Anxiety and brain mitochondria: A bidirectional crosstalk

Michaela D. Filiou\textsuperscript{1,2} and Carmen Sandi\textsuperscript{3}

1 Max Planck Institute of Psychiatry, Munich, Germany
2 Department of Biological Applications and Technology, University of Ioannina, Greece
3 Brain Mind Institute, Ecole Polytechnique Federale de Lausanne (EPFL), Switzerland

Correspondence: m\textasciitilde{filiou}@uoi.gr (M.D. Filiou); carmen.sandi@epfl.ch (C. Sandi)
Abstract

Accumulating data highlight the contribution of brain mitochondria and bioenergetics to psychiatric disorders and stress-related pathologies. Although anxiety has not received much attention in this booming literature, a bidirectional interplay between anxiety and brain mitochondria and metabolism has recently started to emerge. Substantial observations indicate alterations in mitochondria and metabolism in highly anxious individuals and, conversely, anxiety symptoms in humans suffering from mitochondrial disorders. Genetic and pharmacological efforts have made substantial progress at advancing the causal involvement of specific mitochondrial and metabolic factors in anxiety. In this review, we discuss this converging evidence and highlight the relevance to develop a research focus on targeting mitochondria as a promising approach to alleviate anxiety.

Keywords
Psychiatric disorders; Oxidative phosphorylation; Bioenergetics; Anxiolytic drugs; Neurosteroids; Stress.
High anxiety levels represent a major health challenge

Anxiety is a state of uneasiness and enhanced vigilance in the absence of an immediate threat, frequently accompanied by somatic, behavioral, and cognitive distress responses. There is a remarkable variability on the frequency and intensity to which each of us experiences anxiety in the course of our lives. While some tend to remain calm throughout a variety of challenges, others are prone to show attentional biases and exaggerated stress responses towards potentially threatening stimuli [1]. This enduring predisposition is a ‘trait’ (Box 1). High anxiety trait is a risk factor for the development of several psychopathologies, such as anxiety disorders [2] and depression [2, 3].

Despite being unpleasant, anxiety is considered an important evolutionary adaptation, as its characteristic state of alertness can help protecting individuals from potential dangers [4]. However, when its associated responses are excessive or maladaptive, anxiety becomes pathological and it can manifest in a variety of anxiety disorders (Box 1) [5]. Pathological anxiety -as expressed in the widespread nature of anxiety disorders- represents the most common mental health problem, with a lifetime prevalence of over 20% [6], posing a major human and economic burden in modern societies [7]. Moreover, high anxiety is to a great extent comorbid with several neuropathologies [8, 9]. Existing treatments (e.g., cognitive behavioral therapy and anxiolytic drugs) are still suboptimal, resulting in limited remission rate efficiency (approximately 50% in adults), whereas more than a third of anxiety disorders patients are treatment-resistant [10]. Given the limited efficacy of current (psychological and pharmacological) therapies, the need to develop novel treatments based on a greater understanding of the mechanisms underlying high anxiety has been recently voiced [6, 11].

Neuroimaging studies in humans [12, 13] and circuit-based approaches in rodents [11, 14] are driving strong progress on the understanding of how the brain produces anxiety states and associated defensive behaviors. They point at a network of distributed brain regions involved in the processing of anxiogenic stimuli and the regulation of anxiety-related behaviors and physiological responses [14]. Specifically, key regions included in this network are the (extended) amygdala, hippocampus, medial prefrontal cortex (including the anterior cingulate cortex), the hypothalamus, midbrain (e.g., raphe nuclei) and brainstem (e.g., periaqueductal grey). While these studies may help revealing key targets for intervention at the neuronal circuit level, they do not account for the contribution that intracellular organelles may provide to variation in neuronal and circuit function. Here, we examine emerging evidence that brings together mitochondrial function with anxiety from a bidirectional perspective and ask whether there is a causal link between them. Then, we explore the relevance of targeting mitochondrial function and associated metabolic pathways as a mean to modify neural circuits underlying anxiety.
Mitochondrial are small intracellular organelles with multiple functions

Mitochondrial proteins are generated by a coordinated interaction between nuclear and mitochondrial DNA (mtDNA). Vertebrate mtDNA encodes for 37 mitochondrial genes in humans and mice with a hugely varying mitochondrial DNA copy number (mtDNA-cn) per cell [15]. Mitochondria provide most of the organism’s energy in the form of ATP, which is largely generated by oxidative phosphorylation in the inner mitochondrial membrane (Box 2). On its turn, oxidative phosphorylation is a key producer of reactive oxygen species (ROS), mainly accountable for oxidative stress. In addition to their role as powerhouses of the cell, mitochondria exert multiple functions in cellular metabolism, ranging from macromolecule biosynthesis, nutrient catabolism, redox homeostasis and waste management [16]. Mitochondria can also support cellular adaptation to a variety of challenges -such as stress- through their capacities to establish fusion-fission dynamics, to migrate to diverse cell locations and to interact with other organelles (e.g., endoplasmic reticulum). Perhaps not surprisingly, mitochondria are revealing to be crucial for a broad range of neuronal (e.g., neuronal growth and sprouting, synaptic transmission, neuronal plasticity and connectivity) and brain (e.g., neural oscillations, cognition) functions (Box 3).

Mitochondrial dysfunction as a central feature of neuropsychiatric disorders

A mitochondrial etiology of neuropsychiatric disorders has been recently proposed [17, 18], not only because of the increasing experimental evidence linking mitochondrial dysfunction with neuropathologies, but also due to concrete lines of reasoning regarding the role of mitochondria. Specifically, a rather influential argument relies on the fact that the brain has the highest energy consumption (around 20% of our body oxygen and 25 % of our glucose) while just representing 3% of our body’s mass. It, thus, reasons that biological conditions which involve subtle mitochondrial alterations would particularly have a negative impact on brain functions, increasing vulnerability to brain disorders [17]. A second argument relates to the strong capacity of stress to trigger and exacerbate neuropsychiatric disorders and the metabolic-costly nature of the neuronal adaptations in structure and function impinged by these conditions [19]. Consequently, it predicts that individuals with suboptimal mitochondrial function would be particularly vulnerable to stress-associated depletion of the brain’s energy resources and, hence, to the development of psychopathologies (e.g., depression) [20]. A further line of thinking relies on the fact that the stress hormones glucocorticoids are produced and metabolized by mitochondria and, conversely, mitochondrial function is affected by glucocorticoids and other metabolic stress mediators [21, 22]; likewise, mitochondria are crucially involved in the catabolism of catecholamines. In addition, mitochondria critically determine the magnitude of hormonal stress responses [23] and regulate cellular homeostasis during response to
stressful stimuli [24]. Accordingly, mitochondrial functioning would be intimately linked to mechanisms of stress adaptation and regulation [20, 23].

In agreement with these views, compelling genetic, biochemical, molecular, structural and functional evidence, gathered over the last decade, supports a key role for mitochondrial dysfunction in neuropsychiatric disorders [25]. The bulk of these data, including clinical and preclinical studies, has being recently summarized in excellent reviews [17, 25, 26] that have emphasized evidence in schizophrenia [27], bipolar disorder [28] or depression [20]. However, much less attention has been drawn to the potential involvement of mitochondria in anxiety, which is the main focus of this review. Below, we synthesize literature from animal models and clinical studies that highlight different aspects of mitochondrial dysfunction in the context of increased anxiety and those that, conversely, describe anxiogenic phenotypes in the context of mitochondrial disorders.

**Evidence for mitochondrial dysfunction in high anxiety**

In agreement with the importance of bioenergetics for brain function and dysfunction [17, 18, 20, 29] mentioned earlier, emerging evidence implicates brain energy metabolism in anxiety. However, this is not the full story. An increasing number of studies is revealing that several other mitochondrial functions – such as oxidative stress, apoptosis, neurosteroid production, or mitochondrial biogenesis – are also altered in individuals with high anxiety.

**Findings on measurements of brain energy metabolism**

Proteomics- and metabolomics-based studies in mouse lines genetically-selected for differences in anxiety-like behaviors have been instrumental in pointing at a broad range of alterations in mitochondria and brain energy metabolism (for an account of energy production pathways and other mitochondrial functions, see Box 2) as a critical feature of anxiety. Importantly, they have consistently reported differences in brain levels of glycolytic enzymes [30]. Although glycolysis – the process whereby glucose is converted into pyruvate – mainly takes place in the cytoplasm, it is closely linked to mitochondrial energy production (see Box 3). Thus, in the CD1-based mouse lines of high (HAB) and low (LAB) anxiety-related behavior [31-34], analyses in synaptosomes from the cingulate cortex – a brain region critically implicated in anxiety – revealed lower expression of almost all enzymes catalyzing glycolysis in HAB mice [32]. Cortical synaptosomes from HAB mice displayed higher protein expression for subunits of all oxidative phosphorylation complexes, including both nuclear and mitochondrial-encoded gene transcripts [32]. Most probably, these alterations may reflect a compensatory mechanism to the reduced glycolysis capacity in HAB mice, but they might also have undesirable consequences for ROS production, as discussed later.
However, studies in other animal models and brain regions have not confirmed increased expression of oxidative phosphorylation complexes as a general characteristic of the anxious brain [35-38]. For example, in the nucleus accumbens from outbred Wistar rats, high anxious animals display, instead, lower mitochondrial complex I and II protein levels and respiratory capacity, as well as lower ATP levels, than low-anxious animals [36, 38]. Differences in methods, species, subcellular populations studied or other may account for the anxiety-related differences in oxidative phosphorylation in the studies reviewed above. A plausible explanation is that anxiety is reflected in specific adaptations in mitochondrial function in different brain regions (and most probably cell types). In support for this hypothesis, high anxiety in Wistar rats is not only associated with decreased mitochondrial respiratory capacity in the nucleus accumbens, as indicated above, but also with increased capacity in the prefrontal cortex [35], and no changes in the basolateral amygdala [36] or in the ventral tegmental area [38]. The sensitivity of the nucleus accumbens to show anxiety-related differences in brain energy metabolites has also been also confirmed with 1H-NMR spectroscopy approaches in mice [39] and humans [40].

These findings raise the intriguing possibility that anxiety is reflected in adaptations in mitochondrial molecular architecture, and corresponding function, in a cell type- and circuit-dependent manner. Hypothetically, given the fundamental role of mitochondria in the synthesis of the main excitatory—glutamate— and the main inhibitory—GABA—neurotransmitters, mitochondrial adaptations observed in the context of anxiety may contribute to the neural excitation-inhibition imbalance believed to underlie several neuropsychiatric disorders [41]. A further potential implication is that brain region-specific bioenergetic psychiatric disorders [41]. A further potential implication is that brain region-specific bioenergetic changes reflect the divergent patterns of hyperactivation and hypoactivation in specific brain regions commonly reported by neuroimaging studies in the context of anxiety and psychopathology [42].

**Findings on measurements of oxidative stress**

Oxidative stress is the imbalance between free radicals (e.g., ROS) and antioxidant defense. Energy production and oxidative phosphorylation activity are among the most prominent mechanisms of ROS generation (Box 2). Due to technical limitations to measure ROS levels directly, oxidative stress is typically quantified by measuring ROS-related metabolites, markers of oxidative damage (e.g., lipid peroxidation, DNA damage), and/or antioxidant enzymes’ levels or activities.

Findings from studies that measured ROS metabolites or markers of oxidative damage are largely unanimous in reporting increased oxidative stress in highly anxious individuals [43], particularly when inflammation and activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous
system are involved [44]. Thus, in humans, different markers of lipid peroxidation, such as malondialdehyde (MDA) [45] and F2-isoprostanes [46] have been reported to be increased in blood samples from high anxiety patients. Similarly, in rodents, natural and genetically-selected variation in basal anxiety has been consistently associated with markers of oxidative damage in both neurons and glia in several brain regions (e.g., cerebellum, hippocampus, cortex, nucleus accumbens) and in peripheral blood cells [36, 47, 48].

However, although levels of antioxidant enzymes are also frequently altered in patients with anxiety disorders [43, 49] and animal models of anxiety [31, 32], the direction of changes for antioxidant levels with regards to anxiety is less clear. This is best exemplified for the firstly identified anxiety-related antioxidant gene, glyoxalase 1 (Glo1; an enzyme from the glyoxalase system that detoxifies the glycolysis byproduct methylglyoxal) [50]. Although in agreement with the original observations [50], some mouse studies have implicated higher Glo1 expression in higher anxiety [51], other reports have indicated the opposite relationship [30, 33]. A reason for this discrepancy may be related to different set points in the organism corresponding to different anxiety models as well as different mouse strains studied, and suggests that indices of oxidative damage and ROS metabolites may be more reproducible markers for oxidative stress in the context of anxiety.

**Findings on neurosteroid production – The translocator protein 18kDa (TSPO)**

Mitochondria are critically implicated in the synthesis of neurosteroids, allosteric modulators of GABA(A) receptor function, a neurotransmitter system well-known for its crucial involvement in anxiety. By promoting cholesterol transport to the inner mitochondrial membrane, the outer mitochondrial translocator protein (18 kDa) (TSPO; formerly called the peripheral benzodiazepine receptor) critically controls the rate-limiting step in neurosteroidogenesis [52]. In addition, TSPO can modulate Ca²⁺ homeostasis, ROS generation and ATP production [53]. A polymorphism on the TSPO gene has been associated with anxiety-like disorders [54] and suggested to act by altering the rate of corticosterone synthesis [55]. Furthermore, low TSPO expression levels in blood cells have been reported in several anxiety disorders (for a review see [43]).

**Findings from studies measuring mitochondrial DNA copy number**

Mitochondrial biogenesis responds to changes in cellular energetic needs and can respond, as well, to oxidative stress. It is commonly measured indirectly, by quantifying mitochondrial DNA copy number (mtDNA-cn) as a rather inaccurate (due to the high heterogeneity of mtDNA-cn per mitochondrion as well as mitochondrial number per cell) estimate of mitochondrial number. Several studies have consistently shown alterations in mtDNA-cn in blood cells or in saliva samples from psychiatric patients
Several studies have confirmed that the index is affected as well in anxiety. Thus, higher mtDNA-cn has been found in leukocytes from individuals with lifetime anxiety disorder [57] and in saliva samples from anxious adolescents [58]. Stress may be a factor here, as higher mtDNA-cn was also reported in leukocytes from humans that had suffered early life stress [57] and in blood samples from mice submitted to chronic stress [59].

**Clinical evidence linking mitochondria disorders with anxiety**

Mitochondrial disorders -a group of diseases that involves dysfunctional mitochondria due to deleterious mutations in mitochondrial or nuclear genomes- are among those with higher prevalence (approx. 1 in 4,300 people) of inherited neurological disorders [60], including psychiatric disorders. Multiple studies confirm that patients with mitochondrial disorders exhibit psychiatric symptoms and/or have a psychiatric diagnosis at a significantly higher rate than the general population [61, 62]. This has been a critical argument in supporting a causal role for mitochondria in psychiatric disorders. Anxiety has been found among the primary psychiatric manifestations in patients with mitochondrial disorders, along with mood disorders, cognitive impairments and psychosis [63, 64]. Specifically, increased levels of reported anxiety-related symptoms such as phobias and panic attacks with agoraphobia were found in patients with an adenine-to-guanine mutation on mitochondrial transfer RNA, which is associated with diabetes mellitus [65]. Interestingly, a high predisposition to develop high anxiety levels was also reported for mothers of mildly affected children suffering from maternally inherited mitochondrial disorders compared to mothers of children suffering from other metabolic diseases [66]. These findings suggest an increased predisposition to mental illness in matrilineal relatives of individuals with mitochondrial disorder due to sharing the same mtDNA [66].

**Gene targeting confirms a role for mitochondria in anxiety**

**Studies targeting mitochondrial DNA**

Several studies have applied different mtDNA targeting strategies in mice to investigate to what extent mitochondria contribute to specific biobehavioral phenotypes [67], and confirmed that anxiety-like behaviors and physiological responses to stress change with variation in mtDNA. Thus, the generation of congenic mouse strains, in which mitochondria from one mouse strain is substituted with mitochondria from other strains, led to changes in anxiety-like behaviors and glucocorticoid responses to challenges depending upon variation in mtDNA [68, 69]. Similarly, glucocorticoid responses to stress were also affected by inducing specific mtDNA mutations [i.e., in two mtDNA-encoded respiratory chain subunits, NADH dehydrogenase 6 and cytochrome c oxidase subunit] in the same genetic background [23]. Another phenomenon that was found to lead to increased anxiety-like behaviors was
the generation of mouse lines presenting mtDNA heteroplasmy (a mixture of mtDNA copies within the same cell and organism which, under natural conditions, may be due to gene mutations taking place in some, but not all, mtDNA or to mixed inheritance) [70]. Furthermore, modeling the effects of mtDNA damage [e.g., by overexpressing the mutated form of the mtDNA repair enzyme UNG1 (Uracil-DNA glycosylase)] in forebrain neurons led to hypo-anxiety [71]. However, a note of caution should be added regarding specificity for anxiety, as most of the studies summarized here reported, as well, other major behavioral and cellular alterations [70, 71].

**Studies targeting genes involved in oxidative stress**

From the evidence reviewed above, indicating a strong association between oxidative stress markers and anxiety-related behavior, the key question that arises is whether oxidative stress regulatory systems -particularly those engrained in the mitochondria- can modulate anxiety [72], and whether oxidative stress is a cause or a consequence of anxiety [73].

A few studies have addressed this question by genetically targeting genes coding for the transcription of antioxidant enzymes in rodents and testing their effects in anxiety tests. A critical gene in this context is the nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2), as it transcriptionally upregulates various genes involved in antioxidant responses, including those involved in glutathione (GSH) synthesis. A study involving Nrf2 silencing by intraventricular siRNA infusion reported increased anxiety-related behavior [74]. The same effect was achieved through deletion of a gene from the glutathione peroxidase family (Gpx4) in ventral midbrain dopaminergic neurons [75]. Conversely, overexpression of the antioxidant catalase targeted to the mitochondria led, instead, to reduced anxiety-related behavior [76]. Regarding the involvement of the Glo1 gene, several genetic overexpression strategies (i.e., ubiquitous; circumscribed to neurons; or directly targeted to the cingulate cortex) led to increased [51], while its inhibition to decreased [33] anxiety-like behaviors.

**Studies targeting genes that regulate other mitochondrial proteins**

Several studies have reported changes in anxiety-like behaviors when genetically manipulating the expression of specific mitochondrial proteins in mice. For example, TSPO overexpression in the hippocampal dentate gyrus in mice exerted anxiolytic effects, along with increasing hippocampal levels of the neurosteroid allopregnanolone [77]. Conversely, reducing expression of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein in heterozygote mice led to increased anxiety-like behaviors [78]. On its turn, neuronal-specific Bcl-2 overexpression decreased anxiety-related behavior [79]. Another example involves the uncoupling protein 2 (UCP2), a member of the mitochondrial transporter superfamily that uncouples oxidative phosphorylation to ATP synthesis. Its deficiency, either
ubiquitously [80] or specifically in the hippocampus [81] leads, as well, to anxiety-like behaviors. Overall, these studies suggest that perturbation of mitochondrial function in the brain may lead to increased anxiety.

**The pharmacological link between mitochondrial function and anxiety**

Another way to interrogate whether mitochondrial dysfunction and anxiety are causally interrelated is to examine studies involving pharmacological treatments and assessment of anxiety-related behaviors, which we synthesize below.

**Anxiolytic drugs have effects on mitochondrial function**

Anxiolytic drugs are, to a large extent, effective in ameliorating anxiety symptoms. Would they include changes in mitochondrial function among their actions? Although studies addressing this question are scarce, available data suggest that this could be the case. Notably, systemic treatment in rats with the prototypic anxiolytic from the benzodiazepine superfamily, diazepam, was shown to boost mitochondrial respiration in the nucleus accumbens [38] and to decrease levels of mitochondrial phospholipids in the liver [82]. Likewise, several antidepressants with anxiolytic capacity have been reported to affect different mitochondrial parameters. Some of the key examples include reports in rodents for increased mitochondrial antioxidant activity following administration of monoamine oxidase inhibitors [83], selective serotonin reuptake inhibitors (SSRIs) [84] and the atypical antidepressant tianeptine [85]. SSRI treatment effectively attenuated both chronic stress-induced anxiety and changes in mitochondrial proteins [86]. Furthermore, neurosteroids, with established anxiolytic effects, have been shown to closely interact with mitochondria; i.e., they have specific mitochondrial binding sites, regulate Ca²⁺ efflux and boost mitochondrial function [87]. Although it remains to be tested whether mitochondria contribute to the anxiolytic effects of these drugs, these data reinforce the view that mitochondria should attract attention as potential target systems for organelle-directed pharmacological interventions in the context of anxiety.

**Drugs targeting mitochondrial function and oxidative stress can ameliorate anxiety**

Modulating mitochondrial function is emerging as an attractive therapeutic strategy to treat the increased number of disorders (e.g., from cancer to neurodegeneration) involving mitochondrial dysfunction [88], including psychiatric disorders [89]. Although anxiety has not yet received much attention, there are several examples of anxiolytic effects following treatment with drugs that target mitochondrial function broadly. However, the complexity of mitochondrial structure and function, including the difficulty of pharmacologically targeting mitochondrial proteins in a specific manner,
have hindered progress in the development of effective and specific drugs [89, 90]. An overview of the anxiolytic effects of the studies discussed here is shown in Table 1.

**Targeting oxidative stress.** Oxidative stress has received a great deal of attention as a relevant therapeutic target for anxiety. N-acetyl-cysteine [91], a multi-target drug and precursor of the antioxidant GSH, has been shown to exert anxiolytic effects in different mouse strains [92] and to reduce chronic stress-induced increased anxiety levels in zebrafish [93]. When given as adjunctive treatment with SSRIs, NAC ameliorated symptoms in adolescents with anxiety disorders [94]. Methylene blue—a compound with multiple mitochondria-related functions, including antioxidant actions—was reported to decrease age-dependent, anxiety-like behaviors in mice [95].

In order to ensure that mitochondrial processes are selectively targeted, a key strategy is to conjugate compounds with a lipophilic cation [i.e. triphenylphosphonium (TPP+)] that crosses the double mitochondrial membrane and allows organelle-specific accumulation of the desired compound [96]. One such compound with antioxidant capacity, SkQ1, was shown to effectively reduce anxiety-like behaviors in both senescence-accelerated OXYS and Wistar rats [97]. Another very interesting mitochondria-targeted antioxidant compound designed upon this principle is MitoQ, as it was shown to readily cross the blood-brain barrier [98]. MitoQ contains a ubiquinone which is converted to the antioxidant ubiquinol at complex II of oxidative phosphorylation, exerting antioxidant actions in vivo [99]. Recently, its anxiolytic therapeutic potential was proven in mice [100]. Specifically, MitoQ was chronically administered through drinking water to genetically-selected HAB mice that exhibit alterations in mitochondrial markers and increased oxidative stress, as described above [100]. Interestingly, the molecular signatures identified for the anxiolytic effect in brain (e.g., increased expression of Prdx3 Slc25a22, and HK1) and plasma (e.g., higher myo-inositol levels) of HAB mice ‘responders’ to MitoQ treatment (as compared to ‘non-responders’ HAB mice), were reminiscent of the differences previously described when comparing HAB and LAB mice [32, 34]. These data suggest that MitoQ exerts anxiolytic effects by reversing the molecular perturbations observed in high anxiety. Importantly, MitoQ is available over the counter and has been administered in humans in the context of clinical studies with no reported side-effects [101, 102], thus having significant potential for clinical implementation in anxiety disorders.

**Targeting TSPO protein.** Several TSPO ligands with capacity to enhance brain concentrations of neurosteroids (i.e., pregnenolone and allopregnanolone), and thus facilitating GABA(A) receptor activity, have been developed. Some of the developed ligands are characterized by strong anxiolytic effects [91, 103] and exempted from undesirable side-effects typically elicited by conventional benzodiazepines [104]. Their efficacy can be exemplified by the TSPO ligand XBD173 that, in humans,
exerts potent antipanic efficacy without sedation and withdrawal after 1 week of treatment [52]. However, XBD173 binding affinity to TSPO binding was found to be highly variable across different human brains, and XBD173 was reported not to be superior than placebo in a clinical trial phase II of generalized anxiety disorder patients [105] (NCT 00108836).

Targeting mitochondrial protein import. Acetyl-L-carnitine (LAC), an acetylated from of carnitine, is a multi-target molecule that, among other effects, is involved in the regulation of acetyl-CoA uptake into the mitochondria for subsequent fatty acid oxidation (Box 3). Although most preclinical work on LAC in humans and rodents has focused on depression [106], existing evidence supports the interest in further exploring its potential anxiolytic effects. LAC supplementation was shown to induce anxiolytic effects in patients with minimal hepatic encephalopathy [107] and in zebrafish [108]. In the latter model, LAC was, as well, able to prevent the lipid peroxidation induced by acute stress [108]. It should be noted, though, that other mechanisms of action —such as its reported antioxidant properties [109]— may also be implicated in LAC effects. In fact, the promiscuity of mechanisms involved in mitochondrial targeted agents is further supported by a recent study implicating a common mechanistic pathway (i.e., involving the astroglial glutamate exchanger xCT and activation of glutamatergic mGlu2 receptors in ventral hippocampus) for LAC and NAC treatments in the context of stress-resilience and antidepressant-like effects [110].

There are certainly several other ways to molecularly target mitochondria, but its implementation in the context of anxiety has generally not being tested [88, 89]. For example, given the central importance of nicotinamide adenine dinucleotide (NAD⁺) and related co-enzymes in the regulation of nearly all metabolic processes, supplementation with NAD⁺ boosters, such as nicotinamide riboside (NR) or nicotinamide mononucleotide (NMN), are being broadly tested in a wide range of medical conditions [111]. Although its anxiolytic capacity remains to be systematically studied, a recent study in mice delivers promise for anxiolytic effects of NR treatment [112]. Importantly, NAD⁺ is an essential cofactor of Siruins and NAD⁺ boosters can activate the deacetylase Sirtuin 1 (SIRT1) and its downstream targets with a consequent facilitation of mitochondrial function [111]. Activation of this pathway seems to be particularly relevant as genetic deletion of SIRT1, either ubiquitously [113] or specifically in the nucleus accumbens [114] modulates anxiety-like behaviors in mice. A few preclinical studies suggest that resveratrol, a polyphenolic compound that among other actions has been reported to activate SIRT1, may also exert anti-anxiety effects [115, 116].

In addition, we should also note that, although beyond the scope of this review, there are several examples of non-pharmacological treatments (such as exercise [117], nutritional approaches [35, 118]) or endocannabinoids [119, 120]) with concurrent actions in brain mitochondrial activity and anxiety.
In particular, a ketonic diet consisting on medium chain triglycerides (MCTs) was recently shown to ameliorate anxiety symptoms in rats and modify mitochondrial respiratory capacity in the prefrontal cortex [35].

**Concluding remarks and future directions**

Although anxiety has not received much attention in the booming literature establishing a central role for mitochondria in psychiatric disorders, ample data reviewed here allow us to establish the existence of an intrinsic bidirectional relationship between anxiety and brain mitochondrial function. On the one hand, there is strong evidence that mitochondrial disorders and genetic disruptions of mtDNA or essential mitochondrial genes lead to increased oxidative stress [121]. In addition to putative effects that mitochondrial alterations can impinge of neuronal function, increased oxidative stress is as well an important trigger of anxious behaviors (see above). The contribution of high ROS levels to impaired neural function has been well documented [122]. In addition, mitochondrial perturbations can critically disrupt hormonal stress responses [23]. On the other hand, anxious states, particularly when persistent—as it is the case of high trait anxiety individuals and those with anxiety disorders—typically course with oxidative stress [43] potentially linked to their enhanced inflammation levels and heightened activity of the HPA axis and the sympathetic nervous system [44]. High ROS levels in anxiety may be the result of either excessive energy production and oxidative phosphorylation processes, as described for the cingulate cortex in the HAB line of mice [32]. Alternatively, they may be decoupled from ATP production, as described for the nucleus accumbens in high anxious Wistar rats [36] and, thus, reflect abnormal ROS production and/or clearing by the different mitochondrial and non-mitochondrial compartments. Furthermore, highly anxious states may affect mitochondrial function through the actions of glucocorticoids and other metabolic stress mediators [21, 22].

Although progress to convincingly address causality for specific mitochondrial processes in the generation of anxiety has been hindered by technical limitations, the tools to target mitochondria are rapidly improving [90]. New approaches should address determine that mitochondrial changes in anxiety are not an epiphenomenon (see Outstanding questions). It will be important to overcome difficulties in interpreting data in which several mitochondrial interventions may have the same effect due to their common functions (i.e. intervention on glycolysis vs. oxidative phosphorylation affects energy production and respiration in a non-discriminable manner unless explicitly tested) or due to their synergistic action (e.g. altered levels of both mitochondrial and cytoplasmic antioxidants in response to increased oxidative stress). Holistic approaches will be required to fully understand this intrinsic relationship between anxiety and mitochondrial (patho)biology [88]. Hypothetically, given the fundamental role of mitochondria in the synthesis of the main excitatory—glutamate— and the main
inhibitory –GABA– neurotransmitters, mitochondrial adaptations observed in the context of anxiety may contribute to the neural excitation-inhibition imbalance believed to underlie several neuropsychiatric disorders [41]. Importantly, glutamate excitotoxicity is a major trigger of oxidative stress in the brain [123] and high cortical glutamate levels have been documented in highly anxious individuals [124]. Chronic stress promotes anxiety [125] as well as oxidative stress damage [126]. In addition, stress increases brain glutamate levels and shifts the excitatory/inhibitory balance towards increased excitation [40, 127, 128].

Therefore, although the current state of knowledge allows a rather general statement, giving the impression that any intervention that disrupts any of the multiple mitochondrial functions in the brain could eventually result in anxious states. This may indeed be the case for drastic impairments in essential elements of mitochondrial function (anxiety being, thus, a defensive adaptation of the organism to internal sensing of metabolic maladjustment). However, the emerging data indicating brain region-dependent opposite changes in mitochondrial respiratory capacity in anxious animals [35, 36] raise the intriguing possibility that natural variation in anxiety is reflected in adaptations in mitochondrial molecular architecture, and corresponding function, in a cell type- and circuit-dependent manner. Different brain circuits are activated in response to diverse anxiogenic stimuli in an anxiety phenotype-dependent manner [129]; accordingly, the bioenergetic requirements vary across brain regions. Although this degree of specificity may pose a challenging question to the validity of pharmacological or nutritional approaches targeting mitochondrial function as a means to counteract anxious phenotypes, there is emerging evidence indicating that this type of treatments will produce specific interactions with brain region and cellular specificity. For example, the anxiolytic effects of an MCT ketonic diet in rats were observed in parallel with differential effects of the diet in mitochondrial function and protein expression in the prefrontal cortex and nucleus accumbens [35]. Therefore, the contribution of mitochondria in shaping anxiety phenotypes in a region-and cell-type specific manner should be the focus of future investigations. It will also be important to understand which mitochondrial changes represent adaptations to compensate dysfunctions in parallel systems, and which changes represent exhaustion and disease. Furthermore, future studies should extend the characterization of mitochondrial function beyond current approaches to incorporate other relevant phenomena –such as analyses of mitochondria biogenesis, mitophagy, fusion and fission, subcellular location and morphology- to achieve an integrated understanding of how brain mitochondria may shape anxiety phenotypes.
Acknowledgments

This work has been supported by grants from the Hellenic Foundation of Research and Innovation [HFRI Grant, General Secretariat of Research and Technology (GSRT), No. 660] to M.D. Filiou and from the Swiss National Science Foundation (No 31003A_176206 and NCCR SYNAPSY No. 51NF40-158776 and 51NF40–185897) to C. Sandi.
Box 1. Anxiety state, anxiety trait and anxiety disorders

Anxiety is a response to potential threats characterized by increased apprehension and vigilance. It typically involves a range of physiological (e.g., activation of the sympathetic nervous system -with the corresponding increase in hear rate, blood pressure and sweating- and the HPA axis, with resulting increases in glucocorticoid hormones), emotional (e.g., excessive or sustained fear) and behavioral (e.g., behavioral inhibition and avoidance, scanning) reactions.

Anxiety differs from ‘fear’ and from ‘stress’. While anxiety is typically triggered by situations that do not represent immediate danger, fear is the response to a real or imminent threat, and stress the response to a challenging, not necessarily fearful, stimulus. Importantly, high or chronic stress levels can lead to increased anxiety [130].

There are important distinctions to make on how anxiety manifests in individuals:

**Anxiety state** is the transient display of anxiety responses triggered by immediate uncertainty. The Spielberger State-Trait Anxiety Inventory (STAI) ‘state’ subscale (STAI-S) [131] is the most prevalent measurement in human research.

**Anxiety trait** is the stable predisposition of an individual to consider a broad range of situations as potentially dangerous [132]. In most animal species, anxiety trait levels are expressed along a continuum, from mild to strong, across individuals. In humans, anxiety trait is one of the main dimensions of the personality factor neuroticism and frequently measured with the STAI ‘trait’ subscale (STAI-T) [131].

**Anxiety disorders** are the most prevalent mental health conditions, with high levels of distress, chronicity, and functional impairment [133]. The key features are excessive and enduring fear, anxiety and/or the avoidance of perceived threats [6]. According to the Diagnostic and Statistical Manual of Mental Disorders V (DSM V) [134], anxiety disorders consist of 12 different conditions, including generalized anxiety disorder, panic disorder, agoraphobia, and social anxiety disorder. Anxiety disorders have their origin in genetic and environmental factors, such as early life stress [135], substance abuse and microbiota [136]. Note that in the DSM V, post-traumatic stress disorder (PTSD) has been classified not as an anxiety disorder, but in the separate category of Trauma and Stressor-related disorders. For a review on mitochondrial function in vulnerability to PTSD, see [137].
BOX 2. Mitochondrial functions

Mitochondria perform a plethora of functions in the cell/organisms and constitute the epicenter of energy metabolism (for detailed reviews, see [138, 139]. They are better known for their key energetic roles, which are intimately related to the production and regulation of reactive oxygen species (ROS). These two functions are summarized below. In addition, mitochondria exert a wide range of additional functions, including [140] a key regulatory role in calcium buffering; the regulation of apoptotic signaling; and the degradation of damaged mitochondria through autophagic digestion (i.e., mitophagy).

**Energy production.** Cellular respiration takes place in mitochondria through the citric acid cycle (or Krebs cycle) and oxidative phosphorylation. During the citric acid cycle, high energy electron carriers such as NADH and FADH₂, are produced and, then, used by oxidative phosphorylation to create a proton-motive force across the inner mitochondrial membrane. The oxidative phosphorylation comprises five multi-subunit complexes (I-V) that work in a coordinated manner to eventually produce ATP. The metabolic support for mitochondrial energy production largely relies on glycolysis -the major pathway that catabolizes glucose, the main brain fuel- that takes place in the cytoplasm. Pyruvate, the endproduct of glycolysis, is transported to mitochondria and converted to acetyl-CoA. Fatty acids, the building blocks of lipids, are also transported to mitochondria through carnitine, where they are broken down to acetyl-CoA by mitochondrial enzymes. Acetyl-CoA is the entry point of the citric acid cycle, thus allowing both carbohydrates and lipids to enter the cycle and be oxidized so as to produce energy in the form of ATP [141].

**Regulation of oxidative stress** results from cellular states in which pro-oxidants exceed antioxidant capacity, resulting in increased levels of ROS and reactive nitrogen species. Although ROS may have some beneficial effects, when expressed at high levels they can harm lipids, proteins, RNA and DNA, leading to cell oxidative damage and, eventually, death. A main source for the generation of these reactive species is the energy-producing process of mitochondrial oxidative phosphorylation. Antioxidant enzymes include superoxide dismutase, catalase (an enzyme that metabolizes H₂O₂ into H₂O and O₂), glutathione peroxidase (GPX), glutathione (GSH), and several vitamins (A, C and E).
Box 3. Mitochondria are critical regulators of neural structure and function

Neurons have particularly high energy requirements for their broad myriad of functions, from resting state to action potentials. Thus, neurons are highly dependent on ATP production, that is mainly generated by their own mitochondria [142] and supported by astrocytes [143]. The use of energy varies for different cell types and brain regions [144]. The highest energy levels are used on synaptic transmission [145].

Mitochondrial activity modulates neural cell proliferation during development [146] and early lineage progression and aging phenotypes in adult hippocampal neurogenesis [147]. Importantly, neurons are particularly complex cells, with specialized compartments and frequently long projections. Ensuring mitochondria delivery to distant synaptic sites and maintaining their proper function (i.e., neuronal mitostasis) is essential for the appropriate function of neurons and synapses [148]. Mitochondrial dendritic distribution mediates spine and synaptic plasticity [149]. Synaptic mitochondria differ from mitochondria in other neuronal compartments in their protein content [150] and their susceptibility to insults [151].

Changes in brain energy metabolism may have been instrumental for the remarkable development of higher cognitive functions from earlier primates to humans [152]. Higher brain functions, from sensory perception to memory function and consciousness, engage fast neuronal network oscillations in the gamma-frequency band (30–100 Hz) [153], with glucose being the most effective energy substrate [153]. Inherent mitochondrial variability may account for individual differences in the aforementioned functions. Mitochondrial morphology correlates with age-related cognitive function characteristics in higher primates [154]. Dysfunctions in mitochondria-driven processes may causally disrupt brain circuits, affecting behavioral outputs and leading to pathological states [20]. Accumulation of damaged mitochondria in distal parts lead to neurite retraction and consequent synaptic vulnerability [155]. Age-related alterations in calcium regulation, increased ROS production and reduced mitophagy capacity contribute to neurodegeneration [156].
References

27. Ben-Shachar, D. (2017) Mitochondrial multifaceted dysfunction in schizophrenia; complex I as a possible pathological target. Schizophrenia research 187, 3-10
31. Filiou, M.D., et al. (2014) Behavioral extremes of trait anxiety in mice are characterized by distinct metabolic profiles. Journal of psychiatric research 58, 115-122
38. van der Kooij, M.A., et al. (2018) Diazepam actions in the VTA enhance social dominance and mitochondrial function in the nucleus accumbens by activation of dopamine D1 receptors. Molecular psychiatry 23, 569-578
44. Black, C.N., et al. (2017) The association between three major physiological stress systems and oxidative DNA and lipid damage. Psychoneuroendocrinology 80, 56-66
46. Steenkamp, L.R., et al. (2017) Severity of anxiety- but not depression- is associated with oxidative stress in Major Depressive Disorder. J Affect Disord. 219
47. Bouayed, J., et al. (2007) Positive correlation between peripheral blood granulocyte oxidative status and level of anxiety in mice. Eur J Pharmacol 564, 146-149


51. McMurray, K.M.J., et al. (2016) Neuronal overexpression of Glo1 or amygdalar microinjection of methylglyoxal is sufficient to regulate anxiety-like behavior in mice. *Behav Brain Res* 301, 119-123


70. Sharpley, M.S., et al. (2012) Heteroplasmy of mouse mtDNA is genetically unstable and results in altered behavior and cognition. *Cell* 151, 333-343
77. Li, L., et al. (2017) Overexpression of the 18 kDa translocator protein (TSPO) in the hippocampal dentate gyrus produced anxiolytic and antidepressant-like behavioural effects. *Neuropharmacology* 125, 117-128
82. Papapanagiotou, A., et al. (1999) Diazepam treatment in rats induces changes in the concentrations of different phospholipid classes in liver and liver mitochondria. *In Vivo* 13, 259-262
89. Ben-Shachar, D. and Ene, H.M. (2018) Mitochondrial Targeted Therapies: Where Do We Stand in Mental Disorders? *Biological psychiatry* 83, 770-779


Figure caption:

**Elements of mitochondria in anxiety and treatment opportunities.**

Some of the key elements of normative mitochondria (A) include the electron transport chain (ETC), composed of several complexes that, coupled with ATP synthase produce ATP (adenosine triphosphate) through redox reactions. During this process, reactive oxygen species (ROS) are generated and their levels are controlled by an antioxidant machinery, notably including glutathione (GSH). Mitochondrial DNA (mtDNA) is DNA present in mitochondria, responsible to generate a portion of mitochondrial proteins. Translocator protein (TSPO) is an 18 kDa protein mainly found on the outer mitochondrial membrane involved in the generation of neurosteroids and in the regulation of Ca²⁺ and ROS. In high anxiety (B), dysfunctional ETC tend to generate less ATP and more ROS, making mitochondrial function less efficient and toxic to several processes. TSPO levels also diminish and, thus, its capacity to produce anxiolytic neurosteroids and to regulate Ca²⁺ and ROS, whose increased levels are toxic to mitochondria and neurons. There are several opportunities for treatment (C) for the ‘anxious’ mitochondria, such as providing L-acetyl carnitine (LAC), N-acetyl cysteine (NAC), medium chain triglycerides (MCTs) or mitochondria-targeted antioxidants (MTA), which directly or indirectly act on the aforementioned mitochondrial processes, all with high potential to reduce anxiety (for a summary of these studies see Table 1).