

## RESEARCH ARTICLE

# Naked mole rat brain mitochondria electron transport system flux and H<sup>+</sup> leak are reduced during acute hypoxia

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## ABSTRACT

Mitochondrial respiration and ATP production are compromised by hypoxia. Naked mole rats (NMRs) are among the most hypoxia-tolerant mammals and reduce metabolic rate in hypoxic environments; however, little is known regarding mitochondrial function during *in vivo* hypoxia exposure in this species. To address this knowledge gap, we asked whether the function of NMR brain mitochondria exhibits metabolic plasticity during acute hypoxia. Respirometry was utilized to assess whole-animal oxygen consumption rates and high-resolution respirometry was utilized to assess electron transport system (ETS) function in saponin-permeabilized NMR brain. We found that NMR whole-animal oxygen consumption rate reversibly decreased by ~85% in acute hypoxia (4 h at 3% O<sub>2</sub>). Similarly, relative to untreated controls, permeabilized brain respiratory flux through the ETS was decreased by ~90% in acutely hypoxic animals. Relative to carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone-uncoupled total ETS flux, this functional decrease was observed equally across all components of the ETS except for complex IV (cytochrome *c* oxidase), at which flux was further reduced, supporting a regulatory role for this enzyme during acute hypoxia. The maximum enzymatic capacities of ETS complexes I–V were not altered by acute hypoxia; however, the mitochondrial H<sup>+</sup> gradient decreased in step with the decrease in ETS respiration. Taken together, our results indicate that NMR brain ETS flux and H<sup>+</sup> leak are reduced in a balanced and regulated fashion during acute hypoxia. Changes in NMR mitochondrial metabolic plasticity mirror whole-animal metabolic responses to hypoxia.

**KEY WORDS:** Cytochrome *c* oxidase, Citrate synthase, Proton gradient, Mitochondrial membrane potential

## INTRODUCTION

Naked mole rats (NMRs; *Heterocephalus glaber* Rüppell 1842) are among the most hypoxia-tolerant mammals identified. In the laboratory, NMRs tolerate <3% O<sub>2</sub> for several hours (Pamerter et al., 2014), 8% O<sub>2</sub> for days to weeks (Chung et al., 2016), and anoxia for up to 18 min (Park et al., 2017). This hypoxia tolerance is probably the result of adaptations to the putatively hypoxic atmosphere in NMR burrows. Indeed, it is speculated that in the wild, given their deep nests and the large number of animals within the colony, NMRs are likely to encounter chronic hypoxia throughout their lives. Within their burrows, NMRs are likely to

experience a gradient of hypoxic exposure, with the lowest level of O<sub>2</sub> occurring in the densely populated nest areas where the animals huddle in piles and sleep (Brett, 1991). Therefore, these animals probably experience intervals of acute and severe hypoxia while in their nest, lasting for several hours.

NMRs exhibit a range of physiological adaptations that contribute to their hypoxia tolerance, including a high-affinity hemoglobin (Johansen et al., 1976), the ability to metabolize alternative glycolytic substrates in anoxia (Park et al., 2017), dramatic reductions in metabolic rate and ventilation (Pamerter et al., 2015) and body temperature and behaviour (Kirby et al., 2018; Houlahan et al., 2018; Ilacqua et al., 2017) in acute hypoxia, and metabolic remodelling in chronic hypoxia (Chung et al., 2016). Beyond systemic hypoxia tolerance, there is also evidence that NMR brain is tolerant of low O<sub>2</sub> and even ischemic stresses *ex vivo*. For example, NMR brain slices retain synaptic activity in anoxia for up to 30 min, tolerate O<sub>2</sub>–glucose deprivation (OGD) for 24 h, and exhibit blunted neuronal Ca<sup>2+</sup> influx during hypoxia (Nathaniel et al., 2009; Peterson et al., 2012). Conversely, hypoxia-intolerant murine brain slices tolerate only a few minutes of anoxia or OGD and exhibit large-scale deleterious Ca<sup>2+</sup> influx during hypoxia (Nathaniel et al., 2009; Peterson et al., 2012).

In organisms that experience hypoxia, metabolic and cellular adaptations have evolved that scale to match a wide range of O<sub>2</sub> tensions. For example, reduced mitochondrial electron transport system (ETS) respiratory flux and/or adjustments to the H<sup>+</sup> gradient are commonly observed in isolated mitochondria from hypoxia- or anoxia-tolerant reptiles, amphibians and invertebrates (Ali et al., 2012; Galli et al., 2013; Pamerter et al., 2016). Typically, this functional plasticity of mitochondria in hypoxia-tolerant species improves phosphorylation efficiency (i.e. ADP/O ratios) (St-Pierre et al., 2000; Pamerter, 2014). Mitochondria also coordinate neuroprotective responses against low O<sub>2</sub> stress in brains of hypoxia-tolerant species. For example, mitochondrial reactive oxygen species (ROS) generation and Ca<sup>2+</sup> accumulation are regulated in anoxia-tolerant Western painted turtle (*Chrysemys picta bellii*) brains, and these signalling molecules in turn modulate neuronal membrane proteins and limit ion flux and deleterious neurotransmitter release. This prevents excitotoxic cell death during prolonged anoxia and ischemia (Pamerter et al., 2007, 2008, 2011, 2012; Hogg et al., 2015). Conversely, in brains of hypoxia-intolerant organisms, unregulated ROS generation and/or mitochondrial Ca<sup>2+</sup> accumulation initiate cell death pathways during hypoxic or ischemic challenges (Choi, 1992; Pamerter, 2014). Therefore, mitochondria are at the centre of neuronal energy production in normoxia and also the cellular decision between initiating neuroprotective responses versus activating cell death cascades when O<sub>2</sub> is limited.

Despite the central role of mitochondria in cellular signalling during hypoxia and the prominence of NMRs as a mammalian model of hypoxia tolerance, little is known about the function of

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**List of abbreviations**

COX	cytochrome c oxidase
CS	citrate synthase
ETS	electron transport system
NMR	naked mole rat
OGD	oxygen–glucose deprivation
$\Delta\Psi_m$	mitochondrial membrane potential
ROS	reactive oxygen species
SUIT	substrate-uncoupler-inhibitor titration
TPP	tetraphenylphosphonium

NMR mitochondria other than a few ageing studies on muscle mitochondria that do not directly examine ETS function (Holtze et al., 2016; Stoll et al., 2016), while nothing is known regarding the effect of acute *in vivo* hypoxia on NMR brain mitochondrial ETS function. We hypothesized that brain mitochondria from NMRs exhibit metabolic plasticity in the form of altered ETS flux and/or  $H^+$  gradient kinetics following an acute hypoxic episode. We tested this hypothesis by exposing NMRs to 4 h of normoxia (21%  $O_2$ ) or acute hypoxia (3%  $O_2$ ) and examining the impact of this treatment on mitochondrial ETS flux. We found that NMR brain mitochondria exhibit a drastic 90% reduction in total ETS flux after acute *in vivo* hypoxic exposure, which closely matches the 85% whole-animal oxygen consumption rate reduction observed during this hypoxic exposure.

**MATERIALS AND METHODS****Animal husbandry and ethical approval**

All protocols were performed with the approval of the University of British Columbia Animal Care Committee. Animals were group-housed in interconnected multi-cage systems and held at 21%  $O_2$  and 28°C in 50% humidity under a 12 h:12 h dim light:dark cycle. Animals were fed fresh tubers, vegetables, fruit and Pronutro cereal supplement *ad libitum*. Animals were not fasted prior to experimental trials.

**Whole-animal respirometry**

Adult male non-breeding NMRs ( $N=16$ ) were individually placed, unrestrained, into a 450 ml Plexiglas experimental chamber, which was set inside a controlled environmental chamber to maintain ambient temperature at  $\sim 28^\circ\text{C}$ , which matched the housing temperature in the local animal care facility. The temperature of the animal chamber was recorded continuously using an iButton (Maxim Integrated, Chandler, CA, USA). Calibrated rotameters were used to supply an inflowing gas mixture set at a flow rate of  $110\text{ ml min}^{-1}$ . The total airflow ensured that  $O_2$  was not altered by more than 1.5% by the animal's metabolism. Fractional  $O_2$  composition of inspired and expired gas was monitored using an  $O_2$  analyser (Raytech, North Vancouver, BC, Canada). The gas analyser was calibrated before each trial with a pre-mixed gas (21%  $O_2$ , balanced with  $N_2$ ).  $O_2$  consumption was calculated from the product of the constant airflow through the chamber and the difference in  $O_2$  between the inflow and outflowing gases. Whole-animal oxygen consumption data were recorded on WinDaq acquisition software (Dataq Instruments, Akron, OH, USA), and analysed in PowerLab. Once placed within the experimental apparatus, animals were given at least 1 h to acclimate, and then metabolic measurements were collected for either 7 h in normoxia (21%  $O_2$ ; control;  $N=6$ ) or 1 h in normoxia followed by 4 h in hypoxia (3%  $O_2$ ;  $N=10$ ). All control animals were euthanized following the normoxic protocol. Of the population of animals

exposed to acute hypoxia, six animals were euthanized for tissue sampling immediately following the hypoxic exposure, while four animals were permitted to recover in order to obtain recovery data for oxygen consumption rate.

**Permeabilized brain mitochondrial preparation**

Animals were euthanized by cervical dislocation and whole brains were extracted over ice and bisected laterally within 30 s. One half of each brain was frozen in liquid  $N_2$  and stored at  $-80^\circ\text{C}$  for enzyme analysis (see below); the other half was placed into ice-cold homogenization buffer (mM: 250 sucrose, 10 TrisHCl, 0.5  $Na_2EDTA$ ; 1% fatty acid-free BSA; pH 7.4 at  $4^\circ\text{C}$ ) and then minced over ice for 2–3 min until the individual tissue pieces were uniformly smaller than grains of sand. The resulting homogenate was permeabilized with  $4\text{ mmol l}^{-1}$  saponin in homogenization buffer for 45 min, as described previously (Pamenter et al., 2016). Following permeabilization, the cell homogenate was re-suspended in ice-cold BIOPS medium (mmol  $l^{-1}$ : 10 Ca- $K_2$ -EGTA, 5.8 NaATP, 6.6  $MgCl_2$ , 20 imidazole, 20 taurine, 50 potassium 2-(*N*-morpholino)ethanesulfonic acid (K-MES), 15 naphosphocreatine, 0.5 dithiothreitol (DTT); pH 7.1, adjusted with 5 N KOH), rinsed for 2 min on ice, and then re-suspended in BIOPS medium. This rinse procedure was repeated three times. Permeabilized cells were kept on ice until use and all mitochondria were assayed within 2 h of isolation.

**Mitochondrial respiration and membrane potential analysis**

Permeabilized brain mitochondrial respiration was measured with an Oroboros Oxygraph 2-k (O2k) high-resolution respirometry system (Oroboros Instruments, Innsbruck, Austria), as described previously (Galli et al., 2013). Two identical respiration chambers were held at  $28^\circ\text{C}$  in parallel. Permeabilized brain cells were added to each chamber containing 2 ml of respiration solution (mmol  $l^{-1}$ : 0.5 EGTA, 1.4  $MgCl_2$ , 20 taurine, 10  $KH_2PO_4$ , 20 HEPES, 1% BSA, 60 potassium lactobionate, 110 sucrose; pH 7.1, adjusted with  $5\text{ mol l}^{-1}$  KOH). Respiratory flux through the ETS was measured in permeabilized brain cells using a substrate-uncoupler-inhibitor titration (SUIT) protocol as described previously (Galli et al., 2013; Pamenter et al., 2016). State II respiration was used as a proxy for leak respiration because native ATPases prevent the establishment of steady-state state IV respiration in the saponin-permeabilized cell preparation. Respiration values were obtained from steady-state conditions following each chemical addition. Protein content was analysed using the Bradford technique (Bradford, 1976).

**Enzyme activities**

Citrate synthase (CS) and ETS complex maximal activities ( $V_{max}$ ) were assessed using spectrophotometric biochemical assays from whole brain, as described previously (Galli et al., 2013).

 **$H^+$  leak measurements**

$H^+$  flux kinetics across the inner mitochondrial membrane were assessed by simultaneous measurement of  $O_2$  consumption and  $H^+$ -motive force, in the presence of rotenone, succinate and oligomycin, using tetraphenylphosphonium ( $TPP^+$ ) as described previously (Galli et al., 2013), and using an O2k  $TPP^+$  ion-selective electrode (Oroboros). Note that this methodology excludes any contribution of  $\Delta pH$  and therefore resulting measurements of the  $H^+$ -motive force are likely to be slight underestimates of the true value. Mitochondrial matrix volume was not measured because changes in this parameter have minimal impact on  $TPP^+$ -mediated measurements of  $\Delta\Psi_m$  (Rottenberg, 1984). We employed a binding

factor  $b$  for mitochondria of 0.16 (Marcinkeviciute et al., 2000; Galli et al., 2013). The kinetics of  $H^+$  flux were determined by inhibiting the substrate oxidation component by stepwise addition of 0.5 or 1.0  $\mu\text{l}$  aliquots of malonate (2.0 mol  $\text{l}^{-1}$  stock) and measuring the effect on  $\Delta\Psi_m$ . After the final malonate addition, carbonyl cyanide  $p$ -trifluoromethoxyphenylhydrazone (FCCP, 1  $\mu\text{mol l}^{-1}$ ) was added to uncouple mitochondria and determine the degree of electrode drift.  $H^+$  flux curves were fitted using a two-parameter exponential growth curve.

### Statistics

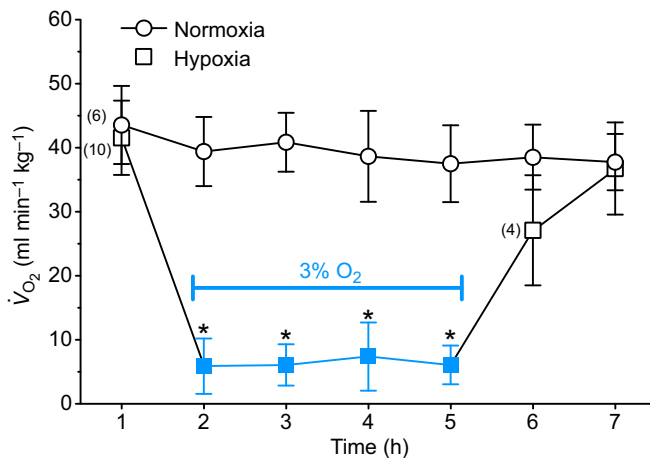
Statistical analysis was performed using commercial software (SPSS 15.0, SPSS, Chicago, IL, USA). For all experiments, individual  $N$  values correspond to a single animal. Values are presented as means  $\pm$  s.e.m. All data were normally distributed with equal variance ( $P > 0.05$ ). For all data, significance was evaluated using a repeated-measures two-way ANOVA to test for significant interactions between the two independent variables: (i) the substrate or inhibitor injected (e.g. ADP versus succinate), and (ii) inhaled  $O_2$  level (normoxia and hypoxia). Bonferonni *post hoc* multiple comparisons tests were run on each of the dependent variables to compare the single point means of interest.  $P < 0.05$  was considered to achieve statistical significance unless otherwise indicated.

## RESULTS

### Whole-animal oxygen consumption rate and mitochondrial ETS flux are markedly reduced by acute hypoxia

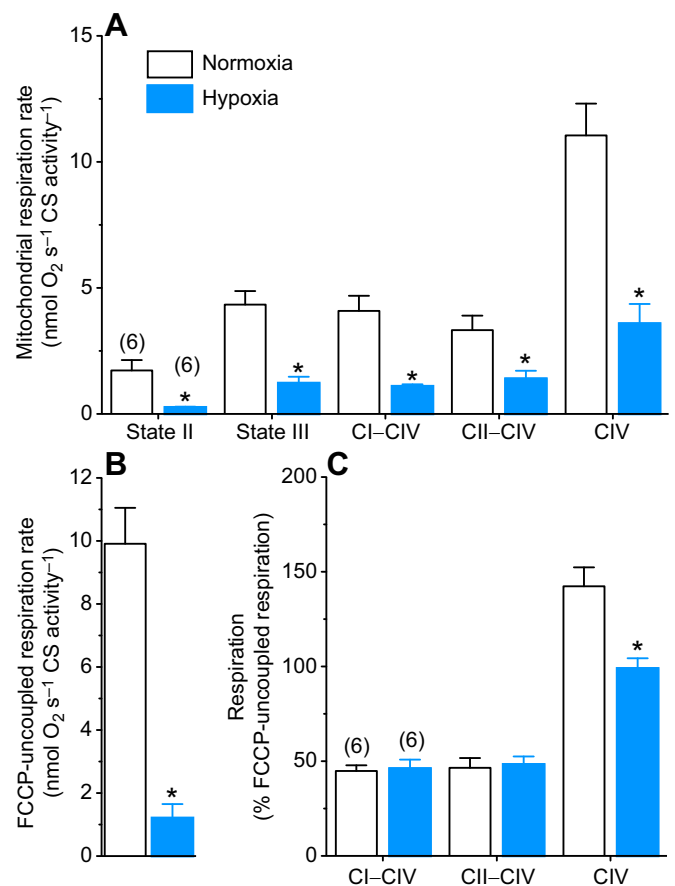
The whole-animal oxygen consumption rate of NMRs in normoxia was  $\sim 40 \text{ ml } O_2 \text{ min } \text{kg}^{-1}$  throughout 7 h of measurement ( $N=10$ ; Fig. 1, open circles), consistent with previous reports of normoxic metabolic rates in this species (Buffenstein, 1996; Pamerter et al., 2015; Chung et al., 2016). Conversely, in acute hypoxia (4 h at 3%  $O_2$ ), the NMR whole-organism oxygen consumption rate decreased by  $\sim 85\%$  (from  $41.6 \pm 5.8$  to  $6.1 \pm 3.0 \text{ ml } O_2 \text{ min } \text{kg}^{-1}$ ,  $N=6$ , Fig. 1, squares) and recovered to pre-treatment levels within 2 h of reoxygenation ( $36.8 \pm 7.2 \text{ ml } O_2 \text{ min } \text{kg}^{-1}$ ,  $N=4$ , Fig. 1).

We next examined mitochondrial respiratory flux in permeabilized brain from normoxic control and acutely hypoxic NMRs using a SUIT protocol. In permeabilized brain isolated from



**Fig. 1. Naked mole rat (*Heterocephalus glaber*) whole-animal oxygen consumption rate is markedly decreased in acute hypoxia.** The graph shows the oxygen consumption rates of individual NMRs exposed to normoxia (21%  $O_2$ , open circles) and then 4 h of hypoxia (3%  $O_2$ , blue squares). Data are means  $\pm$  s.e.m.; numbers in parentheses indicate  $N$ . \*Significant difference from normoxic controls ( $P < 0.05$ ; two-way repeated measures ANOVA).

hypoxia-treated NMRs, respiratory flux through the entire ETS was decreased relative to permeabilized brain from normoxic NMRs (Fig. 2A). Specifically, a two-way repeated measures ANOVA revealed a significant treatment effect between normoxia and hypoxia on ETS respiratory flux. Further analysis with Bonferonni *post hoc* tests revealed specific changes in state II [pyruvate (5.0 mmol  $\text{l}^{-1}$  final concentration; 5.0  $\mu\text{l}$  of 2.0 mol  $\text{l}^{-1}$  stock), malate (2.0 mmol  $\text{l}^{-1}$  final concentration; 5.0  $\mu\text{l}$  of 0.8 mol  $\text{l}^{-1}$  stock) and glutamate (10.0 mmol  $\text{l}^{-1}$  final concentration; 10.0  $\mu\text{l}$  of 2.0 mol  $\text{l}^{-1}$  stock)-fuelled] and III [pyruvate, malate, glutamate and ADP (1.5 mmol  $\text{l}^{-1}$  final concentration; 6  $\mu\text{l}$  of 0.5 mol  $\text{l}^{-1}$  stock)-fuelled] mitochondrial respiration rates, which were 84 and 54% lower, respectively, in permeabilized brain from NMRs exposed to acute hypoxia than from controls (Fig. 2A). Measurements of respiration through the individual components of the ETS revealed that the activity of complexes I, II and IV decreased by 77, 53 and 64%, respectively in hypoxic brain (Fig. 2A). As a result of this consistent downregulation of the ETS, total ETS capacity (as indicated by complex I and II-fuelled, FCCP-uncoupled respiration rates) was drastically reduced by 88% following acute *in vivo* hypoxia (Fig. 2B). Relative to FCCP-uncoupled respiration, only complex IV (cytochrome  $c$  oxidase: COX)-fuelled respiration



**Fig. 2. NMR brain mitochondria exhibit functional plasticity following *in vivo* exposure to 4 h of hypoxia.** (A) Individual complex respiratory rates from animals exposed to normoxia (white bars) or acute hypoxia (3%  $O_2$ ; blue bars). (B) Comparison of complex (C) I and II-fuelled FCCP-uncoupled respiration rates. (C) Summary of ETS complex respiration rates normalized to FCCP-uncoupled maximum respiration. Data are means  $\pm$  s.e.m.; numbers in parentheses indicate  $N$ . \*Significant difference from normoxic controls ( $P < 0.05$ ; two-way repeated measures ANOVA).

[stimulated by *N,N,N,N*-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate] was modified by the low O<sub>2</sub> challenge (Fig. 2C), suggesting a regulatory role for this enzyme in this response.

### ETS complex enzyme activity is unaltered by acute hypoxia

CS  $V_{\max}$  was unchanged between treatments (Fig. 3A). Similarly, the  $V_{\max}$  of ETS complexes I–V were unaltered by acute hypoxia (Fig. 3B).

### NMR permeabilized brain has a lower H<sup>+</sup> leak and respiration rate following acute hypoxia

Next we compared kinetics of the mitochondrial H<sup>+</sup> gradient between treatment groups (Fig. 4). A two-way repeated measures ANOVA revealed that mitochondria from hypoxia-treated NMR permeabilized brain had lower rates of O<sub>2</sub> consumption and a more depolarized  $\Delta\Psi_m$  than mitochondria from control animals. Specifically, state II respiration rate [in the presence of rotenone (0.5  $\mu\text{mol l}^{-1}$  final concentration), succinate (10  $\text{mmol l}^{-1}$  final concentration; 20.0  $\mu\text{l}$  of 1.0  $\text{mol l}^{-1}$  stock) and oligomycin] at the start of the experiment in permeabilized brain from hypoxic NMRs was reduced by ~50% relative to untreated controls, and  $\Delta\Psi_m$  was markedly decreased by ~25 mV at each data point. For example, when H<sup>+</sup> leak kinetics were considered at a common  $\Delta\Psi_m$  of 110 mV, hypoxic NMR permeabilized brain H<sup>+</sup> leak was approximately half that of normoxic NMR permeabilized brain (Fig. 4B). Conversely, the rates of both respiration rate and  $\Delta\Psi_m$  discharge with malonate additions were similar between treatments (Fig. 4C and D), and the kinetic relationship between respiratory flux and  $\Delta\Psi_m$  with titrated additions of malonate was not different between treatment groups as assessed using two-parameter exponential growth curves.

## DISCUSSION

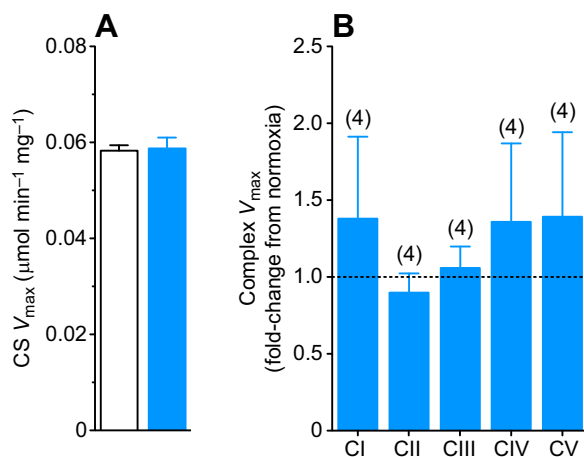
We explored the effect of acute hypoxia on the functional characteristics of hypoxia-tolerant NMR brain mitochondria. To our knowledge, our study is the first to examine any aspect of mitochondrial function in NMR brain. Our study yielded two important findings. First, NMRs markedly decrease their oxygen consumption rate by ~85% during prolonged severe hypoxia.

Second, in hypoxic NMR brain, ETS respiration and H<sup>+</sup> leak are markedly reduced, suggesting a reduced need for aerobically generated ATP during hypoxia. Taken together, these results support our hypotheses that NMR brain mitochondria exhibit functional plasticity and contribute to profound whole-animal metabolic rate suppression during acute *in vivo* hypoxic exposure in this species.

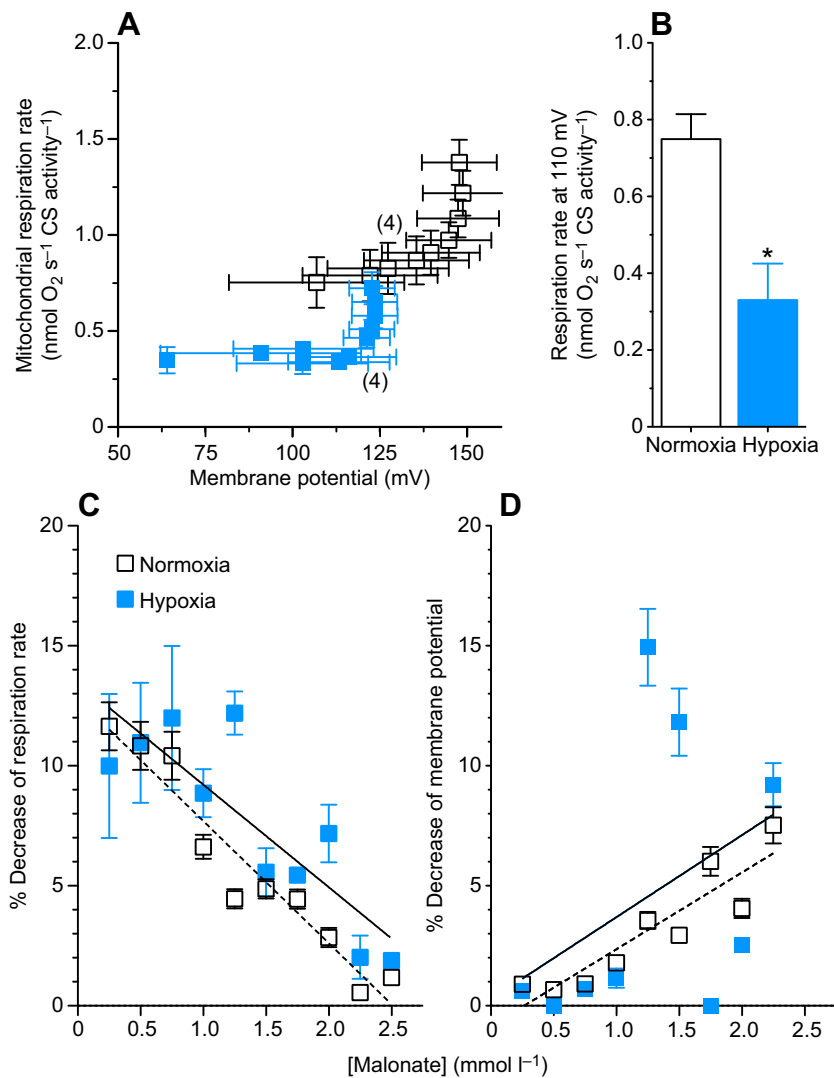
A handful of studies have explored the function of mitochondria in hypoxia-tolerant species and our data agree well with these previous studies. Indeed, although the effect of adaptation through multiple generations of hypoxia acclimation on mitochondrial respiratory flux is not well understood, there is a strong consensus that acute or prolonged hypoxic exposure exerts consistent inhibitory effects on mitochondrial respiration rates in hypoxia-tolerant lowland species. For example: (i) states III and IV respiration are lower in gill mitochondria of intertidal pacific oysters (*Crassostrea gigas*) following both 3 and 12 h of hypoxia (Sussarellu et al., 2013), (ii) state II, III and IV respiration are lower, following 2 weeks of anoxic exposure, in the brain of the anoxia-tolerant freshwater turtle (*Trachemys scripta*), due in part to an ~50% reduction in complex I activity (Pamenter et al., 2016), while (iii) state III respiration is lower due to reductions in complex I and IV activity in hearts of the same species (Galli et al., 2013). Similarly, (iv) states III and IV respiration rates are 20–30% lower in skeletal muscle mitochondria isolated from hypoxia-tolerant frogs (*Rana temporaria*) following exposure to 1 or 4 months of hypoxia (St-Pierre et al., 2000), and (v) in *Drosophila melanogaster* that had been raised under chronic hypoxia (4% O<sub>2</sub>) for >200 generations, state III respiration was lower relative to naïve flies, due primarily to reduced complex II activity (Ali et al., 2012).

An important caveat of these previous studies and also of our current results is that mitochondrial isolation procedures, for both permeabilized cells and also isolated mitochondria, are commonly conducted in standard laboratory atmospheric conditions. As a result, and depending on the isolation protocol, tissues from animals exposed to hypoxia *in vivo* are thus incubated in normoxia for 1–3 h prior to experimentation. However, despite this potentially confounding factor, it is nonetheless notable that mitochondria from normoxia- and hypoxia-treated NMRs exhibit drastically different ETS respiratory flux rates, despite being isolated identically *ex vivo*. These functional differences indicate that the *in vivo* hypoxic exposure had long-lasting effects on mitochondrial function. Similarly rapid responses have been reported in other organisms exposed to short-term hypoxia (Sussarellu et al., 2013). The underlying effectors of these changes are unknown but may involve rapid mechanisms such as post-translational modifications (Stram and Payne, 2016). In particular, redox-related modifications of mitochondrial proteins are a rapidly inducible mechanism to inhibit ETS complexes (Kramer et al., 2015); redox signalling is typically altered by changes in oxygen availability and thus is a good candidate for further studies to elucidate the mechanisms underlying the rapid and sustained downregulation of mitochondrial function.

Notably, similar adaptive responses have been reported in hypoxia-intolerant species acclimated to chronic hypoxia. For example: (i) state II and IV respiration and complexes I and IV activity are all reduced in mouse brain mitochondria following exposure to chronic hypoxia for 21 days (Chávez et al., 1995), and (ii) state III respiration and complex I and II flux rates are lower in skeletal muscle mitochondria of humans acclimated for 4 weeks at altitude relative to pre-acclimatization rates in the same individuals (Jacobs et al., 2012). Such examples of reduced ETS flux as an



**Fig. 3. Complex enzyme maximum activity is not altered by acute hypoxia.** (A) Summary of citrate synthase (CS)  $V_{\max}$  in normoxic (white bars) and acutely hypoxic NMRs (blue bars). (B) Summary of ETS complexes ('C') I–V activity from acutely hypoxic NMR brain tissue normalized to ETS complex activity from normoxic control NMR brain tissue. Data are means  $\pm$  s.e.m.; numbers in parentheses indicate  $N$  ( $P < 0.05$ ; two-way repeated measures ANOVA).



**Fig. 4. The mitochondrial  $H^+$  gradient of NMR brain mitochondria is reduced relative to normoxic control animals.** (A) Hypoxic NMR (blue squares) brain  $H^+$  flux and  $O_2$  consumption are equally coupled but reduced in magnitude relative to normoxic control NMR brain (open squares). (B) Comparison of  $H^+$  flux rates at a common mitochondrial membrane potential of 110 mV. (C) Per cent decrease of mitochondrial respiration and (D) mitochondrial membrane potential with stepwise addition of malonate. Data are means  $\pm$  s.e.m.; numbers in parentheses indicate  $N$ . \*Significant differences between mice and NMR mitochondria ( $P < 0.05$ ; two-way repeated measures ANOVA).

adaptation to hypoxia in low-altitude species are probably associated with reduced metabolic requirements during hypoxic challenges. Indeed, reducing metabolic demand when  $O_2$  is limiting, particularly in brain tissue, is a common strategy employed by hypoxia-tolerant and hypoxia-adapted species (Hochachka et al., 1996; Buck and Pamenter, 2006), including human populations that have lived at altitude for thousands of years (Hochachka et al., 1994). This relationship holds true in our analysis of the effects of acute hypoxia on NMR mitochondrial function.

Interestingly, an opposing phenotype is reported in several studies of high-altitude species that inhabit niches in which hypobaric hypoxia is compounded by a cold environment. For example, high-altitude populations of the Andean torrent duck (*Merganetta armata*) exhibit heightened respiratory capacities in gastrocnemius muscle, which is the primary muscle used for swimming and diving behaviours in this species (Dawson et al., 2016). Similarly, high-altitude deer mice (*Peromyscus maniculatus*) also exhibit higher respiratory capacities in their gastrocnemius muscle mitochondria and also higher mitochondrial volume densities relative to those of low-altitude populations of deer mice (Mahalingam et al., 2017). Conversely, in flight muscles of bar-headed geese (*Anser indicus*) and Tibetan locusts (*Locusta migratoria*), two species adapted to living and exercising at high altitude for thousands of years, mitochondrial respiratory flux is not

different from that of mitochondria isolated from flight muscles of lowland species of geese and locusts, respectively (Zhang et al., 2013; Scott et al., 2015). It should be noted, however, that bar-headed geese face a unique exercise challenge in that they increase their  $O_2$  consumption by 10- to 20-fold while in hypoxia to sustain flight activities at high altitudes, and thus exhibit a remarkable suite of unique adaptive strategies that are difficult to compare directly with other hypoxia-adapted or -acclimated species that do not face a similarly extreme exercise challenge while in hypoxia. Similarly, high-altitude species face a significant thermoregulatory challenge due to the cold climate at altitude and this has a significant impact on mitochondrial physiology. Therefore, high-altitude denizens face a unique set of trade-offs between hypoxia tolerance and thermoregulation that confound determination of an optimal phenotype to enhance mitochondrial function in hypoxia. This compounded ecophysiological stress has probably applied different evolutionary pressures to this species than those applied to lowland species that do not face the same degree of thermoregulatory challenge in their natural environment.

#### COX activity is reduced in hypoxic NMR brain mitochondria

An important observation of our study is that the NMR oxygen consumption rate was  $>85\%$  reduced in acute hypoxia; permeabilized brain from these animals exhibited drastically lower rates of  $O_2$

consumption and a more depolarized  $\Delta\Psi_m$  than control animals. Intriguingly, when normalized to FCCP-uncoupled respiration, only complex IV-fuelled respiration (stimulated by TMPD and ascorbate) was modified by the low  $O_2$  challenge (Fig. 2C), suggesting a regulatory role for this enzyme in this response. COX is the terminal complex of the ETS and is the major site via which  $O_2$  interacts with the ETS. Therefore, COX is ideally positioned to serve as a regulator of ETS flux in acute hypoxia, and in rat hepatocytes reduced ETS respiratory flux during acute hypoxia is triggered by a regulatory effect of molecular  $O_2$  on COX (Chandel et al., 1995). Our observation of reduced COX activity relative to total ETS capacity in hypoxia-treated NMR brain is consistent with examinations of skeletal muscle mitochondria from other hypoxia-tolerant species (Scott et al., 2011; Zhang et al., 2013), and may be due to an upregulation of COX isoform IV-2 in astrocytes and cerebellar granule cells following acute hypoxia in brain (Horvat et al., 2006). In this study, the authors reported that in murine brain cells, *in vitro* exposure to a few hours of hypoxia elevated COX isoform IV-2, which in turn abolished the allosteric inhibition of COX by ATP. This finding indicates that COX may function as an oxygen sensor in mammal brain and can directly modulate ETS function. The rapid mode of action observed in our study suggests that COX may modify ETS function via a similar rapid cellular signalling process in NMRs. Further studies are warranted to examine this hypothesis.

## Conclusions

Mitochondria are the nexus of  $O_2$  consumption and metabolism in the cell, and also coordinate deleterious and neuroprotective responses to low  $O_2$  stress (Pamenter, 2014). NMRs have a low basal metabolic rate and we demonstrate here that they exhibit metabolic arrest in acute hypoxia. The degree to which NMRs are able to decrease their oxygen consumption rate in hypoxia is unprecedented in awake and active mammals. It is notable that the drastic reduction in ETS flux is similar to the magnitude of the reduction in whole-animal oxygen consumption rate observed in this species during the same period of acute hypoxia and no doubt contributes to the ability of this species to suppress metabolic demand in low  $O_2$  conditions. Reducing metabolic rate when  $O_2$  is limiting, particularly in brain tissue, is a common strategy employed by hypoxia-tolerant species (Buck and Pamenter, 2006), and this relationship holds true in our analysis of NMR whole-animal oxygen consumption rate and mitochondrial function during acute hypoxia.

In general, our findings support an emerging consensus in the literature concerning the effects of acute hypoxia exposure on mitochondrial adaptations, at least as this pertains to low-altitude species. The available evidence suggests that common adaptations to hypoxia at low altitude include functional remodelling of mitochondrial metabolism in hypoxia-tolerant and -intolerant species. This remodelling is characterized by some or all of a number of hallmarks, including: decreased overall ETS flux, reductions in mitochondrial volume density, and changes in COX activity and kinetics. Our findings regarding the function of brain mitochondria from hypoxia-tolerant NMRs fit this paradigm. Specifically, NMR brain tissue exhibits rapid plasticity in acute hypoxia including large-scale reductions in respiration and the  $H^+$  gradient. We conclude that NMR brain mitochondrial metabolism is primed to maximize energetic efficiency and support marked and rapid reductions in whole oxygen consumption rate during hypoxia.

## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: M.E.P., G.Y.L., J.G.R., W.K.M.; Methodology: M.E.P., G.Y.L.; Software: J.G.R.; Validation: M.E.P., G.Y.L.; Formal analysis: M.E.P., G.Y.L., J.G.R.; Investigation: M.E.P.; Resources: J.G.R.; Writing - original draft: M.E.P.; Writing - review & editing: M.E.P., G.Y.L., J.G.R., W.K.M.; Supervision: J.G.R., W.K.M.; Funding acquisition: M.E.P., J.G.R., W.K.M.

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