Acidosis overrides oxygen deprivation to maintain mitochondrial function and cell survival

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Sustained cellular function and viability of high-energy demanding post-mitotic cells rely on the continuous supply of ATP. The utilization of mitochondrial oxidative phosphorylation for efficient ATP generation is a function of oxygen levels. As such, oxygen deprivation, in physiological or pathological settings, has profound effects on cell metabolism and survival. Here we show that mild extracellular acidosis, a physiological consequence of anaerobic metabolism, can reprogramme the mitochondrial metabolic pathway to preserve efficient ATP production regardless of oxygen levels. Acidosis initiates a rapid and reversible homeostatic programme that restructures mitochondria, by regulating mitochondrial dynamics and cristae architecture, to reconfigure mitochondrial efficiency, maintain mitochondrial function and cell survival. Preventing mitochondrial remodelling results in mitochondrial dysfunction, fragmentation and cell death. Our findings challenge the notion that oxygen availability is a key limiting factor in oxidative metabolism and brings forth the concept that mitochondrial morphology can dictate the bioenergetic status of post-mitotic cells.
long-term survival of post-mitotic cells, which have a limited regenerative capacity, is essential to ensure continued biological function of an organism. In recent years, it has become apparent that the decline of post-mitotic cells, during aging, neurodegenerative diseases and ischemic disorders, is generally associated with mitochondrial dysfunction\textsuperscript{12}. Mitochondria are essential organelles for energy production, regulation of signalling cascades and cell death\textsuperscript{3}. These organelles form a dynamic interconnecting network through continuous cycles of fission and fusion events\textsuperscript{4}. The regulation of mitochondrial morphology is closely coupled to cell survival and metabolic adaptation during stress\textsuperscript{5–7}. For example, aberrant mitochondrial fission has been observed in many disease and injury models and considered a key contributor to mitochondrial dysfunction and cell death\textsuperscript{8–10}. In these settings, inhibition of mitochondrial fission or enhancing mitochondrial fusion restores cell viability\textsuperscript{11–14}. These observations highlight the importance in the regulation of mitochondrial dynamics as a strategy to promote cellular survival.

A common characteristic of high-energy-demanding post-mitotic cells, such as neurons, muscle and cardiomyocytes, is their dependence on a continuous supply of energy for sustained cellular function and viability. For this reason, contemporary eukaryotic cells are highly dependent on oxygen and functional mitochondria for the efficient generation of ATP through oxidative phosphorylation\textsuperscript{15,16}. In this context, it can be appreciated how limitations in oxygen availability, or hypoxia, have profound physiological effects. Low oxygen levels cause major changes in mitochondrial structure and dynamics, ultimately leading to defective mitochondrial function, reduced ATP supply and activation of cell death pathways\textsuperscript{17–19}. Importantly, a defective mitochondrial function induced by hypoxic stress is observed in diverse complex disorders such as type-2 diabetes mellitus, Alzheimer’s disease, cardiac and brain ischemia/reperfusion injury and tissue inflammation\textsuperscript{17}. The fate of post-mitotic cells subjected to physiological or pathological settings of hypoxia is thus entirely reliant on their ability to respond and adapt to changing environments and stress conditions. Consequently, understanding oxygen sensing and response mechanisms in cells and tissues has been at the forefront of research for many years with the aim of exploiting adaptive strategies to promote cell survival. An essential and often neglected aspect of hypoxia is the accumulation of lactic acid as the end product of glycolysis. Excess H\textsuperscript{+} ions resulting from an increased glycolytic rate are pumped outside the cell, inevitably causing acidification of the extracellular milieu. Physiological levels of acidosis in regions subjected to limited oxygen availability, such as the ischemic penumbra following a stroke, can range within the pH values of 6.0–6.5 depending on the severity of the insult\textsuperscript{20–22}. A long-standing debate in biology is the effect of acidosis on cell survival. Although extracellular acidosis is historically viewed as a mere toxic byproduct of fermentation that is detrimental to cells, it is now clinically recognized as a protective agent when present at mild levels (pH 6.5 and above)\textsuperscript{21,23–35}. In this regard, although several reports have clearly demonstrated the protective nature of mild acidosis, the underlying molecular mechanisms are still poorly understood. Furthermore, the role of mitochondria, being central to cell survival and death, has surprisingly never been addressed in this perspective. Here we show the unexpected observation that mild acidosis triggers massive morphological reorganization of mitochondria in post-mitotic cells, triggered by a dual programme that both activates fusion and cristae remodelling while inhibiting mitochondrial fragmentation. Activation of this reversible homeostatic programme reconfigures mitochondrial bioenergetics to allow for the persistence of efficient ATP production through oxidative phosphorylation despite oxygen limitations. Our work reveals a novel and physiological mechanism that can control the metabolic status of cells and protect mitochondrial-reliant post-mitotic cells following a hypoxic insult, by reprogramming mitochondrial morphology and functional efficiency.

**Results**

**Acidosis triggers mitochondrial elongation during hypoxia.** As mitochondria are central in the cell death that is instigated during hypoxic stress, we investigated mitochondrial morphology in this setting. Cortical neurons were chosen for these experiments since they are a major population affected by hypoxic stress and thus represent a biologically relevant system for the study of adaptive mechanisms in post-mitotic cells. For our studies, we developed an \textit{in vitro} model that mimics the physiological microenvironment found under hypoxic conditions, such as the penumbral region following an ischemic brain injury. This model recapitulates the low oxygen/glucose environment and takes into account that ischemic tissues or hypoxic cells normally acidify their extracellular milieu as a physiological consequence of anaerobic glycolysis. For this, a low glucose media was buffered in a manner to accommodate physiological acidification of the extracellular milieu when neurons are incubated at 1\% O\textsubscript{2} (termed acidosis-permissive media (AP)). The control condition utilizes a low glucose media that maintains a stable neutral pH (pH 7.2) throughout experimentation (termed standard media (SD)). Mitochondria from cultured cortical neurons subjected to hypoxic conditions in a neutral pH environment had severely fragmented mitochondria as observed through immunofluorescence staining of the outer mitochondrial membrane protein Tom20 (Fig. 1a and quantified in Fig. 1b). Mitochondrial fragmentation was observed before signs of cell death or changes in neuronal morphology (Supplementary Fig. 1). Unexpectedly, neurons subjected to hypoxia but allowed to undergo physiological extracellular acidification (measured pH post experiment was 6.5) exhibited massive mitochondrial elongation compared with control (Fig. 1a and quantified in Fig. 1b). Elongated mitochondria were observed in the cell body of cortical neurons and spanned along the axons (Fig. 1c). Mitochondrial elongation was also observed in cultured cerebellar granular neurons (CGNs; Fig. 1d,e) and \textit{in vivo} hippocampal slice preparations (Fig. 1f,g), as well as in differentiated C2C12 myotubes (Supplementary Fig. 2), suggesting that this is a general phenomenon. Interestingly, acidosis-mediated mitochondrial elongation was not observed in proliferative cells, such as primary and transformed mouse embryonic fibroblast cells (MEF), C2C12 myoblasts, Cos7, HeLa and several cancer cell lines, including MCF-7, A549 and P19, suggesting that this response is unique to post-mitotic cells (Supplementary Fig. 2).

The acidosis-dependent alteration in mitochondrial morphology is pH-specific and elongation of mitochondria was only observed at a pH threshold value between 6.65 and 6.45 (Fig. 2a,b and Supplementary Fig. 3), representing mildly acidic conditions as observed in physiological settings of hypoxic stress. In contrast, cells within a neutral (pH 7.2–6.8) or severely acidic (pH 6.0) extracellular environment during hypoxia exhibited significant mitochondrial fragmentation (Fig. 2a,b and Supplementary Fig. 3). Acidosis-mediated mitochondrial elongation is a rapid process whereby the onset of elongation occurred at about 3-h post-treatment (Fig. 2c,d and Supplementary Fig. 4). This opposed the hypoxia-mediated mitochondrial fragmentation that occurs in neutral conditions beginning 3-h following treatment (Fig. 2c and Supplementary Fig. 4). In addition, although acidosis generally occurs as a consequence of increased glycolysis...
during limited oxygen availability, its effect on mitochondrial morphology was in fact independent of oxygen. Incubation of cortical neurons in media set to pH 6.5 in the presence of oxygen (normoxia) resulted in a significant increase in mitochondrial length compared with control (Fig. 2e). To further confirm the direct affect of acidosis on mitochondrial length, the contribution of low glucose levels in the experimental paradigm, which represents a more physiologically relevant setting, was assessed. The effect of acidosis on mitochondrial length was not a consequence of decreased glucose availability since mitochondrial elongation persisted in the presence of high glucose levels (Fig. 2f and Supplementary Fig. 5). Thus, mitochondrial elongation in these settings is directly mediated by acidosis and is not a consequence of an induced starvation response. This is supported by the observation that the limited glucose availability in these experiments was not sufficient to activate autophagy (Supplementary Fig. 6) such as that observed during complete glucose starvation, where there is a marked increase in LC3II, degradation of p62 and loss of outer membrane Tom20 due to mitophagy (Supplementary Fig. 6). These results demonstrate that acidosis alone is sufficient to promote mitochondrial elongation. Acidosis-driven elongation is also a reversible process. Mitochondria remain elongated regardless of oxygen levels, confirming that this process is oxygen-independent (Fig. 2g,h).
and Supplementary Fig. 7). However, mitochondria revert back to a fragmented phenotype following neutralization of the extracellular pH both under hypoxic or reoxygenation conditions (Fig. 2g,h and Supplementary Fig. 7). In fact, the level of fragmentation following reoxygenation in a neutral context was very rapid (within 30 min) and was quite severe (Fig. 2h)
and Supplementary Fig. 7). In order to gain insight as to how extracellular acidosis can relay an intracellular signal to modify mitochondrial morphology, pH changes within the intracellular environment were examined. Analysis of intracellular pH changes using the ratiometric fluorescent indicator BCECF-AM (Supplementary Fig. 8a) showed that a decrease in extracellular pH as used in our studies (pH 6.5) was sufficient to cause a reduction in intracellular pH (Supplementary Fig. 8b) and is consistent with previous reports showing that intracellular pH can be modified by the extracellular milieu36,37. Furthermore, neutralization of the intracellular pH using the Na+/H+ exchanger Monensin (Supplementary Fig. 8c) rapidly reverses the mitochondrial elongation phenotype that was instigated by extracellular acidosis (Fig. 2i and Supplementary Fig. 8d,e). Together, these results demonstrate a pH-dependent regulation of mitochondrial morphology that is oxygen- and glucose-independent and is reversible.

**Acidosis inhibits DRP1-mediated mitochondrial fission.** Mitochondrial fragmentation is a prominent phenotype during hypoxic stress conditions as observed here (Figs 1a,b and 2c) and in previous studies19,38,39. However, physiological hypoxic stress conditions as observed here (Figs 1a,b and 2c) and in previous studies19,38,39. Mitochondrial fragmentation is a prominent phenotype during hypoxic stress conditions as observed here (Figs 1a,b and 2c). Mitochondrial fragmentation is a prominent phenotype during hypoxic stress conditions as observed here (Figs 1a,b and 2c). Mitochondrial fragmentation is a prominent phenotype during hypoxic stress conditions as observed here (Figs 1a,b and 2c).

**Mitochondrial fusion by acidosis depends on the SIMH pathway.** The degree and rate of elongation observed during acidosis suggested the possibility of a pH-dependent enhancement in mitochondrial fusion activity, in addition to suppression of the fission pathway. This was tested in live neurons, infected

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**Figure 3 | Mild acidosis inhibits DRP1-mediated mitochondrial fission.** (a) Colocalization of DRP1 foci at mitochondria by immunofluorescence of DRP1 and Tom20 in cortical neurons using confocal imaging. Arrows show DRP1 foci localized at mitochondria. Scale = 5 μm. (b) Quantification of the number of DRP1 foci colocalized to mitochondria and represented as mean and s.d. (n = 3). (c) Western blot of the indicated proteins from whole cell lysates of cultured cortical neurons following 6-h incubation at the indicated conditions. (d) Quantification of the number of DRP1 foci colocalized to mitochondria following 6-h hypoxic incubation in SD or AP media in the presence or absence of the fission inhibitor Mdivi-1. (e) Western blot of indicated proteins following incubation for 3 h at the indicated conditions and immunoprecipitation of endogenous (endo.) DRP1 using anti-DRP1 antibody.

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with a mitochondrial matrix targeted photo-activatable green-fluorescent protein (GFP) lentivirus (PA-Oct-GFP), following 3 h of incubation at pH 7.2 or 6.5 under normoxic conditions, representing the onset of elongation in the absence of hypoxia-induced fission. The rate of dilution and spread of the photo-converted GFP molecules are used as a measure of fusion activity. Time-lapse imaging of the activated mitochondrial GFP signal demonstrated an increase in mitochondrial fusion in neurons subjected to mild acidosis (Fig. 4a,b and Supplementary Fig. 10). Examination of mitochondrial mass further confirmed that mitochondrial elongation during acidosis is a result of enhanced fusion activity rather than increased mitochondrial biogenesis.

**Figure 4 | Mild acidosis regulates mitochondrial dynamics.** (a) Representative images of mitochondrial fusion over time (indicated in minutes) following activation of exogenously expressed PA-GFP-Oct. Boxes indicate photo-activated regions and arrows indicate spread of the GFP signal within mitochondria (revealed by exogenous expression of Mito-DsRed). (b) Quantification of mitochondrial fusion in cortical neurons as a loss of GFP fluorescence in the activated region. Data represent the mean and s.d. of \( n = 10 \) (control) and \( n = 17 \) (experimental) from three independent experiments. (c and d) Western blot of the indicated proteins from whole-cell lysates of cortical neurons incubated for 6 h at the indicated conditions. Asterisk indicates s-OPA1. (e) Mean and s.d. (\( n = 3 \)) of mitochondrial length during acidosis, of the indicated genotypes, relative to control conditions. (f) Representative images of mitochondrial morphology from cortical neurons of the indicated genotypes following 6-h incubation in MES-buffered media at the indicated conditions. Bottom panel of each condition is a zoom view of mitochondria. Scale = 10 μm. *\( P < 0.05 \); **\( P < 0.001 \) (Student’s t-test).
Figure 5 | Mild acidosis regulates cristae architecture. (a,b) Western blot of OPA1 oligomers and monomers from lysates of BMH crosslinking in live cortical neurons at the indicated conditions. OPA1 western blot has been spliced to better clarify where monomeric and oligomerized Opa1 appear. Graphs represent the mean and s.d. (n = 3) quantification of OPA1 oligomer:monomer ratio. (c–e) Representative EM images of mitochondrial ultrastructure following 6-h incubation at the indicated conditions. Scale = 500 nm. Graphs in (d,e) represent mean and s.e.m (n = 10) for quantification of cristae diameter and cristae number. Ctr = control represents neurons incubated in neurobasal media. (f,g) Representative images and quantification (mean and s.d., n = 4) of CytoC localization following CPT treatment in MES-buffered media at pH 7.2 or 6.5 in normoxia. *P < 0.05; **P < 0.01; ***P < 0.001 (Student’s t-test).

since the levels of resident mitochondrial proteins HSP70 and VDAC were unchanged (Fig. 4c). Mitochondrial elongation during acidosis does not appear to be activated through increased expression levels of core fusion proteins OPA1 and MFN1/2 (refs 46–48; Fig. 4d). Moreover, addition of the protein synthesis inhibitor cycloheximide did not prevent acidosis-mediated mitochondrial elongation or significantly alter mitochondrial length during control conditions, suggesting that acidosis may regulate mitochondrial dynamics at the post-translational level (Supplementary Fig. 11). To further decipher the molecular mechanism enabling enhanced fusion activity, we examined the possibility that acidosis activates the stress-induced mitochondrial hyperfusion (SIMH) pathway7. We found that removal of the key molecular components of the mitochondrial fusion machinery and the SIMH pathway, OPA1, MFN1 and SLP2, rendered neurons unresponsive to acidosis-induced mitochondrial elongation in normoxia (Fig. 4e,f). Together, these data demonstrate that mild acidosis enhances mitochondrial fusion, requires an intact fusion machinery and suggests a specific role for the SIMH pathway in the hyperfusion observed by mild acidosis during hypoxic stress.

Acidosis regulates cristae remodelling during stress. The internal structures of mitochondria, the cristae, are also dynamic and can undergo OPA1-dependent remodelling during stress conditions49,50. OPA1 oligomeric complexes, consisting of both membrane-bound long (l-OPA1) and soluble short forms (s-OPA1) of OPA1 in the intermembrane space, have been associated with inner membrane morphology, tightness of the cristae junctions, as well as sequestration of Cytochrome c (CytoC) within the cristae46,49,51,52. We found that the relative amount of s-OPA1 was altered following 6 h of hypoxic treatment (Fig. 4d) and OPA1-specific oligomeric complexes were disrupted as early as 1 h after treatment (Fig. 5a and Supplementary Fig. 12). However, a mild decrease in the extracellular pH during hypoxia rescued both the levels of s-OPA1 and OPA1 oligomeric complexes (Figs 4d and 5a). Acidosis also increased OPA1 oligomeric complexes under normoxic conditions (Fig. 5b). This suggested that acidosis not only changes mitochondrial length but it may also alter cristae morphology. Examination of mitochondrial ultrastructure using transmission electron microscopy (TEM) revealed that mitochondrial ultrastructure, which is severely disrupted during hypoxia, was preserved by...
acidity (Fig. 5c–e). During hypoxia-neutral conditions, where aberrant mitochondrial fragmentation is observed, there is a significant disruption of mitochondrial ultrastructure leading to a significant increase in cristae diameter as well as a reduction in cristae number (Fig. 5c–e). More importantly, a mild decrease in the pH during hypoxia not only rescued this defect but also resulted in a significant tightness of the cristae diameter and increased cristae number, relative to control (Fig. 5c–e). These data suggest a role for acidity in cristae maintenance and remodelling. This was further confirmed through an indirect examination of cristae remodelling by measuring the degree of CytC release following an apoptotic stimulus, which can provide information related to the tightness of the cristae junctions.

Treatment of neurons with Camptothecin (CPT), a DNA-damaging agent that triggers the apoptotic cell death pathway, revealed that mitochondrial restructuring by acidity renders cells resistant to CytC release (Fig. 5f,g), in the presence of apoptosis signalling as indicated by BAX activation (Supplementary Fig. 13). Together, these data demonstrate that mild acidity modulates mitochondrial dynamics as well as cristae architecture. Furthermore, these data suggest that acidity prevents the intramitochondrial remodelling, as a result of OPA1 oligomer destabilization, that is associated with mitochondrial fragmentation and cell death signalling.

Mitochondrial restructuring protects cells in hypoxia. Several studies have demonstrated the protective effect of mild acidity during ischemic conditions. Importantly, this observation was recapitulated in our model of ischemia. A significant increase in cell death is observed in hypoxia under neutral conditions; however, a physiological decrease in the extracellular pH (pH 6.5) protects neurons from death (Fig. 6a). Interestingly, acidity rendered neurons resistant to other damaging agents such as CPT (Fig. 6b), suggesting that cells subjected to mild changes in extracellular pH can sustain survival under different modes of stress. The observation that mild acidity in our system also promotes mitochondrial remodelling suggests that this may be the underlying mechanism of cellular protection previously observed at similar pH levels. To confirm this hypothesis, we tested neuronal survival during hypoxia–acidosis where we prevented the impact of acidosis on mitochondrial dynamics. The protective effect of acidity was reversed in the absence of the essential fusion machinery. Acute RNA interference-mediated loss of OPA1 expression or genetic ablation of MFN1 prevented acidosis-mediated mitochondrial elongation (Fig. 4e,f) and was sufficient to increase cell death during hypoxia–acidosis even in the presence of acidity (Fig. 6c,d). Moreover, overexpression of the mitochondrial fission protein DRP1, in wild-type neurons, resulted in a significant increase in cell death during hypoxia–acidosis (Fig. 6e). These data demonstrate that the restructuring of mitochondria during stress is a major player in the protective effect of mild acidity.

Reprogrammed mitochondria maintain ATP production in hypoxia. A prominent response to cellular hypoxic stress is mitochondrial fragmentation, and loss of mitochondrial membrane potential and dysfunction. Since acidity prevents hypoxia-induced mitochondrial fragmentation and cell death, we wanted to investigate the status of mitochondrial integrity and function. We found that mild acidity maintains mitochondrial membrane potential during hypoxia (Fig. 7a, and Supplementary Fig. 14). Maintenance of mitochondrial membrane potential requires a protonotive force that is generated by the electron transport chain through respiration or, in pathological situations, by ATP hydrolysis via the F$_{1}$F$_{0}$-ATPase (ATP synthase). A highly polarized membrane potential suggests that either mitochondrial...
It is well established that mitochondrial function and the generation of ATP through oxidative phosphorylation (OXPHOS) is impaired during hypoxic conditions, causing cells to shift to anaerobic glycolysis. Although this shift in metabolism is important for cell survival during acute hypoxic stress, glycolysis represents a much less efficient mode of ATP production, and over the long run it is reasonable to conceive that ATP hydrolysis by the ATP synthase was not a central contributing factor in maintaining membrane potential.

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respiratory function was preserved by mild acidosis or that the membrane potential was maintained by the reversal of the ATP synthase, which would result in ATP consumption. Interestingly, total ATP levels were sustained for an extended period in hypoxia if cells underwent physiological acidification (Fig. 7b), suggesting that ATP hydrolysis by the ATP synthase was not a central contributing factor in maintaining membrane potential.

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for the significant depletion of ATP in the presence of oligomycin, this suggests that cells in acidosis rely less on the glycolytic ATP generation pathway and supports the idea that acidosis preserves mitochondrial function and sustains OXPHOS during hypoxia. This observation was quite surprising considering that oxygen availability is historically viewed as a limiting factor.
for mitochondrial ATP generation through mitochondrial respiration. More importantly, a pH-dependent shift in the bioenergetic status of neurons was observed over time, whereby cells in hypoxia at pH 7.2 relied increasingly on oligomycin-insensitive ATP production (that is, glycolysis) while neurons allowed to undergo physiological acidification relied on mitochondrial-dependent ATP production (Fig. 7f). Thus, acidosis sustains efficient mitochondrial ATP production during prolonged hypoxia (Fig. 7f).

An alteration in the metabolic profile of cells in hypoxia–acidosis implies an adaptation of the electron transport chain (ETC) components to maximize mitochondrial efficiency during limited oxygen availability. Several studies have reported the adverse effect of hypoxia on the expression and activity of ETC complexes as well as supercomplex disassembly. Expression analysis of subunits encompassing the ETC complexes during acute hypoxia revealed maintained expression of Complex I subunits NDUF9 as well as ATP synthase subunit ATP5a by mild acidosis (Supplementary Fig. 15). Blue native PAGE (BN-PAGE) analysis of the respiratory chain complexes showed a destabilization of the monomeric ATP synthase multiprotein complex during hypoxia, which is rescued by acidosis (Fig. 8a,b). Only a minor proportion of the ATP synthase is present in its dimeric form in neurons used during our experiments (Fig. 8c and Supplementary Fig. 16). Nonetheless, longer exposure of BN-PAGE from whole cells or BN-PAGE performed on isolated mitochondria revealed a higher molecular weight band corresponding to dimeric forms of the ATP synthase predominantly in hypoxia–acidosis conditions (Fig. 8c,d and Supplementary Fig. 16), indicating a distinct rise in the stability of the enzyme superassembly. The individual multiprotein complexes of the ETC can further associate into supercomplexes, known as respirasomes. It has been proposed that supercomplex assembly could stabilize the single complexes, enhance the electron flow between complexes and limit generation of oxygen radicals. In addition, a distinct role for cristae shape has been recently shown to affect supercomplex assembly and respiratory efficiency. Examination of higher order respiratory complex assembly by BN-PAGE showed a distinct reorganization of Complex I-containing supercomplexes by acidosis regardless of oxygen levels (Fig. 8e), resulting in loss of monomeric complex I and enhanced supercomplex assembly (complex I supercomplex:monomer ratio; Fig. 8h), as well as an increase in the relative levels (Fig. 8f). Detailed analysis of the respiratory supercomplexes showed maintained assembly of Complexes I, III and IV into supercomplexes in the presence of acidosis during hypoxic conditions (Fig. 8g,h).

We hypothesized that the acidosis-mediated changes to the OXPHOS complexes would be accompanied by an enhancement in the respiratory capacity of mitochondria. To test this hypothesis, the bioenergetic profile of mitochondria was assessed in intact neurons exposed to normoxic conditions at pH 7.2 and 6.5. First, basal cellular and mitochondrial oxygen consumption rate (OCR) was similar in both conditions (Fig. 8i,j). Next, the mitochondrial respiration capacity was determined using the uncoupler FCCP, which stimulates maximal mitochondrial respiration by dissipating the mitochondrial membrane potential. We found that acidosis significantly enhances the ability of mitochondria to increase their maximal respiratory capacity (Fig. 8i,j). In addition, analysis of mitochondrial reserve capacity, which signifies the ability of mitochondria to further engage in the production of ATP through OXPHOS, revealed a significant increase at pH 6.5 (Fig. 8i,j). These data coupled with data obtained from ATP studies provide evidence that acidosis maintains mitochondrial function during hypoxic stress. This is achieved through an adaptive reprogramming of mitochondrial respiratory efficiency that occurs as a result of changes in the mitochondrial ultrastructure and cristae shape, respiratory supercomplex assembly and maintenance of monomeric ATP synthase.

**Discussion**

The ability of post-mitotic cells to adapt and survive physiological or pathological stress, such as that imposed by oxygen deprivation, has been a long-standing question in biology. Here we report that mild extracellular acidosis, a biological consequence of anaerobic metabolism during hypoxia, restructures the mitochondrial network as an adaptive mechanism to enhance mitochondrial function and promote cellular survival. Mitochondrial remodelling by acidosis, through the activation of a dual programme that modulates mitochondrial dynamics and architecture, represents a novel and physiological pathway that sustains mitochondrial integrity and ATP production despite oxygen limitations. Preventing this reversible and homeostatic process results in mitochondrial dysfunction, fragmentation and
cell death. We provide a mechanism underlying the protective nature of mild acidosis, identify a novel physiological regulator of mitochondrial dynamics in post-mitotic cells and propose a role for mitochondrial morphology in the bioenergetic status of cells.

Survival of post-mitotic cells is highly dependent on a sufficient supply of energy to uphold the exhaustive demands incurred by complex molecular networks. The switch to anaerobic metabolism, albeit a critical cellular metabolic adaptation of hypoxic cells, cannot compensate for the loss of mitochondrial respiration during prolonged hypoxia. As a result, cells faced with prolonged hypoxia will inevitably endure energy failure and cell death if not counteracted with an increase in energy supply. Data presented here demonstrate that post-mitotic cells have evolved a rapid and reversible mechanism to uphold efficient ATP production, via mitochondrial remodelling, that is regulated by acidosis. This is, to our knowledge, the first ascribed function for this metabolic product of anaerobic respiration historically associated with clinical resistance during ischemic insults. We propose a model whereby a threshold accumulation of extracellular protons, acquired through an initial increase in the glycolytic rate during hypoxia, would relay a signal back to mitochondria, potentially via changes in intracellular pH, in order to modulate mitochondrial bioenergetics (Fig. 9).

In recent years, mitochondrial structure and dynamics have emerged as a fundamental aspect for biological life. Our work further highlights this concept in demonstrating the protective nature of mitochondrial restructuring during stress. In addition, we provide a link between mitochondrial morphology and cristae architecture with the metabolic state of cells. The data presented in this study suggest that mitochondrial remodelling can instigate a systemic reconfiguration of mitochondrial efficiency to extract more ATP per oxygen molecule. In essence, acidosis-mediated reorganization of mitochondrial efficiency can override oxygen limitations and allow for the persistence of mitochondrial respiration in an anaerobic environment. This observation refines the well-established role of anaerobic metabolism as a limiting factor for oxidative phosphorylation and puts forth the idea that mitochondrial reprogramming can dictate the bioenergetics of the cell. In view of these findings, the capability of post-mitotic cells to sense and adapt to anaerobic conditions by inducing anaerobic mitochondrial respiration should emerge as a central research theme in the study of physiological and pathological situations that relate to oxygen and energy homeostasis.

Methods

Mice, primary neuronal cultures and cell lines. To generate dorsal telencephalon-specific MFN1 conditional mutant mice, floxed MFN1 (Jackson Laboratories) and Emx1-cre heterozygous females were bred with floxed MFN1 homozygous and MFN2 heterozygous males. Telencephalon-specific SLP2 conditional mutants were generated by breeding floxed SLP2 homozygous females with Foxg1-cre male mice (provided by Dr. Sean G Beng). Cortical neurons were cultured from CD1 wild-type female and males (Charles River) and MFN1+/–, MFN1+/-, MFN2+/–, MFN2–/– and SLP2+/–, SLP2+/–, SLP2–/– knockouts at embryonic day 13.5. Mitochondrial length was assessed by staining with Tom20 (BD Biosciences; 1:100) and Bax (Santa Cruz, 1:100). Cells were imaged with a Zeiss (Carl Zeiss, Germany) confocal microscope equipped with a QCam Digital camera (QImaging Corporation) and Zen software.

Cell culture. The plating density for each cell population was chosen to optimize differentiation of C2C12 myoblasts into myotubes. Cortical neurons and CGNs were seeded on plates (with or without coverslips) at a density of 9.0 × 10^6 and 9.0 × 10^6 neurons, respectively, and maintained in Neurobasal media (Gibco) that contained 2% B27 (Gibco), 1% N2 (Gibco), 0.6 mM L-glutamine (Gibco) and 1% Pen-Strep (Sigma). CGNs were plated in four- or 12-well plates with 4 × 10^5 and 8 × 10^5 neurons, respectively, and maintained in DMEM (Wisent Inc.) that contained 10% dialysed BSA (Sigma), 25 mM KCl, 2 mM glutamine (Invitrogen), 25 mM glucose and 0.1 mg ml⁻¹ gentamycin (Sigma). MCF-7 and HeLa cells were cultured from CD1 mice at postnatal day 7 or 8 (ref. 13). All experiments were approved by the University of Ottawa’s Animal Care ethics committee adhering to the Guidelines of the Canadian Council on Animal Care. Neurons were maintained in culture for 3–5 days before experimentation. MCF-7 breast carcinoma, A549 lung carcinoma, P19 embryonic teratocarcinoma, Cos7 African green monkey kidney fibroblasts, HeLa and C2C12 myoblasts were obtained from ATCC (Manassas, VA, USA). Primary and transformed MEFs were generated from CD1 wild-type and MFN1–/–, MFN2–/–, SLP2–/– (shOPA1 sequence; 5′-CTGAAAGTCTGATACTTTATATGGGAAAT-3′) and mouse-specific short-hairpin RNA (shRNA) OPA1 (shOPA1 sequence; 5′-GCGTGACTTATTAGGAGGAAAT-3′) were prepared using the ViralPort lentiviral expression system (Invitrogen)13. For lentiviruses and adenoviruses, neurons were transduced with 2 MOI (multiplicity of infection), with modifications. For acidosis experiments mimicking physiological conditions, SD or AP media was utilized. Buffer-free and low glucose (5.5 mM) medium (DMEM; Gibco) was freshly prepared and supplemented with B27 and N2 for post-mitotic neurons or 5% FBS for replicating cells. The level of physiological acidification of the extracellular environment is proportional to the cellular density and the buffering capacity of the media. NaHCO₃ was added at 10 mM (post-mitotic cells) or 35 mM (replicating cells) and the pH was adjusted with HCl to 7.2 (SD media) or 6.5 (AP media). Air was bubbled into both media at 22 °C, which stabilizes the pH at 7.2. Culture media were aspirated and cells were washed × 2 in buffer-free low glucose DMEM to remove all traces of highly buffered culture media. Cells were then placed in AP or SD media. AP media slowly reverted to its original set pH under hypoxia, whereas the SD medium remained at pH 7.2. For acute acidosis experiments in hypoxia or normoxia, 30 mM of MES was used as a buffer and the pH was set and stabilized at the required value.

Intracellular pH measurements. Changes in intracellular pH were determined utilizing the ratiometric fluorescent intracellular pH indicator BCECF-AM (Invitrogen). Intracellular pH measurements were performed on cultured cortical neurons plated in 96-well black microplates that were treated for 6 h in the
appropriate experimental media and subsequently loaded with BCECF-AM (1 μM) for 30–45 min at 37 °C. A dual-excitation ratio of 480 and 440 nm and fixed emission at 535 nm was used to measure fluorescence. Since these experiments were designed to determine the effect of extracellular acidosis on intracellular pH, the measurements had to be performed while the cells were maintained in the respective experimental media (that is, media at pH 7.2 or 6.5). For this reason, the use of BCECF-AM was first validated by performing a calibration curve, using the high K+–Nigericin technique, to ensure that intra-cellular changes in pH can be detected in the experimental media. MES-buffered DMEM media was supplemented with 130 mM KCl and the pH was set to 6.0, 6.5, 7.0, 7.5 and 8.0 using KOH. The media of cells previously loaded with BCECF-AM (for 30 min at 37 °C) were then incubated in MES-buffered medium supplemented with the ionophore Nigericin (Molecular Probes, 10 μM) and incubated for 10 min to allow for equilibration of the intracellular pH with the controlled extracellular medium. BCECF-AM fluorescence was measured as described above.

**Immunoblot.** For total cell lysates, cells were washed with PBS, lysed with 4% SDS in PBS, boiled for 5 min and the DNA was sheared by passage through a 26-gauge needle. Primary antibodies recognizing Complex I subunit NDUF9 (Innitrogen; 1:1,000), Complex II 70-KDa Fp subunit (Innitrogen; 1: 10,000), ATP5a (Abcam; 1:1,000), Complex IV subunit 1 (Innitrogen; 1:1,000), Complex III core protein 2 (UCQCR2, Abcam; 1:1,000), DLP1 (DRP1, BD Transduction Laboratories; 1:1,000), Phos-DRP1 S637 and S616 (Cell Signaling; 1:1,000), Fis1 (Biovision; 1:500), OPA1 (Abcam; 1:1,000), MFN1 and 2 (Abcam; 1:1,000), VDAC (Abcam; 1:1,000), mitochondrial inner mitochondrial mem-stash protein 70 (mHSP70, Thermoscientific; 1:5,000) and Actin (Santa Cruz; 1:1,000) were used. A secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch) was used and detected using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer). Representative full-gel shots are shown in Supplementary Fig. 17.

**Immunoprecipitation.** Cells were lysed in RIPA buffer (50 mM Tris (pH 7.2), 150 mM NaCl, 10% NP40 and 1 mM sodium orthovanadate with a protease inhibitor cocktail (PIC)). Cell lysates were incubated with anti-DRP1 antibody (BD Transduction Laboratories) for 1 h while tumbling at 4 °C, followed by overnight incubation with Sepharose A/G beads blocked in 2% BSA. Beads were washed 4 × in 1 × Triton-buffered saline and eluted by boiling at 95 °C for 4 min.

**Time-lapse imaging and mitochondrial fusion assay.** Cortical neurons were seeded on 35-mm dishes with coverslips bottoms (MatTek) coated with poly-D-lysine (VWR International) and infected with 2 MOI of the photoactivatable GFP tagged to ornithine carbamyltransferase (PA-GFP-Oct) and Mito-DrRed. Following 2-h incubation in MES-buffered media at pH 7.2 or 6.5, plates were mounted in a temperature-controlled chamber (37 °C) and visualized with an LSM-510 confocal laser-scanning microscope (Axiovert 200), with a ×63 oil immersion objective. Mitochondrial fusion assays were performed as described previously with modifications12. Briefly, PA-GFP-Oct was first pre-imaged and scanned to ensure that there is no spontaneous activation. PA-GFP-Oct was then photolactivated with a 405-nm laser and the spreading of the signal was imaged every 2 min using a 488-nm line for a total of 22 min. Mito-DrRed was excited at 543 nm and used to ensure that the PA-GFP-Oct activation was within the mitochondria. The fusion rate was expressed as a relative measure of pixel intensity at the indicated time over that at t = 0 min (where 0 min represents the signal detected after 2 min of phototoactivation and equilibration of GFP signal). OPA1 crosslinking. In vivo crosslinking reactions were performed at 37 °C with 10 mM BMH crosslinker (Fisher Scientific) for 20 min. Reactions were terminated by adding 0.001% sodium bisulfite and 10 mM BMH crosslinker (Fisher Scientific) for 20 min. Reactions were terminated by adding 0.001% sodium bisulfite and 10 mM BMH crosslinker (Fisher Scientific) for 20 min and terminated immediately with 1% SDS. Cells were then scraped very lightly. Neurons were fixed in 2% gluteraldehyde for 20 min at 4 °C and 1% osmium tetroxide, 10 mM sodium acetate, pH 7.4, 1 mM EDTA, PIC 1:1,000 on ice using a 25-G needle. Following centrifugation at 110 g for 9 min to remove nuclei and cellular debris, the supernatant was centrifuged at 8,600 g for 9 min to pellet mitochondria. This differential centrifugation step was repeated to further purify the mitochondrial fraction. For BN-PAGE analysis, pellets of whole cells or isolated mitochondria were resuspended in digitonin extraction buffer (50 mM imidazole/HCl pH 7.0, 50 mM NaCl, 5 mM 6-aminohexanolic acid, 1 mM EDTA and the appropriate ratio of digitonin). A 1% digitonin ratio was used for Complex-1 and supercomplexes, and 2% was used for ATP synthase assembly. One hundred fifty micrograms were loaded on 5% tricine and 15% dithiogalactose ratio of coomassie dye in 500 mM 6-aminohexanolic acid on 3–13% large acrylamide gradient gels. Gels were transferred to nitrocellulose membranes and the resulting membranes were subjected to immunoblotting.

**Oxygen consumption.** The Seahorse XF24 Extracellular Flux Analyzer (Seahorse Biosciences; North Billerica, MA, USA) was used to measure oxygen consumption in cells. Cortical neurons were seeded onto 24-well Seahorse plates at a density of 1.5 to 1.8 cells per well. Following treatment, cells were washed with modified Kreb’s Ringer Buffer (128 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM CaCl2, 0.1% BSA (fatty acid-free), 10 mM glucose and 1 mM sodium pyruvate, pH 7.4) and placed in Kreb’s Ringer Buffer for 15 min at 37 °C before loading into the XF Analyser. Following measurements of resting respiration, cells were incubated sequentially with 2.5 mM oligomycin (AL, oligomycin-sensitive OCR), 1 μM carbonylcyanide-m-chlorophenylhydrazone (CCCP, to measure the nonphosphorylating OCR; FCCP (1 μM), to measure the maximal OCR; and antimycin A (2.5 μM) and rotenone (1 μM), to measure the extramitochondrial OCR. Each measurement was taken over a 2-min interval followed by 2 min mixing and 2 min incubation. Three measurements were taken for the OCR measurement, three after oligomycin treatment, two after FCCP and two after antimycin A and rotenone.

**Quantification and statistical analysis.** For mitochondrial length measurements, all mitochondria in a field were measured as per condition and a minimum of 1,000 mitochondria were measured for each condition. For cell death studies, a minimum of 300 cells per field (minimum five fields) were scored for each condition at the indicated time points. The data represent mean values ± s.d. from three independent experiments (n = 3) unless otherwise noted. P-values were obtained using two-tailed Student’s t-test.

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Author contributions

All authors reviewed the manuscript. M.K. conceptualized the study, designed and performed experiments, analysed data and wrote the paper; M.T. assisted, performed and analysed experiments; D.P. assisted in the BN-PAGE and Seahorse experiments and interpreted the data; P.K. designed and performed in vivo experiments and interpreted the data; J.M. provided technical assistance and generated tools for the study; J.G. performed SLP2 experiments; R.B. supervised in vivo experiments; S.P.C. provided the SLP2 conditional mice, supervised and performed SLP2 experiments; M.E.H. provided the Seahorse Analyzer, reagents and interpreted the data; D.P. provided reagents and interpreted the data; and R.S.S. supported and directed the research.

Additional information

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