



HHS Public Access

Author manuscript

Cell. Author manuscript; available in PMC 2016 October 22.

Published in final edited form as:

Cell. 2015 October 22; 163(3): 560–569. doi:10.1016/j.cell.2015.10.001.

Mitochondrial ROS Signaling in Organismal Homeostasis

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Summary

Generation, transformation, and utilization of organic molecules in support of cellular differentiation, growth, and maintenance are basic tenets that define life. In eukaryotes, mitochondrial oxygen consumption plays a central role in these processes. During the process of oxidative phosphorylation, mitochondria utilize oxygen to generate ATP from organic fuel molecules but in the process also produce reactive oxygen species (ROS). While ROS have long been appreciated for their damage-promoting, detrimental effects, there is now a greater understanding of their roles as signaling molecules. Here, we review mitochondrial ROS-mediated signaling pathways with an emphasis on how they are involved in various basal and adaptive physiological responses that control organismal homeostasis.

Mitochondria and Associated Homeostatic and Stress Signaling Pathways

Mitochondria are essential organelles present in all but a few mammalian cell types, where they perform multiple functions. They are the sites of the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS), through which large amounts of ATP are generated using the electrochemical gradient generated across the inner of two membranes by the electron transport chain (ETC). However, their critical roles in metabolism go far beyond glucose oxidation via OXPHOS and include fatty acid and amino acid metabolism and biosynthesis of hormones, heme, and iron sulfur clusters. Furthermore, in addition to metabolism, mitochondria are involved in apoptosis, ion homeostasis, and innate immunity, with new roles in cell and organismal biology being discovered at an unprecedented rate.

Mitochondria are complex in composition, form, and function. Though often depicted as small round or oval structures, they are instead usually dynamic, branched networks that

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constantly fuse and divide under control of specific fission and fusion machineries (Mishra and Chan, 2014). Proteomic analyses indicate that mammalian mitochondria contain ~1,200 proteins, with the precise composition varying significantly between cell and tissue types (Calvo and Mootha, 2010). Thirteen of these proteins are encoded by the maternally inherited mitochondrial DNA (mtDNA) located in the matrix, while the rest are encoded by nuclear genes and targeted to the organelle by specific protein import pathways (Shadel and Clayton, 1997). Thus, mitochondrial biogenesis and homeostasis, including mtDNA expression and maintenance, are under strict control of nuclear gene expression programs (Scarpulla, 2008).

The overall status of mitochondria is constantly monitored, allowing their number, morphology, distribution, and activity to be modulated by developmental, physiological, and environmental cues. This requires bidirectional signaling pathways that mediate crosstalk between mitochondria and the nucleus. Pioneering studies in budding yeast revealed that mitochondrial dysfunction leads to so-called “retrograde signaling” events that result in adaptive changes in nuclear gene expression and metabolism mediated by specific transcription factors (Butow and Avadhani, 2004). Mitochondrial retrograde signaling pathways also exist in mammals and are now receiving considerable attention because they drive both beneficial and pathogenic adaptive responses.

Given their complicated nature, mitochondrial stress can manifest in many forms that elicit different stress signals. Reduced OXPHOS capacity or altered ETC function results in energy deprivation (e.g., reduced ATP/energy charge), altered mitochondrial ROS (mtROS) production, or loss of mitochondrial membrane potential that elicit specific stress-signaling responses (Butow and Avadhani, 2004; Sena and Chandel, 2012). Reduced mitochondrial protein import, improper assembly of large enzymatic complexes (e.g., OXPHOS and ribosomes), and altered chaperone activity can cause proteotoxic stress and mitochondrial unfolded protein responses (Haynes et al., 2013; Rugarli and Langer, 2012). As major sites of ROS production, mitochondria are also prone to oxidative damage and stress. Damage, mutation, or depletion of mtDNA causes distinct forms of mitochondrial stress and downstream signaling (Scheibye-Knudsen et al., 2015; West et al., 2015). Finally, altered morphology, dynamics, and distribution can lead to distinct forms of stress and are linked to mitochondrial turnover by autophagy or mitophagy (Labbé et al., 2014). Changes in these parameters and associated stress-signaling responses can occur downstream of physiological (e.g., nutrient limitations, substrate availability, exercise), environmental (exposure to drugs or toxins), and genetic cues. With regard to genetics, the importance of these pathways is underscored by the fact that inherited mitochondrial diseases are caused by mutations in genes encoding proteins involved in each of these processes that can be inherited maternally (mtDNA mutations) or in a Mendelian fashion (nuclear gene mutations) (Nunnari and Suomalainen, 2012). However, much remains to be learned about these stress pathways. For example, the specific sensors and signaling responses to different forms of mitochondrial stress and how they are manifested in different cells and tissues remain largely unknown. Furthermore, the degree of crosstalk between different mitochondrial stress pathways and what determines whether they elicit beneficial or maladaptive responses are not clear.

Mitochondrial ROS Signaling

ROS are formed from one-electron transfers from a redox donor to molecular oxygen (O_2). This initially forms the anionic free-radical superoxide that can be converted to hydrogen peroxide by superoxide dismutase enzymes (Figure 1). Hydroxyl radical is another ROS that can be formed (e.g., by metal-catalyzed oxidation of hydrogen peroxide), but in this Review, “ROS” refers to superoxide and hydrogen peroxide unless otherwise noted. In mitochondria, the orderly flow of electrons down the mitochondrial ETC to complex IV results in their final deposition into molecular oxygen to form water. However, electrons can also react prematurely with oxygen at sites in the ETC to form superoxide/hydrogen peroxide (Murphy, 2009). Complexes I and III are often regarded as the major sites of mtROS production, but more recent studies indicate that at least ten other mitochondrial enzymes also contribute, including complex II (Quinlan et al., 2013). That different sites of mtROS production have distinct signaling roles and the primary production sites likely change under different physiological conditions is likely (Quinlan et al., 2013; Sena and Chandel, 2012).

That hydrogen peroxide has signaling roles in cells was initially elucidated through studies of receptor tyrosine kinase, growth-factor signaling that showed bursts of ROS production by NADPH oxidase (NOX) enzymes. A major mechanism at play in this scenario is the inactivation of redox-sensitive protein tyrosine phosphatases that normally downregulate these receptors (via dephosphorylation) by localized NOX-dependent production of hydrogen peroxide. Several paradigms emerge from these studies that are relevant to mitochondrial hydrogen peroxide acting as a signal (Finkel, 2012). First, many NOX enzymes produce extracellular superoxide that dismutates to hydrogen peroxide that is then transported across the plasma membrane, perhaps in a regulated manner through aquaporin channels, to effect localized redox signaling. In a similar manner, superoxide produced in the mitochondrial inner-membrane space or matrix can be converted to hydrogen peroxide by SOD1 or SOD2, respectively, allowing it to diffuse into the cytoplasm to signal (Figure 1). Whether this involves free diffusion or facilitated diffusion through specific channels in mitochondrial membranes remains unclear. Second, the inactivation of phosphatases by hydrogen peroxide occurs through the modification of specific reactive thiol side chains (e.g., cysteine). It is now recognized that cysteine residues on many proteins can undergo a variety of redox-dependent modifications, including sequential oxidation (to sulfenic, sulfinic, and sulfonic acid), glutathiolation, and S-nitrosation (Go et al., 2015). Like phosphorylation, ubiquitination, and other post-translational modifications, these redox modifications can alter protein structure and function and be regulatory. Therefore, selective oxidation or modification of redox-dependent thiols in regulatory proteins allows for intricate cellular redox-switch control mechanisms (Finkel, 2012; Go et al., 2015). Redox regulatory proteins that associate with or are otherwise selectively tuned to readout mitochondrial hydrogen peroxide production would provide a mechanism for mtROS signaling (Figure 1). Perinuclear clustering of mitochondria has also been postulated to be a mechanism for direct mitochondrial-nuclear signaling via mtROS (Al-Mehdi et al., 2012).

Superoxide is often summarily dismissed as a relevant signaling molecule because of its chemical properties. For example, unlike hydrogen peroxide, it is a negatively charged

molecule and hence not able to easily diffuse across cell membranes, and it does not engage in protein cysteine oxidation reactions conducive to known redox-switch mechanisms of signaling (Winterbourn, 2008). However, as we will discuss, physiologically relevant, superoxide-mediated signaling does appear to exist that is distinct from hydrogen-peroxide-mediated signaling pathways. Although not “freely” diffusible, mitochondrial superoxide can be released from the intermembrane space into the cytoplasm through the voltage-dependent anion channel that spans the outer mitochondrial membrane (Han et al., 2003) (Figure 1). This includes superoxide that is generated in the intermembrane space by complex III, as well as that generated in the matrix by complex I (and potentially by other enzymes) (Lustgarten et al., 2012). However, the latter may require superoxide levels to cross a critical threshold (e.g., when antioxidant defenses are limiting). In the budding yeast, *S. cerevisiae*, mitochondrial superoxide is released into the cytoplasm by a specific isoform of the voltage-dependent anion channel (Por1p) or, in the absence of Por1p, through the TOM protein import complex (Budzińska et al., 2009). Thus, superoxide released from mitochondria can, in principle, participate directly in cytoplasmic signaling processes (Figure 1). Finally, it is possible that mitochondrial matrix superoxide signals to the cytoplasm via second-messenger systems that involved released oxidized components (e.g., heme or FeS clusters) or other redox sensors have yet to be defined (Figure 1).

Mitochondrial ROS Signaling in Organismal Physiology: Lessons from Model Systems

There is now extensive evidence from the study of model organisms supporting an active role for mtROS signaling in organismal physiology and adaptive responses (Hamanaka and Chandel, 2010; Ristow and Zarse, 2010; Yun and Finkel, 2014). The tractability of these genetic model systems has led to elucidation of new molecular details and signaling pathways underlying these responses. In this regard, much has been learned in the context of aging and longevity studies, which will be highlighted here.

In the nematode worm, *C. elegans*, several pathways that extend lifespan involve increased mitochondrial ROS production and signaling. These studies have called into question the “mitochondrial” and “free radical” theories of aging (at least as originally formulated) by implicating ROS as pro-longevity signals as opposed to damaging, pro-aging agents, as they've long been viewed. Ristow and colleagues broke initial ground in this area by showing that reduced glucose availability leads to increased mitochondrial respiration and mtROS production that delays worm aging (Schulz et al., 2007). They and others have subsequently found that increased mtROS is a common downstream event in many conserved longevity-promoting interventions, which has led to the concept of “mitohormesis” (Ristow and Zarse, 2010; Yun and Finkel, 2014). Recent work in this area includes mtROS signaling in the anti-aging effects of reduced insulin/IGF signaling and D-glucosamine supplementation (Weimer et al., 2014; Zarse et al., 2012). Inhibition of the mitochondrial ETC by certain mutations or inactivation of mitochondrial SOD2 increases worm lifespan and has been causally linked to increased mtROS production (Dancy et al., 2014). Hekimi and colleagues have recently shown that this involves a unique form of activation of apoptotic signaling cascades to promote protective stress responses rather than

apoptosis (Yee et al., 2014). Longevity-extending effects of mtROS in worms are also mediated by HIF1 and AMP kinase signaling and are linked to some degree to enhanced immunity (Hwang et al., 2014; Lee et al., 2010). Like in worms, mtROS signaling extends chronological lifespan in *S. cerevisiae*, which, in part, is how reduced TORC1 signaling mediates longevity in this organism (Bonawitz et al., 2007; Pan et al., 2011; Schroeder et al., 2013). Here, the mtROS signal activates the DNA-damage-sensing kinases, Tel1p and Rad35p (yeast orthologs of ATM and Chk2), leading to enhanced subtelomeric silencing via inactivation of the jumonji-family, H3K36 demethylase, Rph1p (Schroeder et al., 2013). This response, vis-à-vis the mtROS-mediated, apoptotic-signaling response in worms discussed above (Yee et al., 2014), suggests that a new paradigm is emerging whereby canonical stress-response pathways (e.g., DNA repair and apoptosis) are utilized differentially to sense mtROS to elicit adaptive, homeostatic responses instead of the emergency and cell death responses for which they were defined originally.

While the above discussion was limited largely to examples from worms and yeast, it is important to note that similar mtROS longevity pathways have been shown to operate in other invertebrates and in mice (Hekimi et al., 2011; Ristow and Schmeisser, 2011). Furthermore, these pathways are not limited to anti-aging responses. For example, mtROS signaling has also been implicated in other homeostatic pathways and processes, including wound healing (Xu and Chisholm, 2014), survival under hypoxia (Schieber and Chandel, 2014), intracellular pH homeostasis (Johnson et al., 2012), cell differentiation (Hamanaka and Chandel, 2010; Hamanaka et al., 2013; Tormos et al., 2011), and innate immunity (West et al., 2011). Accordingly, the remainder of this Review will be devoted to the role of mtROS in whole-body physiology, with the main focus on neuroendocrine control of systemic metabolism in mammals.

ROS Generation and Central Control of Whole-Body Metabolism

The amount of mtROS generated in metabolic processes depends on the fuel load and type (lipid, carbohydrate, protein), as well as the amount, composition, activity, and dynamics of mitochondria in the cell or tissue involved. Because ROS are de facto by-products of mitochondrial oxidative metabolism, it may not be surprising that studies have connected mtROS to neuroendocrine control of metabolism, including feeding behavior, energy expenditure, and glucose homeostasis (Andrews et al., 2008; Benani et al., 2007; Diano et al., 2011; Horvath et al., 2009; Leloup et al., 2006; Long et al., 2014).

Modulation of mtROS Production by Uncoupling Protein 2 in the Brain

The discovery of new members of the uncoupling protein (UCP) family in 1997 (Fleury et al., 1997) and the localization of UCP2 to specific brain areas (Horvath et al., 1999; Richard et al., 1998) initiated studies by several groups to unmask what these proteins might do in neurons and in other brain cells. Regardless of the wealth of information gained in these studies, there remains a great ambiguity about the precise role of these UCPs in cellular functions (Brand and Esteves, 2005).

There is little debate regarding the functional relevance of UCP1 in brown adipose tissue, where it promotes mitochondrial fatty acid oxidation by uncoupling mitochondrial electron

transport from ATP generation under adrenergic and thyroid control (Ricquier, 1998). Under this scenario, the gained energy is dissipated in the form of heat, which is the hallmark of non-shivering thermogenesis (Ricquier, 1998). UCP2 is clearly not an uncoupler, as UCP1 and its precise mode of action remain unclear. Furthermore, none of the cells in the brain that express UCP2 display the aforementioned features of brown adipocytes. At baseline, UCP2 in rodents and primates is expressed predominantly in neurons of basal structures of the brain (Diano et al., 2000; Horvath et al., 1999; Richard et al., 1998). However, UCP2 is induced in many brain sites in response to cellular injury inflicted by physical insults (Bechmann et al., 2002), epileptic seizures (Diano et al., 2003), or ischemia (Deierborg et al., 2008). These seminal observations, together with studies unmasking the bidirectional regulatory relationship between UCP2 and ROS (Arsenijevic et al., 2000; Echtay et al., 2002; Negre-Salvayre et al., 1997), underscore the potential importance of this mitochondrial protein in mtROS control during cellular stress. Pursuit of the physiological functions of UCP2 in normal brain gave further support for this notion.

Initial studies in normal brain implicated UCP2 protein expression in specific subpopulations of neurons that control hunger, energy expenditure, glucose metabolism, and circadian rhythms (Horvath et al., 1999). Simultaneously, but independently, the sites of action of the hunger-promoting peripheral hormone, ghrelin (Cowley et al., 2003), revealed virtually complete overlap of ghrelin action in the brain and UCP2 expression. Eventually it became clear that most cells in the brain that express ghrelin receptors also express UCP2 (Andrews et al., 2008). These observations spurred the interrogation of whether the influence of ghrelin on appetite and food intake is mediated by UCP2 and, if so, via what cellular and intercellular mechanisms (Andrews et al., 2008; Diano and Horvath, 2012; Horvath et al., 2009). Through these investigations, the following chain of events was uncovered regarding UCP2, mtROS, and neuronal activity (Figure 2): (1) ghrelin induces NPY/AgRP neuronal firing via activation of its receptor, GHSR (growth hormone secretagogue receptor), which in turn activates AMP kinase (AMPK); (2) AMPK activation suppresses acetyl CoA carboxylase (ACC) activity, eliminating the inhibitory effect of malonyl-CoA on carnitine palmitoyl transferase 1 (CPT1) activity; (3) CPT1 activation enhances long-chain fatty acid oxidation by mitochondria and the generation of mtROS; (4) ROS, together with fatty acids, promotes UCP2 gene transcription and activity; (5) UCP2, via enhancing proton leak, tempers mtROS production, allowing continuous fatty acid oxidation without oxidative stress burden and transcription of genes that promote mitochondrial biogenesis and activity (e.g., NRF1), enabling continuous support of the bioenergetic needs of sustained firing of NPY/AgRP cells; and (6) activity of NPY/AgRP neurons results in activity-dependent synaptic plasticity and inhibition of POMC neurons. The intracellular signaling of AgRP neurons is supportive of neuronal activation and decreases vulnerability of these neurons to cellular stress. It was also suggested that the long-chain fatty acyl CoAs utilized under these conditions arise from the periphery under the control of the hypothalamic NPY/AgRP neurons (Andrews et al., 2008). Knocking out UCP2 diminished the ability of AgRP neurons to inhibit mtROS production and maintain low levels of cellular ROS, which, in turn, impairs their neuronal functions (Andrews et al., 2008). Consistent with this model, cellular and electric activity of AgRP neurons is restored, together with reversal of impaired feeding behavior, when a ROS-scavenging cocktail

containing L-cysteine is infused into the parenchyma of the hypothalamus (Andrews et al., 2008). These results strongly indicate that behaviors associated with low-energy availability hinge significantly on mtROS signaling associated with lipid metabolism in key neurons that drive these organismal adaptations. However, we recognize that UCP2 likely controls mtROS indirectly, via alteration of mitochondrial fuel utilization, and that other consequences of UCP2 activity in regulating metabolism may also be important (Andrews et al., 2008; Diano and Horvath, 2012; Horvath et al., 2009; Pecqueur et al., 2009; Vozza et al., 2014).

The studies outlined above also suggested the exact opposite scenario for those neurons that support cessation of eating once enough food is consumed (satiety). The hypothalamic neurons that produce pro-opiomelanocortin (POMC) and related peptides are located in the same area as the hunger-promoting AgRP neurons that, when active, suppress POMC neuronal activity. POMC neurons appear to have elevated ROS, as indicated by intracellular dihydroethidium (DHE) staining (Andrews et al., 2008; Diano et al., 2011), when they are active to promote satiety and increased energy expenditure after food consumption (Figure 3). Elevated mtROS production is a logical POMC neuronal activator, since it would likely correlate with mitochondrial activation during full oxidation of glucose, the main fuel of these neurons (Parton et al., 2007). When ROS levels are suppressed chemically, POMC neurons are hyperpolarized and their firing rate declines (Diano et al., 2011). Conversely, when in-slice preparations of POMC neurons are exposed to hydrogen peroxide, they become depolarized and their firing rate is elevated (Diano et al., 2011). These results indicate that it is actually mtROS, rather than glucose itself, that instigate POMC neuronal firing (Diano et al., 2011; Long et al., 2014). Because hypothalamic POMC neurons are involved in both behavioral and autonomic control of energy and glucose metabolism, it is not surprising that hypothalamic ROS control has been tied to all of these processes (Figure 3).

ROS are Satiety Signals

From a behavioral and systemic perspective, under normal conditions, elevated hypothalamic ROS levels are permissive of suppression of eating, increased energy expenditure, and glucose utilization by peripheral tissues (Andrews et al., 2008; Benani et al., 2007; Diano et al., 2011; Leloup et al., 2006; Long et al., 2014). In fact, it is reasonable to conclude that ROS signaling in the brain, as well as in the periphery, is fundamental for appropriate behavioral and autonomic adaptations to energy surplus, for example, after the consumption of a meal. If one interferes with ROS in these physiological processes, both behavioral and autonomic correlates of proper fuel management of the body will be impaired (Andrews et al., 2008; Diano et al., 2011; Horvath et al., 2009; Long et al., 2014). Since mitochondrial perturbations via UCP2 modulate these responses, we conclude that mtROS signaling plays a fundamental and crucial role in physiological regulation of systemic metabolism.

The relentless pursuit of available energy sources in the environment is mandatory for organismal survival, and hence, hunger and hunger-controlled circuits motivate this critical behavior. However, advanced animal species, including humans, also developed the

capacity to maintain energy reserves, for example, in the form of fat or glycogen, so they do not have to continuously feed to survive. This evolutionary advantage, by default, demands that “gauges” and “switches” are in place to shut off feeding when storage capacity is sufficient. The role of the hypothalamic melanocortin system appears to represent both the gauge and the switch, with ROS, likely driven by mtROS production, being the sensor. When cellular ROS reach a threshold, they activate POMC neurons, enabling the cessation of feeding and initiation of storage and utilization of fuels via processes controlled by insulin and leptin (Varela and Horvath, 2012). The control of insulin release itself is regulated by redox events (Bashan et al., 2009). At the same time, elevating ROS in the hypothalamus reduces activity of AgRP neurons that propagate hunger (Andrews et al., 2008) (Figure 2). While it remains unknown what underlies the differential effect of ROS on POMC and AgRP neurons, cell-specific expression of plasma membrane channels could be involved. For example, superoxide can directly alter the activity of potassium channels (Avshalumov and Rice, 2003), which play important roles in glucose sensing by hypothalamic neurons and under regulatory control by UCP2 (Parton et al., 2007). Likewise, it remains unclear how mtROS signals reach neuronal perikarya. In this regard, it is noteworthy that mitochondria in both AgRP and POMC neurons dynamically change their morphology and localization within a short period of time (Andrews et al., 2008; Coppola et al., 2007; Dietrich et al., 2013; Schneeberger et al., 2013). Mitochondrial fission and fusion capacity and the physical interaction between mitochondria and the endoplasmic reticulum may be involved in mtROS production or associated signaling events (Dietrich et al., 2013; Nasrallah and Horvath, 2014; Schneeberger et al., 2013).

It is important to note that ROS are likely not the satiety signal in the hypothalamus under all circumstances. For example, the known effect of cannabinoids on promoting ferocious appetite, regardless of metabolic satiety, is actually mediated by mtROS-driven POMC neurons (Koch et al., 2015). However, under cannabinoid influence, POMC neurons reverse their function and promote appetite because they switch from the release of its satiety-promoting neuropeptide, α -melanocyte-stimulating hormone (α -MSH), to β -endorphin (Koch et al., 2015). This switch is enabled by a mitochondrial adaptive response controlled by UCP2 (Koch et al., 2015) (Figure 3C). Whether this response is specific for this pharmacological situation or relevant to regulation of metabolism under certain physiological circumstances is unknown.

Mitochondrial ROS and Exercise

A key feature of animal species is their need to physically relocate in a rapid and predictable fashion to find food, reproduce, or escape danger. This is accomplished, in *grosso modo*, through behavioral adaptations, which are the sum of coordinating sensory and effector functions via the communication between the nervous system (the sensory component) and the musculoskeletal system (the effector component). Movement and exercise, in general, have long been recognized as key to supporting not only the aforementioned fundamental biological needs, but also tissue health and longevity. Ristow and colleagues showed that suppressing ROS generation during aerobic exercise (likely, in large part, mtROS) diminishes beneficial outcomes on many exercise-related parameters (Ristow et al., 2009). They also argue for the critical relevance of mtROS signaling transients as important

contributors to longevity, as well as mediators of other signaling responses that promote healthspan and longevity (Schmeisser et al., 2013; Zarse et al., 2012). In support of the notion that mtROS transients (and not sustained elevated ROS levels) mediate the benefits of exercise on integrative physiology, UCP2 was found crucial to support exercise-induced synaptogenesis in the dentate gyrus of the hippocampal formation, a key site of spatial learning (Dietrich et al., 2008). This same mechanism is also relevant to hippocampal development (Simon-Areces et al., 2012) and lifespan determination (Andrews and Horvath, 2009).

Short- and Long-Term Effects of ROS

The distinction between physiologically beneficial short ROS bursts and pathological, sustained high ROS levels on cellular and circuit integrity is best illustrated in the response of the hypothalamus to exposure to calorie-dense diets containing high levels of fats and carbohydrates (Diano et al., 2011; Parton et al., 2007). On regular chow diet, which contains <20% fat, ROS levels fluctuate between hunger and satiety states and mice maintain a positive correlation between hypothalamic ROS levels, circulating leptin levels (the adipose hormone that signals to the hypothalamus when sufficient amount of food is consumed), and activity of POMC neurons (Diano et al., 2011). However, when animals are placed on calorie-dense diets (>40% fat), homeostatic control of energy metabolism is gradually deregulated. When this occurs, animals steadily increase their fat stores, which results in steady elevation of circulating leptin. Under homeostatic conditions, this elevated leptin would decrease feeding and enable maintenance of fat stores. This inability of elevated leptin levels to avoid or reverse weight gain is called leptin resistance, for which many cellular and tissue mechanisms have been proposed to explain. One of these mechanisms relates to the aforementioned mtROS control in POMC and AgRP neurons. On a high-fat diet, the positive correlation between hypothalamic ROS, circulating leptin levels, and POMC neuronal activity in mice deteriorates (Diano et al., 2011). Under these conditions, hypothalamic ROS levels plateau and do not follow the robust and steady elevation in circulating leptin concentrations. At the same time, POMC neuronal activity is diminished (Diano et al., 2011). The underlying cause for this dysregulation is tied to PPAR γ -related proliferation of peroxisomes and increased ROS elimination by catalase (Diano et al., 2011; Long et al., 2014). We suggest that leptin affects this process by enabling increased glucose uptake in POMC and AgRP neurons, which is accompanied by increased lipid load. Initially, this will lead to increased mtROS production and crossing cellular ROS thresholds that promote satiety by activating POMC neurons. However, rising leptin levels on high-fat diet will continue to promote glucose uptake by these neurons, which, together with increasing lipid load, will overburden these postmitotic cells. The scenario in which lipid and carbohydrate load is increasing in cells provides a perfect energetic basis for growth. Carbohydrate oxidation will dominate mitochondrial OXPHOS and ATP generation, while long-chain fatty acids are diverted from mitochondria via the malonyl-CoA shuttle for biogenesis of membranes and cell growth. However, in cells whose growth is strictly limited, such as neurons of the adult central nervous system, lipids cannot be continuously utilized for membrane biogenesis and they will accumulate in various intracellular compartments, including the endoplasmic reticulum. The activation of peroxisome

proliferation under these circumstances provides an alternative mechanism through which excess fat within cells can be eliminated via mitochondrial β oxidation that is less coupled to ATP generation. While this is a beneficial process to prevent lipotoxicity, peroxisomal catalase activity will limit ROS generation needed for proper signaling from the hypothalamus to diminish feeding and increase energy expenditure (Diano et al., 2011). These alterations may have multiple negative effects on cellular signaling mechanisms and organelle integrity and function.

A “Fuel Hypothesis” of Cellular Function

While the above details on mtROS-related mechanisms were described in relation to a hypothalamic circuit that controls feeding behavior and peripheral fuel partitioning and utilization, these processes are likely relevant to the functionality and impairment of neurons in various parts of the brain. For example, UCP2-dependent control of mtROS was also found in dopamine neurons in the midbrain substantia nigra, where both normal functioning of these cells and their protection under cellular stress were attributed to this mechanism (Andrews et al., 2005, 2006; Conti et al., 2005). Dopamine cells in this area of the brain are connected to control of fine motor functions and complex motivated behaviors. A role for the peripheral metabolic hormone, ghrelin, was identified to modulate the activity of these neurons and to prevent their impairment and death in models of Parkinson's disease (Abizaid et al., 2006; Andrews et al., 2009). The intracellular signaling pathway that enabled these beneficial effects of ghrelin was related to ROS control by the same machinery as described above in relation to the control of feeding (Andrews et al., 2008). Similar ghrelin action was also found in the hippocampus to promote learning and memory and to ameliorate deficits of animals in a model of Alzheimer's disease (Diano et al., 2006). Because the changing metabolic state (hunger \leftrightarrow satiety) is closely tied to predictable changes in complex behaviors, it is reasonable to suggest that fluctuating ROS levels (mediated by alterations in mtROS output) in all or part of the brain play a critical regulatory role in the synchronization of neuronal circuit activity in support of continuous and appropriate behavioral adaptations. Furthermore, mtROS-controlled neuronal activity in the hypothalamus is sufficient to affect complex behaviors beyond feeding. For example, acute activation of hypothalamic AgRP neurons rapidly alters stereotypic behaviors, locomotion, and anxiety (Dietrich et al., 2015). Finally, we speculate that purposeful alterations in mtROS production to effect cellular redox signaling pathways (Figure 1) will regulate homeostasis in other tissues. In simple terms, it is reasonable to assume that cellular functions in any tissue are determined by fuel availability, uptake, and utilization and that these “fuel” principles drive and orchestrate signaling modalities, including mtROS signaling, to control homeostatic and adaptive responses. For example, intracellular metabolic pathways have distinct and dominant impacts on various immune cell types (Caro-Maldonado et al., 2012; Procaccini et al., 2010), and UCP2-dependent mtROS regulation is connected to both adaptive and innate immune cell functions (Arsenijevic et al., 2000; Horvath et al., 2003; Krauss et al., 2002). How other cells and tissues respond to such signals and the intersection between cell-intrinsic signaling and control by the CNS is an exciting area of future research. In this regard, determining whether the aforementioned CNS processes are mediated by cell-non-autonomous mtROS-mediated signals similar to those documented in *C. elegans*

downstream of ETC disruption and mtROS (Durieux et al., 2011; Schieber and Chandel, 2014) will be important to consider.

Challenges in ROS Research and Ramifications of ROS as Central Controllers of Organismal Homeostasis

In this Review, we have summarized how changes in mtROS production can impact cellular ROS thresholds and redox signaling events that control basal physiological functions and adaptive responses. As such, we argue that these pathways are critical for organismal homeostasis, stress responsiveness, health, and longevity. However, these pathways are far from understood and are in need of more intensive study. Some current impediments and other relevant considerations as the field moves forward in this area are covered below.

At present, it remains very difficult to effectively measure ROS in cells and in vivo. The use of commercially fluorescent ROS probes is widespread but often without the knowledge that these do not always readout specific ROS species faithfully and are prone to other confounding artifacts (Kalyanaram et al., 2012). That is not to say that these are not useful to a degree, but they should not be used as the only line of evidence to implicate ROS in a response. Development of better ROS assays and probes is ongoing (Ezeri a et al., 2014; Logan et al., 2014; Woolley et al., 2013), yet there remains a great need for additional forward progress in this important area.

There are important implications for ROS as physiological signaling molecules that impact therapeutic strategies. Two that immediately come to mind are the use of antioxidants and anti-obesity strategies that target the CNS.

Antioxidants have long been considered potential therapeutics for a number of conditions involving oxidative stress. In general, trials using these have failed, likely, in part, because of unintentional inhibition of important basal and adaptive ROS signaling pathways. It has even been argued that taking antioxidants as daily dietary supplements might also perturb these ROS pathways in ways that are not beneficial or even harmful (Ristow, 2014). Similarly, strategies that target activation of POMC neurons (to promote satiety) as an anti-obesity/anti-diabetic strategy might also be confounded due to perturbation of ROS signaling circuits that we have described herein (Dietrich and Horvath, 2012). That is, we assert that many compounds that activate POMC neurons will, by default, upregulate ROS production and signaling that governs their activity (Diano et al., 2011). If this scenario is maintained for a prolonged period of time (hours, days, months), weight loss may be accomplished, but sustained ROS levels could have a multitude of unintended detrimental consequences. As these examples point out, previously held views of ROS as just damaging agents that need to be eliminated are out of date, and a new appreciation of their signaling roles is important to consider going forward. The fact that mitochondria are major producers of ROS also highlights the importance of better understanding what controls their activity and rate of mtROS production, both in terms of redox signaling and oxidative stress. In this regard, the greater recent appreciation of mitochondria as important signaling hubs (Chandel, 2014; West et al., 2011) has begun to transcend older, over-simplified views of these organelles as just sites of intermediary metabolism and ATP production.

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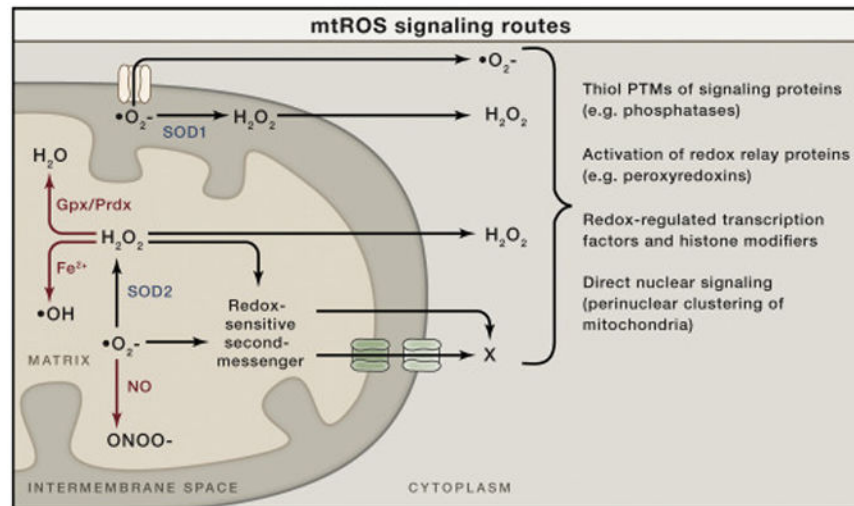


Figure 1. Mitochondrial ROS Signaling Basics

Superoxide (O_2^-) is generated on both sides of the inner mitochondrial membrane and hence arises in the matrix or the intermembrane space (IMS). Superoxide can be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase enzymes (SOD1 in the IMS or SOD2 in the matrix). The resulting hydrogen peroxide can cross membranes and enter the cytoplasm to promote redox signaling. Superoxide is not readily membrane permeable but may be released into the cytoplasm through specific outer membrane channels, as shown (see main text). In addition to signaling in the cytoplasm directly, both superoxide and hydrogen peroxide could, in principle, oxidize or modify other molecules in mitochondria that can be released into the cytoplasm to signal (redox-sensitive second messenger; X). These mitochondrial ROS (mtROS) can generate signaling responses and changes in nuclear gene expression in multiple ways (shown to the right). There are other fates of mtROS that would prevent signaling (or potentially enact other signaling and damage responses). For example, superoxide can react with nitric oxide (NO) to form peroxynitrite (ONOO^-). This would prevent its conversion to hydrogen peroxide, could cause damage by the highly reactive peroxynitrite, and could potentially limit NO availability for its own type of signaling. Hydrogen peroxide can be eliminated enzymatically by glutathione peroxidase (Gpx) in the matrix or peroxyredoxins (Prdx) in the matrix and elsewhere in the cell. Peroxyredoxins can also promote redox signaling by promoting disulfide bond formation in target proteins. Finally, in the presence of transition metals, hydrogen peroxide can generate damaging hydroxyl radicals (OH).

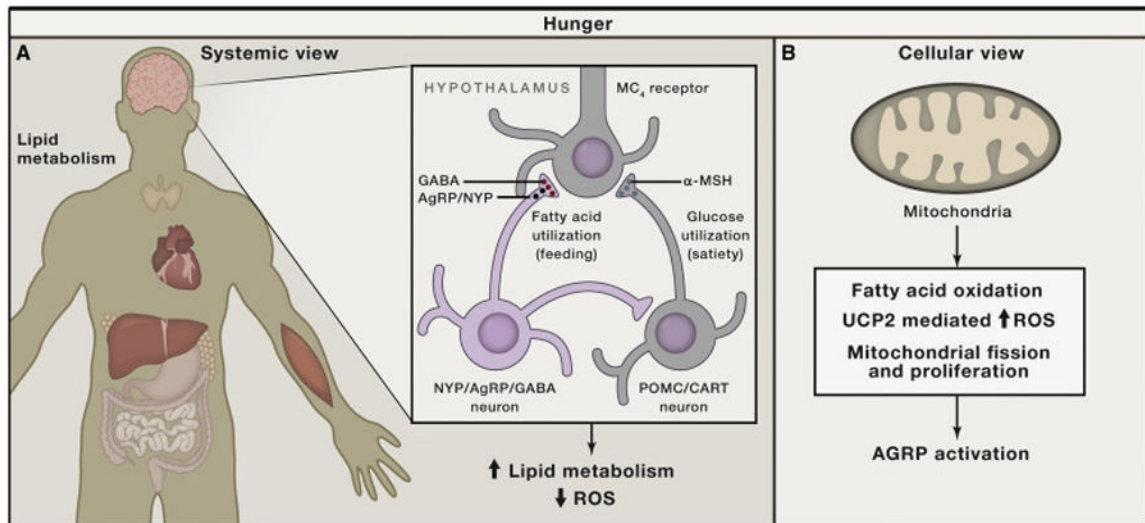


Figure 2. Schematic Illustration of Hypothalamic Control of Negative Energy Metabolism with Low ROS

(A) In the brain, the hypothalamus contains neuronal populations that control hunger (negative energy balance) and satiety (positive energy balance). Hunger state is promoted by neurons (green) that produce Agouti-related peptide (AgRP) and neuropeptide Y (NPY), as well as GABA. When these neurons are active (hunger, calorie restriction, starvation), systemic metabolism is shifting to lipid metabolism with an overall lower level of mtROS production in all tissues.

(B) The activation of AgRP neurons during negative energy balance is promoted by pathways enabling long-chain fatty acid oxidation in the mitochondria, which is enabled by maintenance of low mtROS generation by engagement of UCP2 and mechanisms that propagate fission and/or proliferation of mitochondria (NRF1, Sirt1, and PGC1 α).

(C) UCP2 is believed to function as a conditional mitochondrial uncoupler in the presence of long-chain fatty acids and, as such, reduces mtROS production and overall cellular ROS levels.

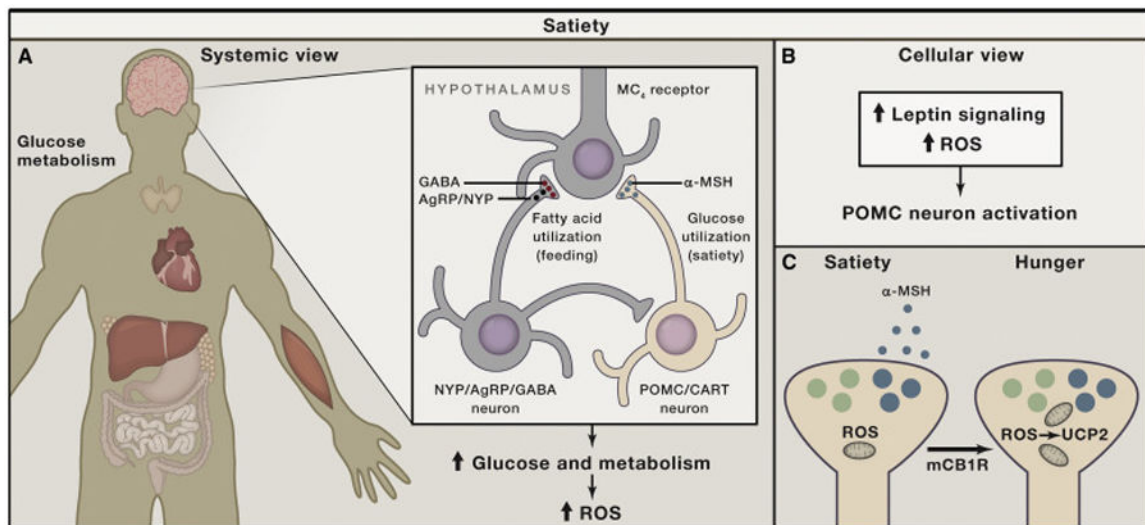


Figure 3. Schematic Illustration of Hypothalamic Control of Positive Energy Metabolism with Elevated ROS

(A) Satiety (feeling full) is promoted by hypothalamic neurons (blue) that produce pro-opiomelanocortin (POMC)-derived peptides, such as α -MSH, which, in turn, act on melanocortin-4-receptor-containing neurons. When these neurons are active, systemic metabolism is shifting toward glucose utilization, with enhanced mtROS production contributing to increased cellular ROS in various tissues.

(B) The activation of POMC neurons after a meal is accomplished by ROS, in part driven by increased mtROS production, and is supported by intracellular leptin (Jak/Stat) and insulin (PI-3K/PTEN) signaling, involving altered K-ATP channel activity.

(C) Recent studies indicate that, under unique circumstances (e.g., activation of cannabinoid receptors), POMC neurons, while still driven by ROS, will become promoters of hunger rather than satiety because they shift to releasing appetite-stimulating opiates (β -endorphin) through UCP2-dependent mitochondrial adaptations.