at an elevation of 4300 m (barometric pressure of 60 kPa, an O_2 partial pressure of ∼12.5 kPa) (n = 5 *Epas1*$^{H/H}$, 3 female, 2 male; n = 7 *Epas1*$^{H/L}$, 4 female, 3 male; n = 6 *Epas1*$^{L/L}$, 3 female, 3 male). After acclimation, this second subset of mice was subjected to another series of acute hypoxia response measurements, and the mice were then killed and sampled for carotid body morphology and haematology. Specially designed hypobaric chambers were used for chronic hypoxia exposures, as described previously (Ivy & Scott, 2017a; Lui et al., 2015; McClelland et al., 1998). Mice were temporarily returned to normobaric conditions twice per week for cage cleaning (which took <20 min).

Ventilatory and metabolic responses to hypoxia

We measured hypoxia responses using two protocols (conducted in random order and separated by at least 2 days). Measurements were made in unrestrained mice using the barometric plethysmography and respirometry techniques employed in previous studies (Ivy & Scott, 2017a, 2018). One protocol was used to test acute

ventilatory sensitivity to hypoxia. Mice were placed in a whole-body plethysmography chamber (530 mL) that was supplied with normoxic air (21 kPa O_2, balance N_2) at 2 L min^{-1}. Mice were given 20–40 min to adjust to the chamber until relaxed and stable breathing and metabolism were observed. Breathing was then measured every minute for an additional 5 min at 21 kPa O_2, then during 5 min of acute exposure to hypoxia and elevated CO_2 (10 kPa O_2, 3 kPa CO_2, balance N_2), and then finally during 5 min of recovery in normoxia (21 kPa O_2, balance N_2). The use of elevated inspired CO_2 in this protocol was used to avoid the respiratory hypocapnia/alkalosis that can occur during exposure to hypoxia in absence of inspired CO_2, as in previous studies (Bishop et al., 2013; Fielding et al., 2018; Hodson et al., 2016). Indeed, the chosen level of inspired $CO_{2\,h}$as been shown to minimize the fall in arterial PCO_2 and rise in arterial pH during exposure to hypoxia in mice (Ishiguro et al., 2006). As a result, the responses to hypoxia in this protocol probably provide a good reflection of ventilatory sensitivity to acute hypoxia in absence of the confounding effects of CO_2/pH disruption. Metabolism was not measured in these tests of acute ventilatory sensitivity because we were focused on the initial ventilatory response to hypoxia, before any appreciable changes in whole-animal metabolism were expected to have occurred, and because of the high chamber flow rates used to rapidly change inspire O_2 and CO_2.

We used a second protocol to measure the ventilatory and metabolic responses to progressive stepwise hypoxia under poikilocapnic (uncontrolled CO_2) conditions. Mice were placed in the same plethysmograph as described above, and given 20–40 min to adjust to the chamber with 21 kPa O_2 supplied at 600 mL min^{-1}. Measurements were then recorded for 20 min at 30 kPa O_2 (hyperoxia), after which mice were exposed to 20 min stepwise reductions in inspired P_{O_2} to 21, 16, 12, 10 and 8 kPa. In this stepwise poikilocapnic hypoxia protocol, breathing and metabolism were measured during the last 10 min of exposure at each step when stable measurements had been reached. For each protocol, incurrent gas composition was set by mixing dry compressed gases using precision flow meters (Sierra Instruments, Monterey, CA, USA) and a mass flow controller (MFC-4; Sable Systems, Las Vegas, NV, USA). Body temperature (T_b) was measured every 5 min using thermosensitive passive transponders (micro LifeChips with Bio-therm technology; Destron Fearing, Dallas, TX, USA) that were implanted subdermally on the left side of the abdomen close to the leg ∼2 weeks before normoxic measurements were conducted.

Breathing and metabolism were measured as follows. Breathing frequency and tidal volume were measured in both protocols using whole-body plethysmography (Ivy & Scott, 2017a, 2017b), and total ventilation was calculated as the product of breathing frequency and tidal volume. Total ventilation and tidal volume data are reported in volumes expressed at body temperature and pressure saturated. Metabolism was measured in the stepwise poikilocapnic hypoxia protocol as recommended by Lighton (2008). Gas composition was measured continuously in incurrent and excurrent air flows that were subsampled at 200 mL min^{-1}; incurrent air was continuously measured for O_2 fraction (FC-10; Sable Systems), dried with pre-baked drierite and analysed for O_2 and CO_2 fraction (FC-10 and CA-10; Sable Systems). These data were used to calculate rates of O_2 consumption ($\dot{V}_{O_2}$), expressed at standard temperature and pressure, using established equations (Lighton, 2008). The air convection requirement is the quotient of total ventilation and $\dot{V}_{O_2}$. All data was acquired using a PowerLab 16/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA). Arterial O_2 saturation was measured using MouseOx Plus pulse oximeter collar sensors and data acquisition system (Starr Life Sciences, Oakmont, PA, USA). This was enabled by removing fur around the neck ∼2 days before the experiments.

Haematology

Blood was collected for haematology and Hb-O_2 affinity assays, both before and after chronic exposures. Blood Hb content was measured using Drabkin's reagent (Sigma-Aldrich, Oakville, ON, Canada) in accordance to the manufacturer's instructions, and haematocrit was measured by spinning blood in a heparinized capillary tube at 12 700 ***g*** for 5 min. Oxygen dissociation curves were generated at 37°C for all mice using a Hemox Analyzer (TCS Scientific, New Hope, PA, USA) using 10 μL of whole blood in 5 mL of buffer (50 mmol L^{-1} HEPES, 10 mmol L^{-1} EDTA, 100 mmol L^{-1} KCl, 0.1% bovine serum albumin, and 0.2% anti-foaming agent; TCS Scientific). Red cell O_2 affinity (P_{50}, the P_{O_2} at which Hb is 50% saturated with O_2) was calculated using Hemox Analytic Software (TCS Scientific).

Immunohistochemistry of the carotid bodies

We examined carotid body morphology using similar approaches to those we have used previously in deer mice (Ivy & Scott, 2017a). Mice were killed as described above, and the bifurcations of the carotid artery were dissected, removed and fixed in 4% paraformaldehyde for 48 h. Tissues were then incubated in 24% sucrose solution for cryoprotection, frozen in embedding medium (Cryomatrix; Thermo Fisher Scientific, Waltham, MA, USA) and cryosectioned at 10 μm thickness using a CM-1860 cryostat (Leica, Wetzlar, Germany). Every second section through one entire carotid body was

mounted on slides (Superfrost Plus Fisherbrand; Thermo Fisher Scientific), which amounted to 10–31 sections per animal. Slides were then air-dried and stored at −80°C. Sections were immunostained with rabbit anti-tyrosine hydroxylase antibody (TH; dilution 1:2000; AB152; Millipore, Billerica, MA, USA), mouse anti-neurofilament (NF; dilution 1:100; MAB1615, Millipore) and mouse anti-growth-associated protein-43 (GAP-43; dilution 1:2000; G9264, Sigma-Aldrich), followed by detection with the fluorescent secondary antibodies AlexaFluor488 goat anti-rabbit (dilution 1:400; A11034, Life Technologies, Mississauga, ON, Canada) and AlexaFluor594 goat anti-mouse (dilution 1:400; A11032, Life Technologies) along with DAPI (4′,6-diamidino-2-phenylindole; dilution 1:100 000, Sigma-Aldrich). Sections were imaged using a microscope (Olympus, Tokyo, Japan) with Northern Eclipse software (Elite, version 8.0; Empix Imaging, Mississauga, ON, Canada). The staining of neurons (NF and GAP-43) and type I cells (TH) was used to delineate the outer border of the carotid body, such that the projected area of the carotid body could be determined for each section (NIS Elements documentation software, version 4.30.02; Nikon, Tokyo, Japan). Total carotid body volume was then calculated as the sum of the volumes in each section (calculated as the product of projected area and section thickness), multiplied by 2 to account for the fact that we only kept every second section (Ivy & Scott, 2017a; Saiki et al., 2006). The number of type I cells in each section were counted using ImageJ, version 1.47 (NIH, Bethesda, MD, USA) and the total number of type I cells was similarly calculated as twice the sum of the number counted across all sections.

Statistical analysis

Two- or three-factor ANOVA was carried out as appropriate to test for the main effects of acclimation environment, genotype and/or inspired P_{O_2}, as well as their interactions. When the main effects or interactions from ANOVA were significant, Holm–Sidak post tests were used to test for pairwise differences between groups. Specifically, when the main effect or interactions with acclimation environment (e.g. acclimation × genotype, acclimation × P_{O_2}) were significant, we conducted pairwise comparisons between acclimation environments within each genotype and inspired P_{O_2} (or just within each inspired P_{O_2} when mice of all genotypes were considered altogether). When the effects of genotype were significant, we conducted pairwise comparisons between genotypes within each environment and inspired P_{O_2}. In all cases, the P values from the comparisons made using these Holm–Sidak post tests are reported. P values from ANOVA for main effects and interactions are also reported. Values are reported as the mean ± SD, often along with individual values, and the reported N reflects the number of individual animals. All statistical analysis was conducted with R, version 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria). $P < 0.05$ was considered statistically significant.

Results

Ventilatory chemosensitivity to hypoxia

Ventilatory chemosensitivity increased after chronic exposure to hypoxia in deer mice, as occurs in many

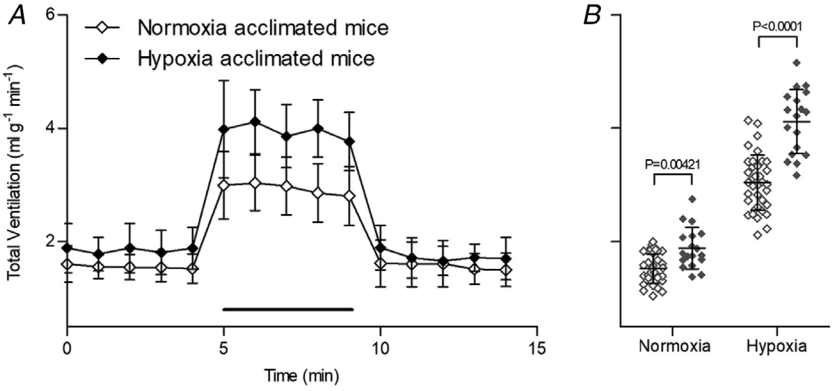

Figure 1. Chronic hypoxia increased ventilatory sensitivity to acute hypoxia when deer mice of all genotypes were considered altogether
A, changes in total ventilation over time in normoxia (21 kPa O_2; no line) and hypoxia (10 kPa O_2 with 3 kPa CO_2; black line), shown as the mean ± SD, to illustrate the treatment used to measure ventilatory sensitivity. *B*, total ventilation immediately before (minute 4) and just after (minute 6) the transition from normoxia to hypoxia was analysed statistically, with data shown as individual values, as well as the mean ± SD. Holm–Sidak *post hoc* tests carried out in two-factor ANOVA were used to compare acclimation groups for data in (*B*), for which P values are shown (two-factor ANOVA results: acclimation, $P < 0.0001$; P_{O_2}, $P < 0.0001$; acclimation × P_{O_2}, $P < 0.0001$). N (N females, N males) were: 35 (19, 16) normoxia-acclimated mice and 18 (10, 8) hypoxia-acclimated mice.

Table 1. Breathing frequency and tidal volume in tests of ventilatory chemosensitivity when deer mice of all genotypes were considered altogether

	Acclimation environment		
	Normoxia	Hypoxia	*P*
	Breathing frequency (min^{-1})		
Normoxia	144.3 ± 24.8	157.2 ± 30.2	NS
Acute hypoxia	270.0 ± 46.5	292.8 ± 60.6	NS
	Tidal volume ($\mu l\ g^{-1}$)		
Normoxia	10.85 ± 2.15	12.29 ± 3.09	0.0668
Acute hypoxia	11.56 ± 2.58	14.59 ± 3.52	**<0.0001**

Values are the mean ± SD for measurements in normoxia (21 kPa O_2) and acute hypoxia (10 kPa O_2 with 3% CO_2). *N* as in Fig. 1. *P* values above are for pairwise comparisons between acclimation environments within a measurement condition, conducted using Holm–Sidak *post hoc* tests when effects of acclimation environment were significant in ANOVA ('NS' denotes when acclimation effects were not significant and post tests were not carried out). Two-factor ANOVA results were as follows. Breathing frequency: acclimation, $P = 0.0627$; inspired P_{O_2}, $P < 0.0001$; acclimation × P_{O_2}, $P = 0.4924$. Tidal volume: acclimation, $P = 0.0021$; P_{O_2}, $P < 0.0001$; acclimation × P_{O_2}, $P = 0.0523$.

other species (Aaron & Powell, 1993; Bishop et al., 2013; Sato et al., 1992), when mice of all genotypes were considered altogether (Fig. 1 and Table 1). Ventilatory chemosensitivity was determined using a brief exposure to hypoxia to avoid confounding changes in metabolism, along with elevated levels of CO_2 in inspired air to avoid secondary hypocapnia (see Methods). In general, acute exposure to hypoxia elicited a strong increase in total ventilation of ∼2-fold (Fig. 1*B*), which arose from large increases in breathing frequency and more modest increases in tidal volume (Table 1). Chronic acclimation to hypoxia increased total ventilation in normoxia ($P = 0.00421$) and led to a much greater ventilatory response to acute hypoxia ($P < 0.0001$) (Fig. 1*B*), which was associated with a significant main effect of acclimation environment and a significant environment × P_{O_2} interaction in the two-factor ANOVA ($P < 0.0001$). The effects of chronic hypoxia on breathing and ventilatory chemosensitivity were driven largely by increases in tidal volume, which was greater in acute hypoxia after acclimation to chronic hypoxia ($P < 0.0001$) (Table 1).

The effect of chronic hypoxia on ventilatory chemosensitivity was attenuated in mice with the *Epas1*$^{H/H}$ genotype (Fig. 2 and Table 2). There was a significant main effect of genotype on total ventilation ($P = 0.0270$), which was detected in the three-factor ANOVA used to test for overall effects of genotype, acclimation environment, and P_{O_2}. This effect of genotype was not driven by variation among normoxia-acclimated mice, in which total ventilation in normoxia and in response to acute hypoxia was similar between *Epas1* genotypes (Fig. 2*B*). However, total ventilation during acute hypoxia was significantly lower in *Epas1*$^{H/H}$ mice than in *Epas1*$^{H/L}$ mice ($P = 0.00260$) and *Epas1*$^{L/L}$ mice ($P = 0.0148$) in comparisons conducted among hypoxia-acclimated mice (Fig. 2*D*). As a result, the hypoxic ventilatory response (calculated as the absolute change in total ventilation from normoxia to acute hypoxia) increased after chronic hypoxia acclimation in *Epas1*$^{L/L}$ mice ($P = 0.0168$) and *Epas1*$^{H/L}$ mice ($P < 0.0001$), but not in *Epas1*$^{H/H}$ mice ($P = 0.0900$) (Fig. 2*E*). The attenuated increase in total ventilation in *Epas1*$^{H/H}$ mice was associated with significant differences in breathing frequency between genotypes ($P = 0.000782$, main effect of genotype in the three-factor ANOVA) (Table 2), but no significant differences in tidal volume (Table 2).

Ventilatory and metabolic responses to progressive hypoxia

We next aimed to determine whether *Epas1* genotype affected the ventilatory response to progressive stepwise hypoxia during poikilocapnic conditions, in which increases in breathing can augment CO_2 release and thus induce respiratory hypocapnia. This was motivated by previous studies suggesting that *Epas1* knockout has much less pronounced effects on the hypoxic ventilatory response measured under poikilocapnic conditions compared to isocapnic conditions (Hodson et al., 2016). All mice increased total ventilation in response to stepwise hypoxia as a result of increases in breathing frequency, offset by small declines in tidal volume (Fig. 3). However, there was a clear difference between genotypes in the effects of chronic hypoxia acclimation, as reflected by a significant interaction between genotype and acclimation environment for breathing frequency in the three-factor ANOVA ($P = 0.00312$). In particular, chronic hypoxia augmented breathing frequency at 12 kPa O_2 ($P = 0.00830$ and 0.0345), 10 kPa O_2 ($P = 0.000200$ and 0.00420) and 8 kPa O_2 ($P < 0.0001$) in mice with *Epas1*$^{H/L}$ (Fig. 3*B*) and *Epas1*$^{L/L}$ (Fig. 3*C*) genotypes, but chronic hypoxia had no effect on breathing frequency in *Epas1*$^{H/H}$ mice (Fig. 3*A*). There was also a significant genotype × acclimation interaction for total ventilation in the three-factor ANOVA ($P = 0.0191$), although total ventilation in severe hypoxia increased after chronic hypoxia in *Epas1*$^{H/H}$ mice ($P = 0.00311$ at 8 kPa O_2) (Fig. 3*G*), *Epas1*$^{H/L}$ mice ($P < 0.0001$ at 8 kPa O_2, $P = 0.0170$ at 10 kPa O_2) (Fig. 3*H*) and *Epas1*$^{L/L}$ mice ($P < 0.0001$ at 8 kPa O_2) (Fig. 3*I*). Chronic exposure to hypoxia increased arterial O_2 saturation in hypoxia across genotypes, but there was also a significant genotype × acclimation

interaction for this trait in the three-factor ANOVA ($P = 0.00115$). In particular, increases in arterial O_2 saturation were significant across a broader range of inspired P_{O_2} in *Epas1*$^{H/L}$ (8–16 kPa O_2) (Fig. 3*K*) and *Epas1*$^{L/L}$ mice (8–12 kPa O_2) (Fig. 3*M*) than in *Epas1*$^{H/H}$ mice (8 kPa O_2 only) (Fig. 3*J*). Therefore, despite the potential confounding effects of respiratory hypocapnia, the high-altitude *Epas1* variant was still observed to have a blunted response to chronic hypoxia.

Epas1 genotype also affected metabolism and thermoregulation in chronic hypoxia, which may have contributed to some of the variation in breathing during progressive stepwise hypoxia. Exposure to severe hypoxia can elicit metabolic depression that tends to reduce ventilation (Dzal & Milsom, 2019; Ivy & Scott, 2017a, 2018; Olson et al., 2001; Tattersall et al., 2002), as reflected by lower rates of O_2 consumption in severe acute hypoxia compared to normoxia. However, there was a significant genotype × acclimation interaction for O_2 consumption rate in the three-factor ANOVA ($P = 0.00111$). In particular, O_2 consumption rate increased after chronic hypoxia in *Epas1*$^{H/H}$ mice across a broad range of inspired P_{O_2} (8–21 kPa O_2), but only increased in deep hypoxia in *Epas1*$^{L/L}$ mice and was

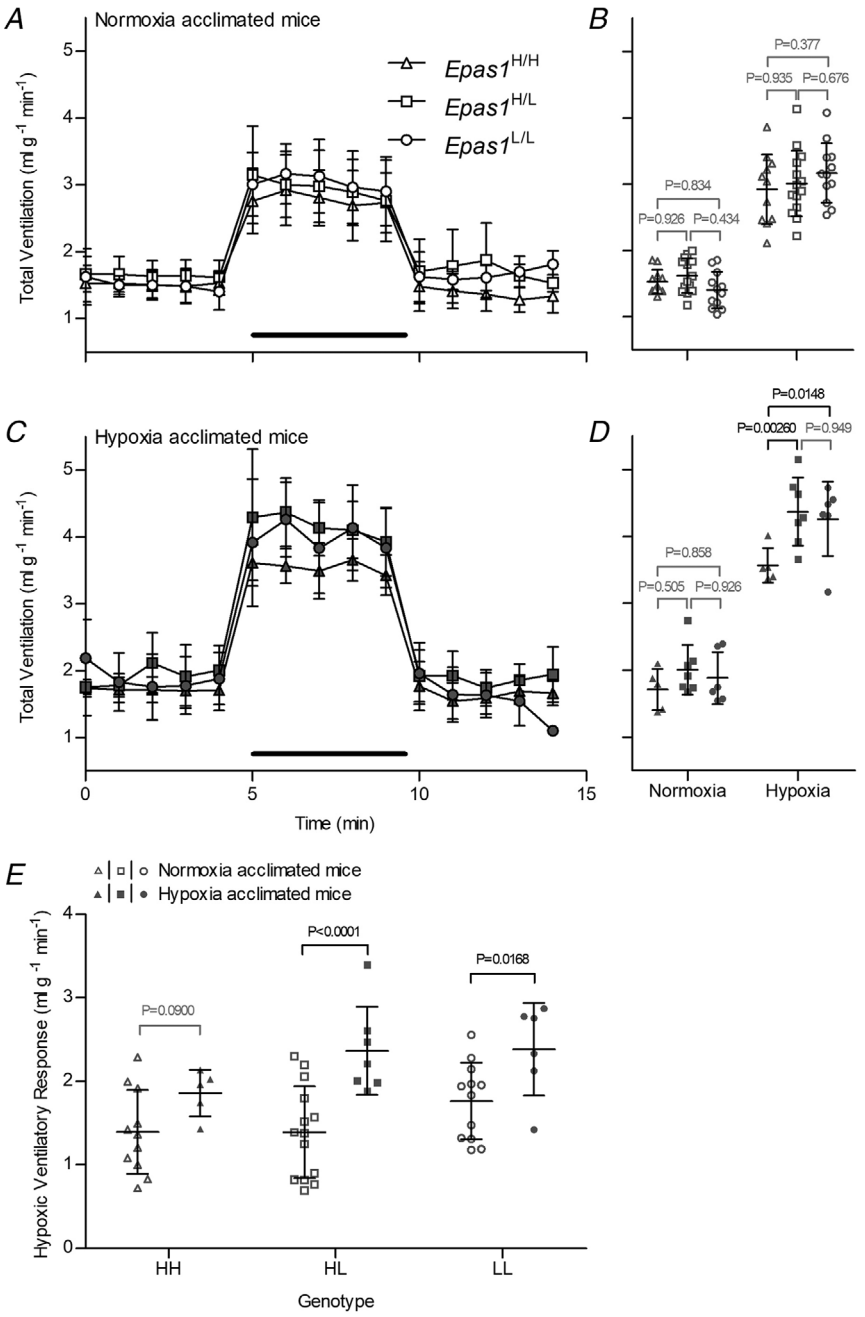

Figure 2. The effects of chronic hypoxia on ventilatory sensitivity to acute hypoxia was attenuated in deer mice with the *Epas1*$^{H/H}$ genotype
A and *C*, changes in total ventilation over time in normoxia (21 kPa O_2; no line) and hypoxia (10 kPa O_2 with 3 kPa CO_2; black line), shown as the mean ± SD, to illustrate the treatment used to measure ventilatory sensitivity. *B* and *D*, total ventilation immediately before (minute 4) and just after (minute 6) the transition from normoxia to acute hypoxia were analysed statistically, with data shown as individual values, as well as the mean ± SD. Holm–Sidak *post hoc* tests carried out in ANOVA were used to compare *Epas1*$^{H/H}$ with the other genotypes for data in (*B*) and (*D*), for which *P* values are shown (three-factor ANOVA results: genotype, $P = 0.0270$; acclimation, $P < 0.0001$; P_{O_2}, $P < 0.0001$; genotype × acclimation, $P = 0.0627$; genotype × P_{O_2}, $P = 0.0853$; acclimation × P_{O_2}, $P < 0.0001$; genotype × acclimation × P_{O_2}, $P = 0.411$). *E*, the hypoxic ventilatory response (i.e. the absolute change in total ventilation from normoxia to acute hypoxia) is shown as individual values and the mean ± SD. Holm–Sidak *post hoc* tests were used to compare acclimation groups within each genotype for data in (*E*) (two-factor ANOVA results: genotype, $P = 0.0621$; acclimation, $P < 0.0001$; genotype × acclimation, $P = 0.328$). *N* (*N* females, *N* males) were: 10 (6, 4) normoxia-acclimated *Epas1*$^{H/H}$ mice, 13 (7, 6) normoxia-acclimated *Epas1*$^{H/L}$ mice, 12 (6, 6) normoxia-acclimated *Epas1*$^{L/L}$ mice, 5 (3, 2) hypoxia-acclimated *Epas1*$^{H/H}$ mice, 7 (4, 3) hypoxia-acclimated *Epas1*$^{H/L}$ mice and 6 (3, 3) hypoxia-acclimated *Epas1*$^{L/L}$ mice.

Table 2. Breathing frequency and tidal volume of deer mice with different *Epas1* genotypes in tests of ventilatory chemosensitivity

Acclimation environment	Measurement condition	Genotype			*P*
		Epas1$^{H/H}$	*Epas1*$^{H/L}$	*Epas1*$^{L/L}$	
		Breathing frequency (min^{-1})			
Normoxia	Normoxia	135.97 ± 24.39	149.70 ± 28.94	145.74 ± 19.40	0.748, 0.900
	Hypoxia	239.91 ± 26.76	270.58 ± 39.13	297.01 ± 53.99	0.132, **0.0014**
Hypoxia	Normoxia	145.10 ± 31.38	157.00 ± 31.59	167.50 ± 29.05	0.932, 0.698
	Hypoxia	250.67 ± 32.89	327.32 ± 72.57	287.52 ± 42.71	**0.0023**, 0.294
		Tidal volume (μl g^{-1})			
Normoxia	Normoxia	11.57 ± 1.70	11.15 ± 2.26	9.83 ± 2.17	NS
	Hypoxia	12.24 ± 2.12	11.46 ± 2.38	11.06 ± 3.21	NS
Hypoxia	Normoxia	12.17 ± 3.37	13.04 ± 3.04	11.53 ± 3.28	NS
	Hypoxia	14.44 ± 2.08	14.10 ± 4.41	15.27 ± 3.78	NS

H, highland allele; L, lowland allele. Values are the mean ± SD for measurements in normoxia (21 kPa O_2) and acute hypoxia (10 kPa O_2 with 3% CO_2). *N* as in Fig. 2. *P* values above are for pairwise comparisons of *Epas1*$^{H/L}$ and *Epas1*$^{L/L}$ to *Epas1*$^{H/H}$, respectively, within each acclimation environment and measurement condition, conducted using Holm–Sidak *post hoc* tests when effects of genotype were significant in ANOVA ('NS' denotes when genotype effects were not significant and post tests were not carried out). Three-factor ANOVA results were as follows. Breathing frequency: genotype, $P = 0.000782$; acclimation, $P = 0.0270$; P_{O_2}, $P < 0.0001$; genotype × acclimation, $P = 0.308$; genotype × P_{O_2}, $P = 0.0960$; acclimation × P_{O_2}, $P = 0.550$; genotype × acclimation × P_{O_2}, $P = 0.0861$. Tidal volume: genotype, $P = 0.368$; acclimation, $P = 0.000111$; P_{O_2}, $P = 0.0210$; genotype × acclimation, $P = 0.554$; genotype × P_{O_2}, $P = 0.483$; acclimation × P_{O_2}, $P = 0.161$; genotype × acclimation × P_{O_2}, $P = 0.810$.

not increased by chronic hypoxia in *Epas1*$^{H/L}$ mice (Table 3). As a result, chronic hypoxia led to declines in air convection requirement (the ratio of total ventilation to O_2 consumption rate) at 10 kPa O_2 in *Epas1*$^{H/H}$ mice ($P = 0.0184$), but chronic hypoxia had no effect in *Epas1*$^{L/L}$ mice and it increased air convection requirement at 8 kPa O_2 in *Epas1*$^{H/L}$ mice ($P = 0.00463$) (Table 4). There was also a significant genotype × environment interaction for body temperature in the three-factor ANOVA ($P < 0.0001$). Chronic hypoxia increased body temperature during acute exposure to deep hypoxia in *Epas1*$^{H/H}$ mice ($P = 0.000416$ at 10 kPa O_2; $P < 0.0001$ at 8 kPa O_2) and *Epas1*$^{L/L}$ mice ($P = 0.00351$ at 8 kPa O_2), but chronic hypoxia had no effect on body temperature in *Epas1*$^{H/L}$ mice (Table 5). Therefore, *Epas1* genotype also appears to affect metabolism and thermoregulatory control in chronic hypoxia.

Carotid body morphology

Hypoxia acclimation enlarged the carotid bodies in *Epas1*$^{L/L}$ mice, but had no significant effects in *Epas1*$^{H/H}$ or *Epas1*$^{H/L}$ mice (Fig. 4). There was a significant main effect of hypoxia acclimation on carotid body volume in the two-factor ANOVA ($P = 0.0211$). This effect was driven by a significant increase after chronic hypoxia in *Epas1*$^{L/L}$ mice (~2-fold; $P = 0.00807$), but no significant increases in *Epas1*$^{H/L}$ mice or *Epas1*$^{H/H}$ mice (Fig. 4*G*). There was also a significant main effect of hypoxia acclimation on the number of type I cells in the carotid body ($P = 0.00104$), which was again driven by a significant increase after hypoxia acclimation in *Epas1*$^{L/L}$ mice ($P = 0.00113$) but not in *Epas1*$^{H/H}$ or *Epas1*$^{H/L}$ mice (Fig. 4*H*).

Haematology and Hb-O_2 binding affinity

Whole-blood Hb content and haematocrit increased after chronic hypoxia acclimation in all mice, with no changes in the Hb-O_2 affinity measured in erythrocytes (Fig. 5). There were strong main effects of acclimation environment on Hb content ($P < 0.0001$) (Fig. 5*A*) and haematocrit ($P < 0.0001$) (Fig. 5*B*) in the two-factor ANOVA. Both traits increased after hypoxia acclimation in *Epas1*$^{L/L}$ mice ($P = 0.00901$ and $P < 0.0001$, respectively), *Epas1*$^{H/L}$ mice ($P < 0.0001$) and *Epas1*$^{H/H}$ mice ($P < 0.0001$). Individual values for Hb P_{50} varied across the range exhibited by populations of deer mice from high and low altitudes (Fig. 5*C*) (Ivy, Greaves et al., 2020), although there was no significant variation in Hb P_{50} detected by ANOVA across groups.

Discussion

Recent research suggests that the HIF pathway has been a target of selection in high-altitude deer mice and in high-altitude natives of many other species of mammals and birds (Ai et al., 2014; Beall et al., 2010; Buroker et al., 2012; Gou et al., 2014; Graham & McCracken, 2019; Li et al., 2013; Petousi et al., 2014; Qu et al., 2013; Schweizer et al., 2019; Simonson et al., 2012; Song

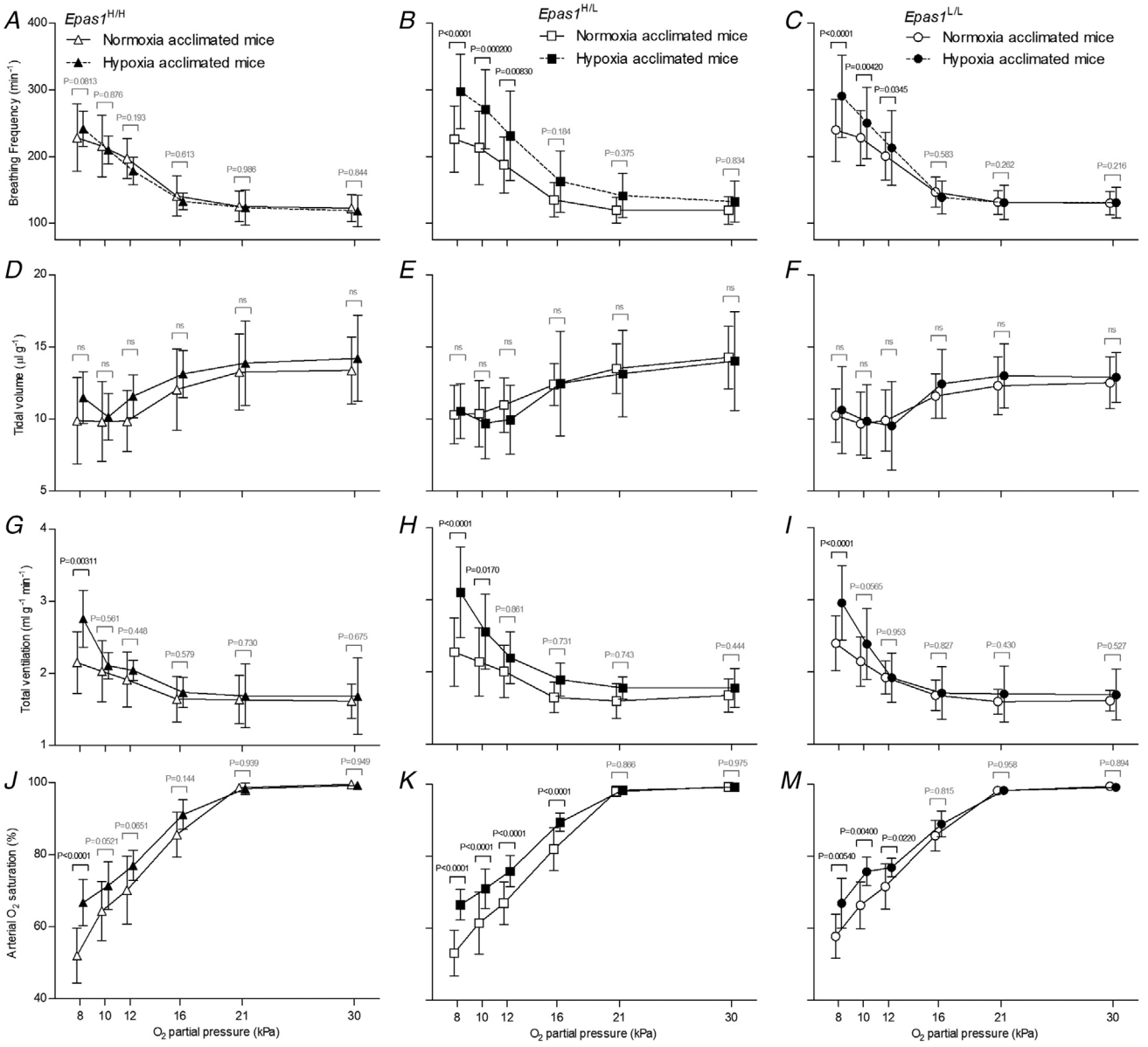

Figure 3. The effects of chronic hypoxia on the ventilatory response to stepwise hypoxia under poikilocapnic conditions were attenuated in deer mice with the *Epas1*$^{H/H}$ genotype
Values are the mean ± SD and data are offset on the *x*-axis to assure that error bars are clearly visible. Holm–Sidak *post hoc* tests were used to compare between acclimation groups within each P_{O_2} for each genotype when the effects of acclimation environment were significant in ANOVA, for which *P* values are generally shown ('ns' denotes when acclimation effects were not significant and *post hoc* tests were not carried out). *A–C*, breathing frequency (three-factor ANOVA results: genotype, $P = 0.348$; acclimation, $P < 0.0001$; P_{O_2}, $P < 0.0001$; genotype × acclimation, $P = 0.00312$; genotype × P_{O_2}, $P = 0.460$; acclimation × P_{O_2}, $P < 0.0001$; genotype × acclimation × P_{O_2}, $P = 0.667$). *D–F*, tidal volume (three-factor ANOVA results: genotype, $P = 0.603$; acclimation, $P = 0.0852$; P_{O_2}, $P < 0.0001$; genotype × acclimation, $P = 0.562$; genotype × P_{O_2}, $P = 0.605$; acclimation × P_{O_2}, $P = 0.596$; genotype × acclimation × P_{O_2}, $P = 0.902$). *G–I*, total ventilation (three-factor ANOVA results: genotype, $P = 0.260$; acclimation, $P < 0.0001$; P_{O_2}, $P < 0.0001$; genotype × acclimation, $P = 0.0191$; genotype × P_{O_2}, $P = 0.276$; acclimation × P_{O_2}, $P < 0.0001$; genotype × acclimation × P_{O_2}, $P = 0.955$). *J–M*, arterial O_2 saturation (three-factor ANOVA results: genotype, $P = 0.241$; acclimation, $P < 0.0001$; P_{O_2}, $P < 0.0001$; genotype × acclimation, $P = 0.00115$; genotype × P_{O_2}, $P = 0.124$; acclimation × P_{O_2}, $P < 0.0001$; genotype × acclimation × P_{O_2}, $P = 0.826$). *N* (*N* females, *N* males) were: 10 (6, 4) normoxia-acclimated *Epas1*$^{H/H}$ mice, 13 (7, 6) normoxia-acclimated *Epas1*$^{H/L}$ mice, 12 (6, 6) normoxia-acclimated *Epas1*$^{L/L}$ mice, 5 (3, 2) hypoxia-acclimated *Epas1*$^{H/H}$ mice, 7 (4, 3) hypoxia-acclimated *Epas1*$^{H/L}$ mice and 6 (3, 3) hypoxia-acclimated *Epas1*$^{L/L}$ mice.

Table 3. O_2 consumption rates of deer mice with different *Epas1* genotypes during progressive stepwise hypoxia in poikilocapnic conditions

P_{O_2} (kPa)	Normoxia acclimated mice	Hypoxia acclimated mice	*P*
	Epas1$^{H/H}$ genotype		
30	0.036 ± 0.007	0.037 ± 0.011	0.286
21	0.041 ± 0.007	0.048 ± 0.006	**0.00310**
16	0.042 ± 0.005	0.049 ± 0.004	**0.00170**
12	0.041 ± 0.005	0.050 ± 0.004	**<0.0001**
10	0.038 ± 0.006	0.047 ± 0.006	**<0.0001**
8	0.035 ± 0.004	0.045 ± 0.006	**<0.0001**
	Epas1$^{H/L}$ genotype		
30	0.039 ± 0.014	0.040 ± 0.005	0.715
21	0.044 ± 0.013	0.046 ± 0.006	0.910
16	0.045 ± 0.008	0.048 ± 0.007	0.318
12	0.046 ± 0.011	0.050 ± 0.006	0.231
10	0.042 ± 0.010	0.048 ± 0.005	0.0605
8	0.040 ± 0.010	0.045 ± 0.006	0.0815
	Epas1$^{L/L}$ genotype		
30	0.042 ± 0.009	0.038 ± 0.010	0.160
21	0.046 ± 0.008	0.048 ± 0.013	0.465
16	0.047 ± 0.008	0.047 ± 0.009	0.936
12	0.045 ± 0.008	0.048 ± 0.008	0.141
10	0.043 ± 0.008	0.050 ± 0.009	**0.0102**
8	0.039 ± 0.007	0.047 ± 0.007	**0.00220**

H, highland allele; L, lowland allele. Values are the mean ± SD. *N* as in Fig. 3. *P* values above are for pairwise comparisons between normoxia- and hypoxia-acclimated mice within a genotype and measurement P_{O_2}, conducted using Holm–Sidak *post hoc* tests following ANOVA. Three-factor ANOVA results: genotype, $P = 0.313$; acclimation, $P < 0.0001$; P_{O_2}, $P < 0.0001$; genotype × acclimation, $P = 0.00111$; genotype × P_{O_2}, $P = 0.976$; acclimation × P_{O_2}, $P = 0.000133$; genotype × acclimation × P_{O_2}, $P = 0.935$.

Table 4. Air convection requirements of deer mice with different *Epas1* genotypes during progressive stepwise hypoxia in poikilocapnic conditions

P_{O_2} (kPa)	Normoxia acclimated mice	Hypoxia acclimated mice	*P*
	Epas1$^{H/H}$ genotype		
30	45.69 ± 6.21	47.15 ± 13.9	0.223
21	39.80 ± 5.63	34.92 ± 5.64	0.516
16	39.03 ± 5.08	35.34 ± 2.87	0.679
12	46.64 ± 5.14	40.77 ± 2.09	0.121
10	53.60 ± 8.46	45.00 ± 2.78	**0.0184**
8	61.13 ± 8.31	61.35 ± 6.09	0.617
	Epas1$^{H/L}$ genotype		
30	47.89 ± 19.4	45.09 ± 6.94	0.390
21	39.24 ± 12.3	38.92 ± 4.69	0.719
16	37.56 ± 4.76	29.83 ± 5.95	0.838
12	44.99 ± 6.44	44.53 ± 7.31	0.219
10	51.57 ± 6.95	53.76 ± 11.86	0.789
8	58.25 ± 8.61	68.12 ± 9.07	**0.00463**
	Epas1$^{L/L}$ genotype		
30	39.00 ± 6.64	45.60 ± 6.35	**0.0337**
21	35.00 ± 4.34	36.20 ± 6.96	0.808
16	36.20 ± 3.72	36.91 ± 5.53	0.943
12	43.67 ± 4.03	39.95 ± 4.61	0.138
10	50.53 ± 3.75	48.50 ± 7.84	0.371
8	61.74 ± 6.43	62.43 ± 5.77	0.949

H, highland allele; L, lowland allele. Values are the mean ± SD. *N* as in Fig. 3. *P* values above are for pairwise comparisons between normoxia- and hypoxia-acclimated mice within a genotype and measurement P_{O_2}, conducted using Holm–Sidak *post hoc* tests following ANOVA. Three-factor ANOVA results: genotype, $P = 0.632$; acclimation, $P = 0.516$; P_{O_2}, $P < 0.0001$; genotype × acclimation, $P = 0.681$; genotype × P_{O_2}, $P = 0.757$; acclimation × P_{O_2}, $P = 0.00273$; genotype × acclimation × P_{O_2}, $P = 0.203$.

et al., 2016; Yi et al., 2010). Although there is a growing appreciation of the important role played by HIF-2α in the hypoxic chemoreflex and ventilatory responses to chronic hypoxia, the physiological implications of natural genetic variants in the HIF pathway remain poorly understood. Here, we show that deer mice homozygous for the high-altitude variant of the HIF-2α gene *Epas1* (*Epas1*$^{H/H}$) exhibit reduced ventilatory chemosensitivity and no carotid body growth after chronic exposure to hypoxia, in strong contrast to mice with the ancestral low-altitude variant. *Epas1* genotype also modulated the effects of chronic hypoxia on metabolic rate and body temperature depression in hypoxia. However, *Epas1* genotype had no significant effects on the increases in blood Hb content or haematocrit that were exhibited in chronic hypoxia in deer mice. These findings provide further support for the key role of *Epas1* in ventilatory chemosensitivity in chronic hypoxia, as well as the genetic mechanisms underlying the evolution of hypoxia resistance in high-altitude natives.

Mice with the *Epas1*$^{H/H}$ genotype exhibited reduced ventilatory chemosensitivity after acclimation to chronic hypoxia. Chronic hypoxia tended to increase the ventilatory response to acute hypoxia (Fig. 1), although this effect of chronic hypoxia was attenuated in *Epas1*$^{H/H}$ mice (Fig. 2*E*) in association with differences between genotypes in breathing frequency but not tidal volume (Table 2). Chronic hypoxia also augmented breathing frequency during severe poikilocapnic hypoxia in *Epas1*$^{L/L}$ and *Epas1*$^{H/L}$ mice, but not in *Epas1*$^{H/H}$ mice (Fig. 3). Two lines of evidence suggest that these observations result from a reduction in HIF-2α-mediated signalling within glomus cells of the carotid body in *Epas1*$^{H/H}$ mice. First, VAH is strongly attenuated in mice in which HIF-2α signalling was reduced by

Table 5. Body temperatures of deer mice with different *Epas1* genotypes during progressive stepwise hypoxia in poikilocapnic conditions

P_{O_2} (kPa)	Normoxia acclimated mice	Hypoxia acclimated mice	*P*
	Epas1$^{H/H}$ genotype		
30	37.82 ± 1.35	38.31 ± 0.43	0.938
21	37.45 ± 1.43	38.07 ± 0.31	0.654
16	37.15 ± 1.26	38.01 ± 0.28	0.189
12	36.57 ± 1.19	37.55 ± 0.56	0.0756
10	35.70 ± 1.15	37.34 ± 0.58	**0.000416**
8	34.82 ± 0.94	36.59 ± 1.22	**<0.0001**
	Epas1$^{H/L}$ genotype		
30	37.85 ± 1.44	38.08 ± 0.78	0.707
21	37.73 ± 1.35	37.69 ± 0.73	0.954
16	37.62 ± 1.23	37.52 ± 0.83	0.880
12	37.01 ± 1.28	37.07 ± 1.19	0.920
10	36.19 ± 1.41	36.42 ± 1.41	0.699
8	35.16 ± 1.53	35.61 ± 1.42	0.458
	Epas1$^{L/L}$ genotype		
30	38.15 ± 0.99	37.92 ± 1.26	0.663
21	37.93 ± 1.07	37.68 ± 1.31	0.599
16	37.67 ± 0.92	37.67 ± 1.09	0.820
12	37.27 ± 1.07	37.40 ± 0.90	0.537
10	36.41 ± 1.36	36.64 ± 1.33	0.360
8	35.48 ± 1.51	36.39 ± 0.76	**0.00351**

H, highland allele; L, lowland allele. Values are the mean ± SD. *N* as in Fig. 3. *P* values above are for pairwise comparisons between normoxia- and hypoxia-acclimated mice within a genotype and measurement P_{O_2}, conducted using Holm–Sidak *post hoc* tests following ANOVA. Three-factor ANOVA results: genotype, $P = 0.982$; acclimation, $P = 0.175$; P_{O_2}, $P < 0.0001$; genotype × acclimation, $P < 0.0001$; genotype × P_{O_2}, $P = 0.850$; acclimation × P_{O_2}, $P = 0.000240$; genotype × acclimation × P_{O_2}, $P = 0.869$.

pharmacological inhibition (Cheng et al., 2020) or by acute inducible knockout using Cre-Lox recombination (Hodson et al., 2016). The effects of reducing HIF-2α signalling appear to be specific to glomus cells because similar results were observed when HIF-2α was acutely knocked out only in cells expressing tyrosine hydroxylase (TH, an enzyme involved in catecholamine synthesis that is highly expressed in glomus cells), but not when knocked out in cells expressing glial fibrillary acidic protein (a marker of glial cells, including type II cells in the carotid body) (Fielding et al., 2018). Second, the $^{Thr}755^{Met}$ variant of HIF-2α in high-altitude deer mice reduces transcriptional activity by disrupting the interaction of HIF-2α with CREB-binding protein (Song et al., 2021), which would be expected to reduce HIF-2α signalling. This may also explain the modest differences in breathing frequency between genotypes among normoxia-acclimated mice (Table 2) because HIF-2α-mediated signalling occurs in normoxia in carotid body glomus cells, where it appears to be important for inducing the expression of genes responsible for O_2 sensing and hypoxic chemosensitivity (Macias et al., 2018; Moreno-Domínguez et al., 2020).

The effects of *Epas1* genotype on total ventilation were less pronounced during the progressive stepwise hypoxia protocol (Fig. 3), which would have coincided with respiratory hypocapnia and metabolic depression. This is consistent with previous studies in which variation in the magnitude of the hypoxic ventilatory response was more clearly seen when there was compensation for the secondary respiratory hypocapnia that occurs with increased ventilation (Hodson et al., 2016; Howard & Robbins, 1995). Ventilation is also strongly influenced by changes in metabolism during prolonged hypoxia exposure (Olson et al., 2001; Sprenger et al., 2019; Tattersall et al., 2002). The effects of hypocapnia and metabolism on total ventilation can arise via changes in tidal volume (Ivy & Scott, 2018), offsetting the effects of hypoxia on total ventilation. Nevertheless, this progressive hypoxia protocol was valuable for examining the potential role of *Epas1* genotype on the metabolic and thermoregulatory responses to hypoxia. Exposure to severe hypoxia reduced aerobic metabolism and body temperature (Tables 3 and 5), as we have previously observed in highland and lowland populations of deer mice (Ivy & Scott, 2017a, 2018), and as observed in other small mammals (Dzal & Milsom, 2019; Ivy & Scott, 2017b; Ivy, Sprenger et al., 2020; Tattersall et al., 2002). However, chronic exposure to hypoxia increased metabolism and attenuated body temperature depression in *Epas1*$^{H/H}$ mice but not in *Epas1*$^{H/L}$ mice. Such effects of *Epas1* genotype on metabolism probably masked the effects of differences in ventilatory chemosensitivity on total ventilation during progressive hypoxia. Nevertheless, the effects of chronic hypoxia on the breathing frequency response to progressive hypoxia were completely absent in *Epas1*$^{H/H}$ mice, in contrast to *Epas1*$^{H/L}$ and *Epas1*$^{L/L}$ mice. Therefore, the effects of chronic hypoxia on breathing frequency were attenuated in *Epas1*$^{H/H}$ mice in both experimental tests of the ventilatory responses to acute hypoxia. An important issue for future consideration is whether the influence of *Epas1* on VAH differs between sexes, considering the influence of sex on ventilation in chronic hypoxia (Joseph et al., 2000), although we lacked sufficient numbers of males and females to evaluate such sex differences here.

Mice with the *Epas1*$^{H/H}$ genotype did not exhibit significant carotid body growth in response to chronic hypoxia, unlike the strong carotid body growth and hyperplasia of glomus cells that occurred in *Epas1*$^{L/L}$ mice (Fig. 4). These findings also parallel research showing that reductions in HIF-2α signalling, either via pharmacological means or via inducible knockout

of HIF-2α (broadly or only in cells expressing TH), attenuates carotid body hyperplasia (Cheng et al., 2020; Fielding et al., 2018; Hodson et al., 2016). However, the relationship between carotid body growth and ventilatory chemosensitivity in chronic hypoxia remains unclear. VAH has several time domains that are underpinned by distinct mechanisms (Powell et al., 1998; Robbins, 2007) and increases in the O_2 sensitivity of the carotid bodies probably arise in part from mechanisms other than glomus cell proliferation (e.g. increased excitability of individual glomus cells) (Hempleman, 1995, 1996). Therefore, it remains to be determined whether there is a direct link between the effects of HIF-2α on glomus cell proliferation, carotid body O_2 sensitivity, and the hypoxic ventilatory response.

HIF-2α also appears to play an essential role in the survival and growth of carotid body glomus cells during development in normoxia. In house mice (*Mus*), embryonic deletion of *Epas1* restricted to cells expressing TH eliminates glomus cells at 8–12 weeks of age and abolishes the ventilatory response to hypoxia (Macias et al., 2018). This is in stark contrast to deer mice with the *Epas1*$^{H/H}$ genotype, in which glomus cell abundance was not reduced (Fig. 4) and the hypoxic ventilatory response was normal (Fig. 2*B*) compared to other genotypes among mice raised and held in normoxia. This could suggest that HIF-2α signalling is not completely eliminated and some functional roles of HIF-2α are maintained in *Epas1*$^{H/H}$ mice. Alternatively, this could suggest that additional genetic loci contribute to carotid body development in deer mice compared to *Mus*. Future work examining the development of the carotid bodies and ventilatory chemosensitivity between genotypes would be useful for examining this issue and for better defining the general phenotypic effects of variation in *Epas1*.

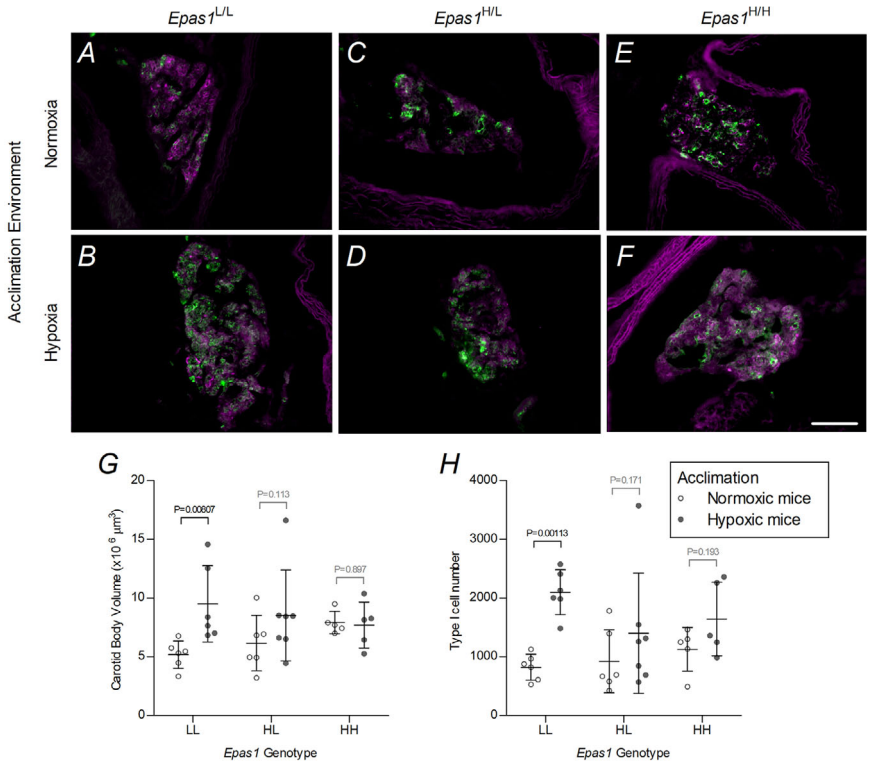

Figure 4. The effects of chronic hypoxia on carotid body growth were attenuated in deer mice with either the *Epas1*$^{H/H}$ or *Epas1*$^{H/L}$ genotype
A–F, fluorescence immunohistochemistry was used to identify type I cells (tyrosine hydroxylase, TH, in green) and neurons (neurofilament, NF, and growth-associated protein 43, GAP-43, in magenta). Scale bar = 100 μm. *G* and *H*, values for each individual animal are shown as circles along with the mean ± SD of these values. Holm–Sidak *post hoc* tests carried out in two-factor ANOVA were used to compare between acclimation groups within each genotype, for which *P* values are shown. *G*, total volume for a single carotid body (two-factor ANOVA results: genotype, $P = 0.893$; acclimation, $P = 0.0211$; genotype × acclimation, $P = 0.144$). *H*, total number of type I (glomus) cells for a single carotid body (two-factor ANOVA results: genotype, $P = 0.459$; acclimation, $P = 0.00104$; genotype × acclimation, $P = 0.216$). *N* (*N* females, *N* males) were: 6 (3, 3) normoxia-acclimated *Epas1*$^{L/L}$ mice, 6 (3, 3) normoxia-acclimated *Epas1*$^{H/L}$ mice, 5 (3, 2) normoxia-acclimated *Epas1*$^{H/H}$ mice, 6 (3, 3) hypoxia-acclimated *Epas1*$^{L/L}$ mice, 7 (4, 3) hypoxia-acclimated *Epas1*$^{H/L}$ mice and 5 (3, 2) hypoxia-acclimated *Epas1*$^{H/H}$ mice.

Haematological responses to chronic hypoxia were not altered by the high-altitude *Epas1* variant in deer mice (Fig. 5), consistent with previous findings in wild deer mice at high altitude (Schweizer et al., 2019). This contrasts the findings obtained in Tibetan humans, in which sequence variants in and around the *Epas1* gene are strongly associated with reduced blood Hb content at high altitude (Beall et al., 2010; Yi et al., 2010). This reduced blood Hb content does not result from a reduction in total Hb mass, but rather from an increase in plasma volume, suggesting that the erythropoietic response of Tibetan humans is similar to that of lowlanders (Stembridge et al., 2019). It remains unclear whether changes in plasma volume were the direct phenotypic target of selection acting on *Epas1* in Tibetan humans, or whether they were a secondary consequence of effects of the *Epas1* variant on other physiological traits that affect O_2 supply and function of tissues responsible for controlling plasma volume (Simonson, 2015; Storz & Scott, 2019; Storz et al., 2010). Nevertheless, the discrepancy between our results and these previous findings suggests that there may be some differences in the physiological effects of selection on *Epas1* across high-altitude taxa. Broad inducible knockout of *Epas1* blunts hypoxia-induced increases in haematocrit in mice (Hodson et al., 2016), suggesting that the high-altitude *Epas1* variant of deer mice does not completely eliminate HIF-2α-mediated signalling across the body.

Because we examined the effects of genetic variation in *Epas1* in F_2 interpopulation hybrids, it is important to consider the possibility that multiple genetic differences contribute to the physiological variation associated with *Epas1* genotype. *Epas1* is a very large gene (>70 kb) that contains several single-nucleotide polymorphisms (SNPs) in deer mice, but only three SNPs in close proximity exhibit significant differences in allele frequency between high- and low-altitude populations (Schweizer et al., 2019). Linkage disequilibrium to these three SNPs does not appear to extend beyond the boundaries of the *Epas1* coding region (Schweizer et al., 2019). Therefore, it is improbable that there is an unsampled causal variant in a nearby gene that has caused the physiological variation associated with *Epas1* genotype observed here. Of the three SNPs within *Epas1* that exhibit allele frequency differences between high- and low-altitude populations, the non-synonymous DNA substitution in the 14th exon that encodes the $^{Thr}755^{Met}$ variant in high-altitude mice has been shown to have strong effects on HIF-2α-mediated signalling (Song et al., 2021), as described earlier. There is an additional SNP exhibiting population differences in allele frequency in the 14th exon, but this is probably not the causal variant because it is a synonymous DNA substitution (Schweizer et al., 2019). The third SNP exhibiting population differences in allele frequency is located in the 3′ untranslated region (Schweizer et al., 2019). Although it remains possible that

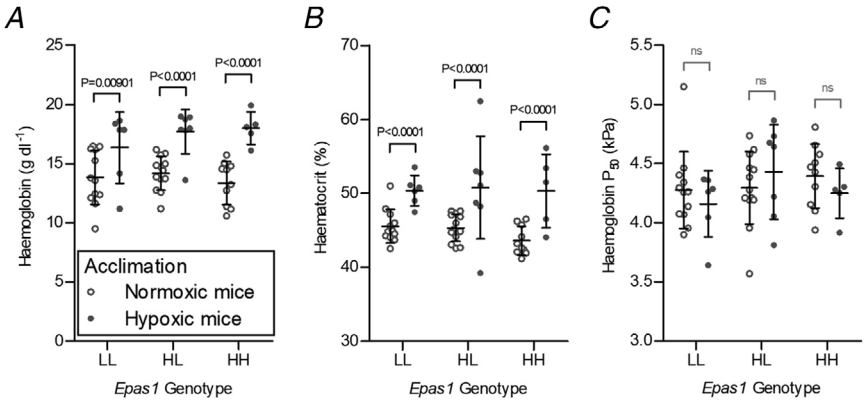

Figure 5. *Epas1* genotype did not alter the increases in blood Hb content or haematocrit in response to chronic hypoxia, and there was no significant variation in Hb-O_2 affinity across genotypes or acclimation groups

Individual values, as well as the mean ± SD, are shown. Holm–Sidak *post hoc* tests were used to compare between acclimation groups within each genotype when the effects of acclimation environment were significant in ANOVA, for which *P* values are generally shown ('ns' denotes when acclimation effects were not significant and *post hoc* tests were not carried out). *A*, whole-blood Hb content (two-factor ANOVA results: genotype, $P = 0.492$; acclimation, $P < 0.0001$; genotype × acclimation, $P = 0.301$). *B*, haematocrit (two-factor ANOVA results: genotype, $P = 0.644$; acclimation, $P < 0.0001$; genotype × acclimation, $P = 0.798$). *C*, Hb-O_2 affinity measured as the O_2 pressure at 50% saturation (P_{50}) in intact erythrocytes (two-factor ANOVA results: genotype, $P = 0.404$; acclimation, $P = 0.732$; genotype × acclimation, $P = 0.412$). *N* (*N* females, *N* males) were: 10 (6, 4) normoxia-acclimated *Epas1*$^{H/H}$ mice, 13 (7, 6) normoxia-acclimated *Epas1*$^{H/L}$ mice, 12 (6, 6) normoxia-acclimated *Epas1*$^{L/L}$ mice, 5 (3, 2) hypoxia-acclimated *Epas1*$^{H/H}$ mice, 7 (4, 3) hypoxia-acclimated *Epas1*$^{H/L}$ mice and 6 (3, 3) hypoxia-acclimated *Epas1*$^{L/L}$ mice.

the latter SNP could affect *Epas1* gene regulation and thus contribute to some of the physiological variation between *Epas1* genotypes, the known effects of the $^{Thr}755^{Met}$ mutation on HIF-2α-mediated signalling make this the most probable causal variant.

Because the causal variant lies within *Epas1*, our findings suggest that sequence variation in the HIF-2α gene may be partly responsible for the absence of VAH in natural populations of deer mice native to high altitude (Ivy & Scott, 2017a, 2018). Chronic hypoxia leads to robust increases in total ventilation during stepwise hypoxia under poikilocapnic conditions and in carotid body volume in low-altitude deer mice, but these changes do not occur in high-altitude deer mice (Ivy & Scott, 2017a, 2018). These observations could not be explained by clear population differences in CO_2 sensitivity that might have influenced the ventilatory response to poikilocapnic hypoxia (Ivy & Scott, 2018). Here, we show that the highland variant of *Epas1* contributes to reducing VAH and carotid body growth in high-altitude deer mice. The magnitude of the differences between *Epas1* genotypes observed here are not as great as the previously observed differences between natural populations, which may reflect the fact that effects of *Epas1* genotype were examined in interpopulation hybrids with a mixed genomic background. Nevertheless, the pattern of variation observed here suggests that *Epas1* genotype is a strong (if not sole) contributor to the evolved changes in VAH and carotid body growth in high-altitude mice. Our findings therefore provide an environmentally relevant context for the role of *Epas1* in carotid body O_2 sensing, and shed light on the physiological and genetic mechanisms of hypoxia tolerance in high-altitude natives.

The intriguing question that arises is whether the attenuated VAH observed in high-altitude deer mice is advantageous at high-altitude? Although high-altitude populations of deer mice do not express VAH, they exhibit a strong increase in effective ventilation compared to low-altitude populations (slower and deeper breaths) that is insensitive to chronic hypoxia (Ivy & Scott, 2017a, 2018; Ivy, Greaves et al., 2020, 2021), in contrast to the interpopulation hybrids studied here. It is possible that the combination of evolved changes in control of breathing in high-altitude deer mice serves to maintain O_2 uptake in chronic hypoxia (by virtue of the stable increase in effective ventilation) at the same time as attenuating some of the maladaptive effects of amplifying the hypoxic chemoreflex (e.g. persistent sympathoadrenal activation) (Scott et al., 2019; Storz & Scott, 2019). Consistent with this possibility, high-altitude deer mice also appear to have reduced catecholamine release from the adrenal medulla (Scott et al., 2019), which results at least in part from reduced expression of enzymes involved in catecholamine synthesis in mice with the highland *Epas1* variant (Schweizer et al., 2019). Therefore, the high-altitude variant of *Epas1 m*ay be important for restraining the hypoxic chemoreflex, avoiding some of its detrimental side effects, whereas other mechanisms assure high alveolar ventilation to help safeguard respiratory gas exchange.

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Additional information

Data Availability Statement

Physiological data have been deposited in Mendeley Data (doi:10.17632/sxv2bjpzhx.1).

Competing interests

The authors declare that they have no competing interests.

Author contributions

G.R.S. and Z.A.C. conceived the experiment. C.M.I. and J.P.V. raised mice. C.M.I. acquired, analysed and interpreted the data. All authors drafted and revised the manuscript. All authors approved the final version of the manuscript submitted for publication, agree to be accountable for all aspects of the work, and qualify for authorship.

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Keywords

high-altitude adaptation, hypoxic ventilatory response, oxygen chemosensitivity, peripheral chemoreceptor, ventilatory acclimatization to hypoxia

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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