

Differential proteomics analyses reveal anxiety-associated molecular and cellular mechanisms in cingulate cortex synapses

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Abstract: Selectively inbred animal models for anxiety traits provide useful insights for the elucidation of the relevant pathophysiological mechanisms of anxiety disorders by modeling molecular pathology in a defined genetic background. However, little is currently known about the functional characteristics that distinguish high anxiety-related (HAB) from low anxiety-related (LAB) behaviors. Analytical integration of cingulate cortex (CC) synaptosomal proteomes of HAB and LAB mice revealed that the synaptic environment in the cingulate cortex of HAB animals is dominated by the stabilization and enlargement of existing excitatory dendritic spines, associated with increased high-frequency stimulation of excitatory glutamatergic synapses, enhanced control over the modulation of synaptic strength and relatively weakened inhibitory GABAergic control together with increased spontaneous synaptic activity in non-glutamatergic network members. This is coupled with increased oxidative phosphorylation (OXPHOS), enhanced fatty acid oxidation and ATP production in synaptic mitochondria. The mitochondrial effects of increased oxidative and ionic stress appear to be controlled through at least seven different mechanisms, while the mechanisms attached to the maintenance of mitochondrial structural integrity and protein homeostasis are significantly reinforced. Overall, this analysis describes a context characterized by excitatory long-term potentiation (LTP) maintenance, low de novo spine generation, significant neurotransmission imbalances and structural as well as metabolic adaptations to persistent synaptic mitochondrial Ca^{2+} loading and oxidative stress associated with the HAB phenotype

Keywords: Anxiety Disorders, Cingulate Cortex, Trait Anxiety Mouse Model, Proteomics, Molecular Mechanism

1. Introduction

The wide spectrum of neuropsychiatric conditions collectively known as “anxiety disorders” [1] are highly comorbid with depression [2] and are the most common psychiatric conditions encountered in the general population [3]. Despite considerable advances in understanding the pertinent symptomatology, the neurological and functional mechanisms of anxiety-related behavior are not completely understood. This impedes the search for potential biomarkers that could help define vulnerability to these disorders, characterize disease expression and clinical progression, improve initial clinical diagnosis and decision-making and inform treatment response and efficacy. In this respect, animal models can

help elucidate the relevant disease mechanisms by modeling the pathophysiology in a defined genetic background.

Selective bidirectional inbreeding of CD1 mice according to the time spent on the open arms of the elevated plus-maze gave rise to HAB/ LAB mouse model of trait anxiety [4].

Several divergent characteristics have been described for HAB and LAB mice. HAB mice sleep more than LAB or normal anxiety-related behavior (NAB) mice, with sleep patterns characterized by heavy fragmentation, reduced maintenance of wakefulness, and frequent intrusions of REM sleep. They also exhibit a dramatic increase in electroencephalogram (EEG) delta power during the entire light-dark cycle and more particularly after 6 h sleep

deprivation, a characteristic also found in patients presenting sleep-maintenance insomnia with depression attributes [5]. In HAB mice, chronic mild stress results in reduced central amygdala/lateral amygdala activity. Inversely, LAB mice that experienced chronic mild stress show increased anxiety levels and central amygdala/lateral amygdala activity. Hence variations in the physiological constitution of the lateral to central activity circuitry give rise to a differential regulation of neuronal signal flow through the input-output network of the amygdala [6].

Comparing behavioral extremes of trait anxiety in a non-hypothesis driven manner at the molecular level will allow the identification of phenotype-specific mechanism associated with anxiety traits and shed light on the underlying pathobiology. In a recent multi-omics study of the CC, synaptosomal proteome comparison of HAB and LAB mice revealed protein alterations in energy metabolism, mitochondrial pathways, oxidative stress, and neurotransmission [7]. Here, we implemented a novel analytical integration of the proteomics data to enable the detailed identification of the mechanisms that, at least in part, help explain the origins of the HAB phenotype.

2. Materials and Methods

The proteomics data used for the current study, together with their origins and methods of acquisition were

presented previously [7-10].

Data integration. All integration tasks (data acquisition, structuring and mining; pathways construction, integration and visualization) were conducted using the “Decius” configuration of the CADITM analytical platform [11], proprietary to BM-Systems.

3. Results and Discussion

3.1. Synapses

3.1.1. Reinforcement of Existing Glutamatergic Synaptic Structures

The vast majority of structural synaptic proteins over-represented in CC synaptosomes obtained from HAB animals address glutamatergic post-synaptic densities (Fig.1).

Variants of several of these proteins, such as the AMPA receptors (AMPA: Gria2/Glur2, Gria3/Glur3, x1.35 and x1.55, respectively), the MAGUK membrane-associated guanylate kinases Psd95 and Psd93 (x1.4 each), the contactin-associated protein Caspr1/Cntnap1 (x1.35) and the synaptic scaffolding (adapter) proteins Shank1 (x1.55) are all associated with multiple psychiatric disorders such as autism spectrum disorder, attention deficit-hyperactivity disorder, bipolar disorder, major depressive disorder, and schizophrenia [12].

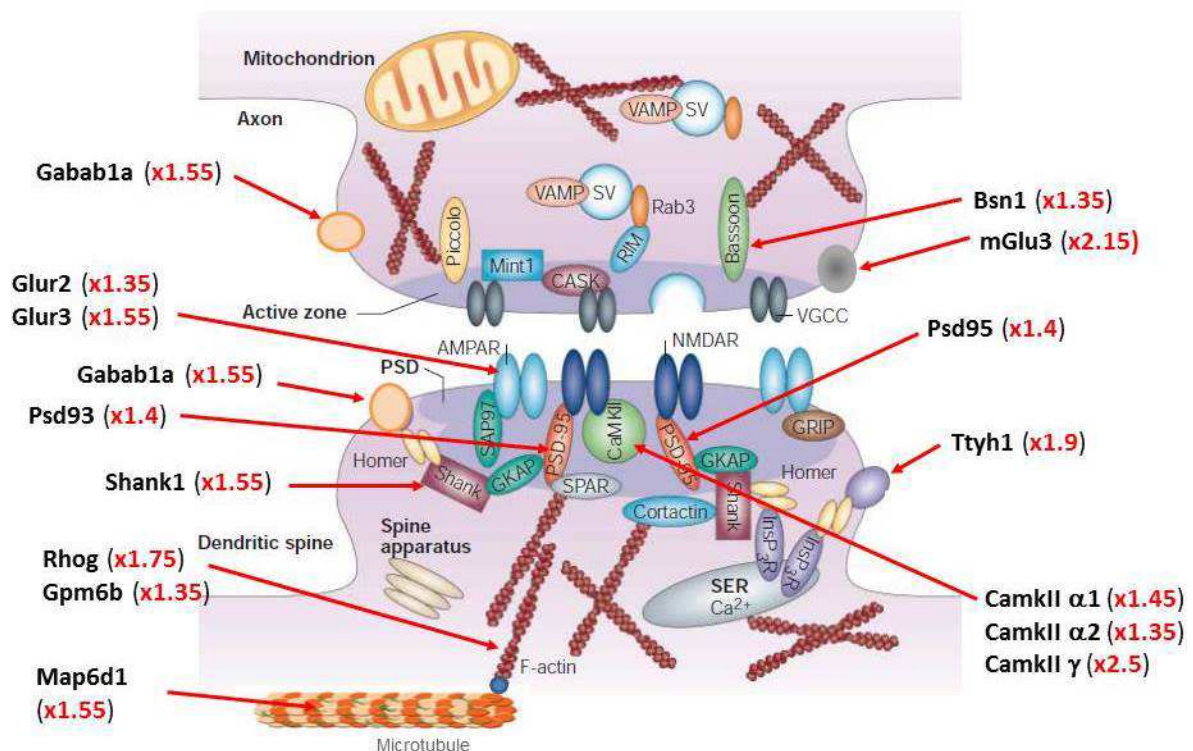


Fig. 1. Synaptic proteins over-represented in cingulate cortex (CC) synaptosomes obtained from high anxiety-related behavior (HAB) animals. The figures in red represent the fold over-representation levels as compared to those observed in LAB animals (modified from [159]). For details, see text.

In addition to the role of high Ca^{2+} permeability AMPARs in Hebbian and homeostatic synaptic plasticity, substantial evidence also points to an involvement of these

receptors in pathological synaptic plasticity. The Glur2 subunit shows low permeability to Ca^{2+} and Glur2-lacking AMPARs can either be switched off by a fear-inducing

stimulus [13], or rapidly expressed during cocaine administration [14] and fear conditioning [15], and are required for drug craving behavior following prolonged cocaine withdrawal [16]. The over-representation dominance of Glur3 (x1.55) over Glur2 (x1.35) suggests possible synaptic transmission scaling [17, 18].

Amongst the over-represented post-synaptic components are Map6d1 (x1.55), a calmodulin-regulated neuronal protein that binds and stabilizes microtubules [19, 20], Ttyh1 (x1.9), a Ca^{2+} -independent, volume-sensitive large conductance Cl^- channel [21] and RhoG (x1.75), which facilitates the translocation of the Rac guanine nucleotide exchange factor (RAC-GEF) complex from the cytoplasm to the plasma membrane [22]. The mechanisms modulated by these proteins result in the induction of long branched filopodia [23] and the formation of new, recurrent excitatory circuits that contribute to increased network excitability [24]. Of particular note here is the over-representation of the CamKII $\alpha 2$ (x1.35), $\alpha 1$ (x1.45) and γ (x2.5) isoforms, central to the regulation of glutamatergic synapses and of LTP/ long-term depression (LTD)-like changes in vivo.

By enhancing NMDA channel function and the subsequent activation of AMPA receptors through relief of the Mg^{2+} block [25, 26], CamKII is necessary for LTP induction, is persistently activated by stimuli that elicit LTP, and can, by itself, enhance the efficacy of synaptic transmission [27].

The auto-phosphorylation of CamKII γ at Ser26, a residue located within the ATP binding site, terminates the sustained activity of the enzyme, inhibiting its auto-phosphorylation at Thr287 by blocking ATP binding [28]. Therefore, the over-representation of this isoform could constitute an important negative feedback mechanism for switching off CamKII activity and regulating LTP in glutamatergic synapses [29, 30].

Few pre-synaptic components are over-represented in CC synaptosomes from HAB animals. Amongst these is the highly over-represented Grm3/mGlu3 (x2.15), a member of the group II, Gi/o-coupled metabotropic glutamate receptors involved in pre-synaptic inhibition [31], the pharmacological activation of which corrects a schizophrenia-like phenotype induced by prenatal stress in mice [32]. Within neuronal networks, activation of Grm3 (reduction of glutamatergic neurotransmission) results in increased spontaneous synaptic activity in non-glutamatergic network members [31].

The over-representation of Cend1 (x1.4), Srcin1/P140Cap (x1.35) and Bsn1 (x1.35) further suggests a deregulated pre-synaptic over-activation of excitatory synapses. Cend1 is a neuron-specific protein that acts as an up-stream effector underlying altered synapse morphology (smaller active zones together with smaller pools of clustered vesicles) in excitatory synapses [33]. Cend1 up-regulation strongly enhances responses to high-frequency stimulation and reduces short-term depression [34]. Srcin1 is an interacting partner of

synaptophysin promoting calcium-dependent neurotransmitter release [35] while the scaffolding protein Bsn1 is involved in organizing the pre-synaptic cytoskeleton, facilitating neurotransmitter release from a subset of brain glutamatergic synapses [36].

Taken together, these patterns of synaptic component over-representation point towards a situation dominated by high-frequency stimulation of excitatory glutamatergic synapses coupled with increased spontaneous synaptic activity in non-glutamatergic network members.

In addition, there is a striking lack of evidence for increased synaptogenesis or de novo dendritic spines generation, indicating impaired learning capacities of HAB mice.

3.1.2. Non-Glutamatergic Synaptic Components

Gabbr1a, the 1A isoform of GABA type B receptor subunit 1 expressed in glutamatergic terminals [37], which is reported to be the main contributor for trans-synaptic inhibition of glutamate release via pre-synaptic GABA receptors [38], is over-represented in HAB CC synaptosomes (x1.55). The functional receptor is a dimer composed of GABAB1a and GABAB2 coupled to Gi/o subunits. GABA-mediated activation of the receptor inhibits N type (Cav2.2) or P/Q type (Cav2.1) Ca^{2+} channels, resulting in the inhibition of pre-synaptic glutamate release [39]. However, the GABAB1a heteroreceptor is also located on post-synaptic glutamatergic neurons where, also via the Gi/o subunits, its activation triggers the opening of inward rectifying K^+ channels, resulting in hyperpolarization and slow inhibitory post-synaptic potentials [40, 41]. Thus, GABAB1a receptors on dendritic spines counteract the excitatory synaptic currents elicited by glutamate, promoting voltage-sensitive block of NMDA receptor, inhibiting the activity of PKA, necessary for phosphorylation and up-regulation of NMDA receptors, and that of voltage-gated L-type (Cav1) Ca^{2+} channels.

Strikingly, Gng3, the G-protein $\gamma 3$ subunit of a Gi/o protein complex that is required for GABA type B receptor-regulated neuronal excitability [42], is over-represented (x1.45) in HAB CC synaptosomes.

The extent of GABAB receptor signaling upon GABA release may be further determined by activity of the neuron-specific K^+-Cl^- co-transporter Slc12a5/KCC2, which mediates the electrophysiological effects of GABA by driving the post-synaptic switch of GABA from excitation to inhibition [43], as well as by GABA diffusivity and uptake via GABA transporters. Both Slc12a5, the K^+-Cl^- co-transporter which maintains chloride homeostasis in neurons, and Slc6a1/GAT1, the sodium and chloride-dependent GABA transporter that terminates the action of GABA through high affinity sodium-dependent reuptake into pre-synaptic terminals [43, 44], are over-represented (x1.55 and x1.65, respectively). However, while HAB CC synaptosomes provide evidence of increased glutamate catabolism to 2-oxoglutarate (Gdh over-represented, see below), there is no evidence of

increased GABA synthesis from glutamate (Gad2 is not over-represented), GABA vesicle loading activity (Slc32a1 is not over-represented) or increased GABA catabolism (Gabt is not over-represented).

The overall effects of the above mechanisms appear to lead to considerable structural and functional reinforcement of existing glutamatergic post-synaptic densities together with enhanced control over the modulation of synaptic strength and relatively weakened GABAergic control. Importantly, GABAB1a, Slc6a1/GAT1 and Slc12a5 are the only non-glutamatergic synaptic components present amongst the over-represented synaptosomal proteins in HAB CC.

3.1.3. Stabilization of Existing Excitatory Dendritic Spines and LTP Maintenance

The structural plasticity of excitatory dendritic spines is regulated through integrin-mediated attachment to extracellular matrix (ECM). Cleavage of these ECM components induces integrin receptors signalling, provoking actin cytoskeleton modifications with subsequent dendritic spine enlargement and LTP maintenance [45, 46].

It is striking to note that the main mechanisms attached to excitatory dendritic spine stabilization through integrin-mediated ECM attachment are present in the over-represented synaptosomal proteins. Ttyh1, an integrin-associated membrane protein mainly found in invaginations of dendritic spines [47, 23] is over-represented in HAB CC synaptosomes (x1.9). Ttyh1 expression silencing results in the swelling of synaptic spines, while Ttyh1 over-expression causes intense neurogenesis and the formation of numerous filopodia-like protrusions [47].

Ociad1 and Ociad2, also over-represented in HAB synaptosomes (x1.9 and x1.75, respectively), induce increased cell adhesion through integrin clustering [48, 49]. Increased expression of Ociad1 is mediated, in a time and dose-dependent manner, by lysophosphatidic acid (LPA) [48], the synthesis of which is carried out by acylglycerol kinase (Agk1), one of the most highly over-represented HAB CC synaptosomal proteins (x2.65). LPA signals through at least six different receptors (LPAR1-6) that can couple to three different heterotrimeric G proteins, Gi, Gq and G12/13, which, in turn, lead to Rho/Rho-kinase activation [50, 51]. RhoG, over-represented in HAB synaptosomes (x1.75), is a significant contributor to the stimulation of actin polymerization, which increases spine size and shape [52, 53]. Large spines are more stable than smaller ones and may be resistant to modification by additional synaptic activity [54].

LPAR1 signaling leads to activation of RalA [55] also over-represented in HAB synaptosomes (x1.45). RalA acts as a GTP sensor and potentiates the exocyst-mediated, GTP and activity-dependent growth of post-synaptic membranes [56], a key control point for post-synaptic plasticity, while acting as an inhibitor of G-protein coupled receptors desensitisation [57]. Non-degradative mono-ubiquitination increases RalA activity [58], while acetylation of ubiquitin

acceptor sites inhibits non-degradative ubiquitination [59]. Notably, the cytoplasmic deacetylase Sirt2, one of the most over-represented proteins in HAB synaptosomes (x2.3), deacetylates α -tubulin [60], thus decreasing the levels of stable and long-lived microtubules and negatively modulating neurite extension and energy/molecule transport into protruding nerve fibres while possibly maintaining RalA activity by favouring its non-degradative mono-ubiquitination. Elevated Sirt2 activity increases neuronal electrical excitability and potentiates the rewarding effects of cocaine [61].

Thus, the overall effects of the above mechanisms may lead to sustained stabilization of existing glutamatergic dendritic spines together with substantial LTP maintenance. The over-represented mechanisms also suggest the possibility that post-synaptic spines could now act as “signal amplifiers” where weak pre-synaptic inputs would result in much stronger (amplified) post-synaptic signals, thereby attenuating signal versus noise discrimination.

3.1.4. Potentiation of Glutamate Clearance, Maintenance of Synaptic Ca^{2+} Homeostasis and Increased Excitation Threshold

Chronic low-level exposure to extracellular glutamate has a disproportionately toxic effect on neurons. Most glutamate released during neurotransmission is primarily transported into astrocytes [62]. However, <10% of glutamate arising from presynaptic depolarization is taken up by neurons [63].

Although glutamatergic neurons express both EAAT4 (Slc1a6) and EAAT3 (Slc1a1), the neuronal EAAT4/Slc1a6 transporter is responsible for maintaining low extracellular glutamate levels in between neuronal firing events [64] and has a 20-fold greater affinity for glutamate than EAAT1/Slc1a3, EAAT2/Slc1a2 or EAAT3/Slc1a1 [64, 65].

In the HAB CC synaptosomes, Slc1a2/EAAT2 and Slc1a1/EAAT3 are over-represented (x1.9 and x2.0, respectively), suggesting increased spill over, as opposed to burst-associated, glutamate clearance.

Activation of AMPARs, and subsequently of NMDARs, gives rise to significant Ca^{2+} influx into post-synaptic structures. Rapid Ca^{2+} efflux from neurons occurs through two main systems: electrochemically driven $\text{Na}^+/\text{Ca}^{2+}$ exchangers (VGCCs) with a low Ca^{2+} affinity, and calmodulin-activated, plasmalemmal-specific Ca^{2+} -ATPase (PMCA) extrusion pumps with a high Ca^{2+} affinity [66, 67]. Three PMCA isoforms, Atp2b3/PMCA3 (x1.35), Atp2b4a/PMCA4a (x1.35) and Atp2b2/PMCA2 (x1.55) are over represented in the HAB cingulate synaptosome.

PMCA interacts with synaptic signaling complexes in glutamatergic post-synaptic structures and play a key role in Ca^{2+} transients amplitude down-modulation following AMPARs activation (recovery of depolarization-mediated Ca^{2+} elevation) [68]. Atp2b2, which has high basal activity, fast Ca^{2+} activation kinetics and high V_{max} , associates with rapid clearance of Ca^{2+} in fast-spiking synapses that face

severe transient calcium loads [69].

Following depolarization, the Na^+/K^+ -ATPase pumps, which restore the sodium gradient, also maintain the ionic gradient responsible for neuronal resting potential, thereby modulating depolarization thresholds [70]. The ATP1 α 2 (catalytic subunit), β 3 and β 2 (regulatory subunits) isoforms of the dimeric Na^+/K^+ -ATPase pump are all over-represented in HAB CC synaptosomes (x1.4, x1.75 and x1.35, respectively).

Mice heterozygous for α 2 isoform deficiency display increased anxiety-related behaviour and impaired spatial learning [71]. Increased presence of β 3 subunits results in increased number of functional Na^+/K^+ pumps on synaptic structures and thus in more negative resting membrane potentials [70].

Cumulatively, these mechanisms are suggestive of rapid over-repolarization of glutamatergic synapses.

3.1.5. LTP Modulation through Depolarization-Induced Alkalinization and Perisynaptic ATP Hydrolysis

Following AMPAR activation, elevation of external K^+ elicits a Ca^{2+} -dependent acid transient followed by a large net alkaline shift. This depolarization-induced alkalinization mechanism, which also improves neuronal excitability [72], is governed by Cl^- -dependent and Cl^- -independent HCO_3^- transporters [73], and in particular by Slc4a8/Ndcbe (x1.75) together with Slc4a7/Nbcn1 (x1.9) or Slc4a10/Nbcn2 (x2.0) and the electrogenic Slc4a4/Nbce1 (x1.35) transporters. The HCO_3^- necessary to maintain this mechanism is generated by the carbonic anhydrase 2 (CA2; x1.9) shuttle involved in the regulation of Na^+/K^+ -ATPase and PMCA pumps activities. A second neuro-protective mechanism is mediated by the extracellular hydrolysis of ATP released together with glutamate by pre-synaptic glutamatergic buttons. Excitatory dendritic spines express a variety of P2X purinoceptors. Upon adenosine binding, these receptors function as ligand-gated ion channels mediating synaptic transmission [74, 75]. Entpd2, the enzyme necessary for the hydrolysis of perisynaptic ATP into adenosine, is highly over-represented (x2.0) in HAB CC synaptosomes.

3.2. Mitochondria

3.2.1. Significantly Increased Electron Transport Chain and Oxidative Phosphorylation Protein Expression

In neurons, most of the ATP requirements are provided by the concurrent intra-mitochondrial activities of the tricarboxylic acids (TCA) cycle and the respiratory chain (OXPHOS) system.

Respiratory chain complex I is a very intricate structure composed of seven sub-complexes. Out of the 48 proteins that constitute complex I [76, 77], 33 are highly over-represented in CC synaptosomes of HAB mice: 11 Ndufa subunits, with over-representation levels oscillating between x2.0 and x2.3, except for Ndufa7 and Ndufa10 (x2.85 and x2.65, respectively); nine Ndufb subunits (x2.1 to x2.5); one Ndufc subunit out of 2 (Ndufc2 = x2.2); eight

Ndufs subunits (x2.1 to x2.5), including Ndufs3 (x2.15) which is regarded as a potential hypoxia marker [78], two Ndufv subunits out of 3 (x1.9 to x2.5) and two mitochondrially encoded subunits (MT-ND4 = x2.2 and MT-ND5 = x2.5).

Three out of four complex II subunits [79, 80] are over-represented in HAB CC synaptosomes: Sdha (x1.75), the flavoprotein subunit of succinate dehydrogenase responsible for transferring electrons from succinate to ubiquinone (coenzyme Q), converting FAD to FADH_2 in the process; Sdhb (x2.0), the iron-sulfur protein (three Fe-S clusters) subunit of succinate dehydrogenase which accepts electrons from FADH_2 that are then transferred to the Fe-S clusters, and Sdhc (x1.9), one of the two membrane-anchoring subunits which transfers electrons from the iron clusters of Sdhb to the ubiquinone pool, generating QH_2 used by complex III as a co-enzyme in proton translocation.

Complex III (cytochrome b-c1 reductase/ Coenzyme Q/ cytochrome C reductase) has a dimeric structure with each monomer containing 11 subunits [81]. Nine complex III subunits are over-represented in HAB CC synaptosomes. These include Uqcrrf1 (x2.15), the cytochromes b (x1.75) and C1 (x2.15), the two core proteins Uqcrc1 and Uqcrc2 (x2.15 each), and the three components required for ubiquinone binding and redox-linked proton pumping (Uqcrb, x2.0), contact establishment between ubiquinone and cytochrome b (Uqcrq, x1.75), and cytochrome c1 (Uqcr10, x1.75) stabilization. Cytochrome c (Cycs), the primary electron acceptor used as co-enzyme by complex IV, is also over-represented (x1.75).

Complex IV (cytochrome c oxidase), the terminal oxidase of the ETC, is composed of 13 subunits [82] nine of which are over-represented in HAB CC synaptosomes, ranging from x1.7 to x2.3. These include mt-Co2 (x1.75), a mitochondrially-encoded subunit which transfers electrons from cytochrome c via its binuclear copper A center to the bimetallic centre of catalytic subunit 1; Cox7a2l (x2.0), a nuclear-encoded subunit which may be a regulator mediating higher level of energy production; Cox5a (x1.9), the nuclear-encoded haem A-containing chain of cytochrome c oxidase, and Cox6b1 (x1.9), a nuclear-encoded subunit which connects the mitochondrially encoded mt-Cox1 and mt-Cox 2 monomers into their physiological dimeric form [83].

Complex V (F0F1 ATP synthase) utilizes the proton gradient, generated across the inner mitochondrial membrane by the activities of ETC complexes I, III and IV, to generate ATP. All five major components of the F1 complex [84, 85], including the α (Atp5a1, x2.15), β (Atp5b, x2.15) and OSCP (Atp5o, x2.3) catalytic core and the γ stem (Atp5c1, x2.5) subunits, as well as components critical in linking F0 and F1 complexes (Atp5f1, x2.3; Atp5h x2.15; Atp5j, x1.75) and maintaining the ATP synthase population in mitochondria (Usmg5, x1.75), are over-represented.

In view of the consistent increase in OXPHOS observed

[86], with Mcp2 being over-represented in HAB CC synaptosomes (x2.5). The influx of inorganic phosphate from the cytoplasm into the mitochondrial matrix is mediated by the mitochondrial transporter Slc25a3 which co-transport phosphate with H^+ [87] and is over-represented in HAB CC synaptosomes (x1.9). The export of intra-mitochondrial ATP in exchange for cytoplasmic ADP is carried out by the transporters Slc25a4 and Slc25a5, both highly over-represented in HAB CC synaptosomes (x2.15 and x2.3, respectively).

Hence, the pattern of over-representation observed above may be indicative of a mitochondrial situation dominated by enhanced substrate delivery and ATP export together with significantly strengthened compartmented pH control in a context of large and fluctuating energy demands (Fig.2).

Pyruvate, the end product of glycolysis and a major substrate for the TCA cycle, occupies a central node in mitochondrial functions. It is produced in the cytoplasm before being transported into the mitochondria for further metabolism in the TCA cycle. Pyruvate import is mediated by the mitochondrial pyruvate carriers Mpc1 and Mpc2

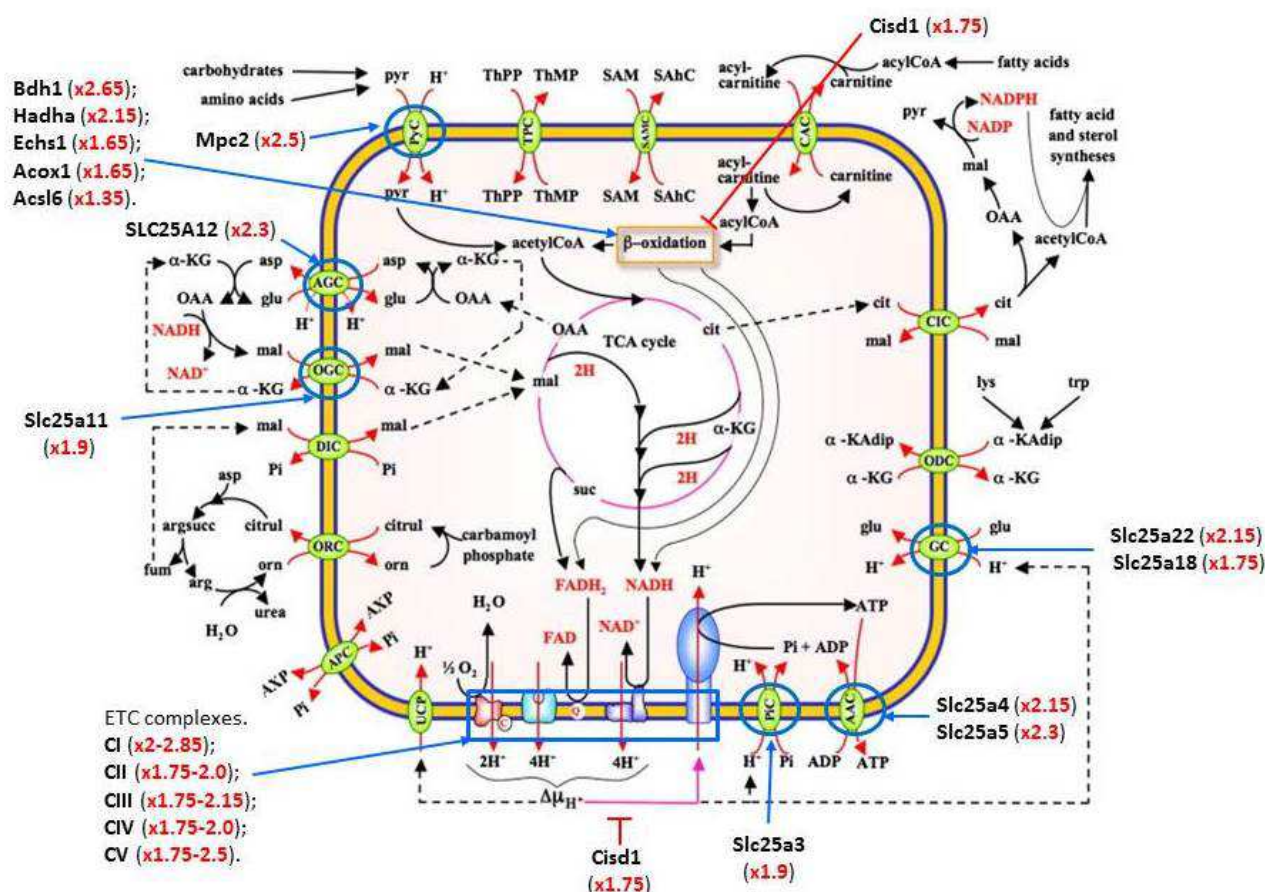


Fig. 2. Mitochondrial metabolic functions significantly amplified in HAB CC synaptosomes. The diagram shows 15 carriers catalyzing metabolite transport through the inner mitochondrial membrane. These carriers are involved in OXPHOS (AAC/Slc25a4-25a5; PiC/Slc25a3, UCP); oxidation/reduction pathways (AGC/Slc25a12; OGC/Slc25a11, DIC; CIC; CAC; PyC/Mpc2); homeostasis of the intra-mitochondrial adenine nucleotide pool (APC); methylation of mtDNA, mtRNA and some intra-mitochondrial proteins (SAMC); import of the essential coenzyme thiamine pyrophosphate (ThPP) required for pyruvate and oxoglutarate-dehydrogenase complex activities; and, with partial overlap, amino acid metabolism (AGC/Slc25a12; ORC; GC/Slc25a22-25a18; ODC). Although not showing all the implicated metabolic pathways in which the identified carriers are involved, the diagram indicates the metabolic functions that are presumably amplified through over-represented enzymes (β -oxidation) and ETC complexes. The over-representation levels (red figures in parentheses) are indicated for each component (modified from [160]).

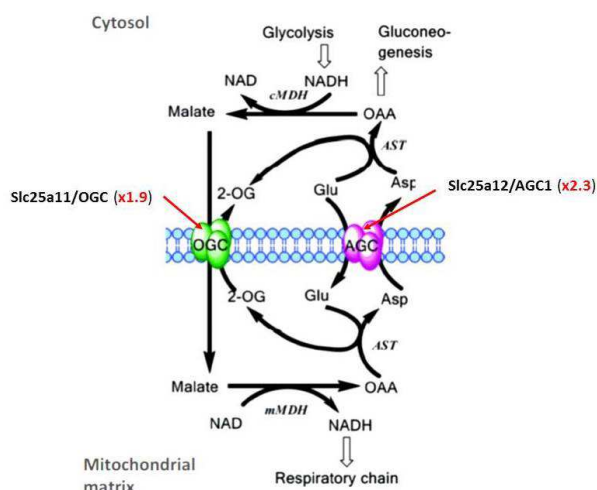


Fig. 3. The malate-aspartate shuttle. AGC1 (Slc25a12, x2.3), the only aspartate/glutamate carrier isoform expressed in synapses, exchanges intra-mitochondrial aspartate (Asp; efflux) for extra-mitochondrial glutamate (Glu; influx). In the cytosol, aspartate aminotransferase (AST) converts Asp to oxaloacetic acid (OAA) in a reaction coupled to the conversion of Glu into oxoglutarate (2-OG). The produced OAA is then converted by cytosolic MDH (cMDH) in a NADH-dependent reaction that generates malate and NAD. In this reaction, NADH in the cytosol (inter-membrane space) passes two reducing equivalents to oxaloacetate, producing malate. The generated cytosolic malate generated is then transported into the mitochondrial matrix by OGC (Slc25a11, x1.9) in exchange for intra-mitochondrial 2-OG which arises from mitochondrial MDH (mMDH)-mediated conversion of incoming malate into OAA, passing two reducing equivalents to NAD⁺ and generating NADH in the process, followed by AST-mediated dual conversion of OAA into Asp and intra-mitochondrial Glu into 2-OG. In this process imported cytoplasmic malate passes two reducing equivalents to NAD⁺ and the resulting mitochondrial NADH can then pass electrons directly to the ETC complex I in the inner membrane. The AST-mediated dual conversions reduce the availability of cytosolic Glu while increasing the cytosolic levels of 2-OG and the other side, increase the intra-mitochondrial levels of both Asp and 2-OG, act as feedback loops regulating the activity balance between AGC and OGC which govern the shuttle's dynamics. Here, the electrogenic AGC1 transporter provides directionality so that reducing equivalents are transferred into the mitochondrial matrix (modified from [161]).

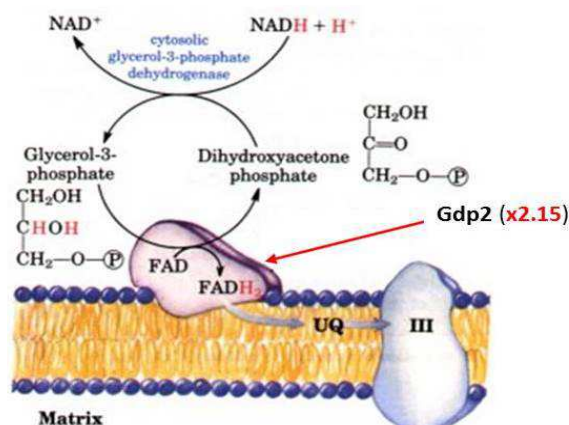


Fig. 4. The glycerol-3-phosphate (G3P) shuttle. Dihydroxyacetone phosphate (generated from fructose metabolism; [162]) in the cytosol accepts two reducing equivalents from cytosolic NADH in a reaction catalyzed by cytosolic glycerol-3-phosphate dehydrogenase (Gpd1, not over-represented). A membrane-bound mitochondrial GPD isozyme (Gpd2,

x2.15), located on the outer face of the inner membrane, transfers two reducing equivalents from glycerol-3-phosphate in the intermembrane space to ubiquinone, regenerating dihydroxyacetone phosphate in the process. Note that this shuttle does not involve membrane transport systems (modified from [163]).

3.2.3. Mechanisms Maintaining Redox Balance and Energy Production Homeostasis

Mitochondria modulate the cytosolic redox status by oxidizing NADH and FADH₂ and generating reactive ROS while participating to pH control and phosphate trafficking.

However, under conditions of high energy demands, the NADH generated by glycolysis and lactate dehydrogenation in the cytosol must be transferred to the mitochondrial matrix, while cytosolic NADH is utilized to maintain adequate intra-mitochondrial FADH₂ levels. These homeostatic tasks are carried out by the malate-aspartate shuttle (Fig.3) which transfers reducing equivalents from cytosolic NADH to NAD⁺ inside mitochondria and requires aspartate/glutamate Slc25a12/AGC1 carrier and oxoglutarate carrier Slc25a11/OGC activities [88], and the glycerol-3-phosphate (G3P) shuttle (Fig.4), which transfers reducing equivalents from cytosolic NADH to FAD inside mitochondria by converting G3P to dihydroxyacetone phosphate and requires Gpd2 activity [89, 90].

In neurons, transported glutamate is stoichiometrically converted into aspartate [91]. Neuronal mitochondria are provided with the Agc1/Slc25a12 transporter, which performs the calcium-dependent exchange of cytoplasmic glutamate with mitochondrial aspartate across the mitochondrial inner membrane [92], and with the 2-oxoglutarate OGC/Slc25a11 transporter, which imports cytosolic malate into and exports 2-oxoglutarate out of the mitochondria. Agc1/Slc25a12 activity is boosted by cytosolic calcium and elevated AGC activity together with altered Ca²⁺ homeostasis play a key interactive role in the cascade of signaling events leading to autism [93].

Both the Slc25a12 and Slc25a11 transporters are highly over-represented in HAB CC synaptosomes (x2.3 and x1.9, respectively) and are at the core of the mechanism that powers the malate-aspartate shuttle which transfers NADH reducing equivalents to the mitochondrial matrix while metabolizing glutamate.

However, increased activity of the malate-aspartate shuttle, which diverts substrates from the TCA cycle, implies a mechanism to maintain the supply of 2-oxoglutarate and subsequent activity of the TCA cycle, necessary for ETC functions and ATP production. When extra-cellular glutamate levels are high (0.5-1.0 mM), glutamate dehydrogenase can shift glutamate metabolism toward the ATP-generating oxidative deamination of glutamate into 2-oxoglutarate and its subsequent entry into the TCA cycle [94]. Gdh1, the enzyme that deaminates glutamate into 2-oxoglutarate, generating NH₄⁺ in the process, is over-represented (x1.75) in HAB synaptosomes.

However, this mechanism requires a mitochondrial glutamate transporter independent of the malate-aspartate

shuttle. Slc25a22, highly over-represented in HAB synaptosomes (x2.15), which co-transport glutamate with H^+ across the inner mitochondrial membrane, fulfils this requirement.

Thus, since Gpd2, Gdh1, Slc25a12/Agc1, Slc25a11/OGC and Slc25a22 are over-represented in HAB CC synaptosomes (x2.15, x1.74, x2.3 and x1.9 and x2.15, respectively), both the G3P and malate-aspartate shuttles are utilized by HAB CC neurons, suggesting large enhancement of intra-mitochondrial substrate oxidation and ATP production rates [95].

3.2.4. Mechanisms Coupling Intracellular ATP

Utilization and Mitochondrial ATP Regeneration

The creatine kinase isoforms are coupled with both ATP producing (mitochondrial and glycolytic) and ATP consuming (ions pumping) processes. At least 80% of the total energy produced in mitochondria is exported by phosphocreatine molecules and mitochondrial creatine kinases (MtCKs) functionally couple phosphocreatine production to OXPHOS [96].

ATP is hydrolyzed by cellular ATPases pumps into extra-mitochondrial ADP and inorganic phosphate (Pi). In the presence of creatine and ATP, MtCKs, functionally coupled to ATP synthase via adenine nucleotide translocases (ANTs), and non-mitochondrial creatine kinases (MMCK/MBCK), coupled to the glycolytic enzymes phosphoglycerate kinase and pyruvate kinase, produce intra- and extra-mitochondrial ADP, respectively. The phosphoenolpyruvate and pyruvate kinase system continuously regenerate extra-mitochondrial ATP consumed by intracellular ATPase-mediated reactions. Intra-mitochondrial ADP produced by MtCKs forms micro-compartments within the mitochondrial intermembrane space and is re-imported into the matrix via ANTs functionally coupled with MtCKs. This rapidly activates respiration up to maximal rate [97, 96]. MtCKs are located in the inter-membrane space as well as in the cristae space where they form part of proteolipid complexes. At inter-membrane space sites, these complexes contain adenine translocators (Slc25a4/ANT1; Slc25a5/ANT2; Slc25a6/ANT3 and Slc25a31/ANT4) while in the inner membrane MtCKs co-localize with voltage-dependent anion channel (Vdac1 to 3) [98, 99].

ATP delivery to ATPase pumps is facilitated through MtCKs and the glycolytic system, which includes hexokinase. Pi transfer is catalysed by the near-equilibrium Slc25a3-Gadph shuttle and H^+ removal is facilitated by the CA2 shuttle which catalyses the reversible hydration of CO_2 to bicarbonate (HCO_3^-) [100, 101]. In this system, glutamate transporter (Slc1a1 activity) supplies the intracellular Na^+ necessary for Na^+/K^+ -ATPase activity [102] and subsequent stimulation of glycolysis [103] while mitochondrial acylglycerol kinase (Agk) activity promotes the insertion of ANTs in the inner mitochondrial membrane [104].

Both MtCK1 and MtCK2 are over-represented in HAB

CC synaptosomes (x1.75 and x1.9, respectively), together with the ANTs Slc25a4 (x2.15) and Slc25a5 (x2.3), the three VDAC isoforms (x1.65 each) and the mechanisms necessary to maintain active equilibrium in the above system [105], such as the glutamate transporter Slc1a1 (x2.0), Agk1 (x2.65), the hexokinase 1a isoform (HK1a, x1.65), the Pi transporter Slc25a3 (x1.9) and CA2 (x1.9).

3.2.5. Evidence for Significant Mitochondrial Stress

Under conditions of high metabolic load, mitochondrial stress, in the form of reduced redox potential (increased ROS production) may develop, the control of which requires the implementation of coordinated mechanisms [106]. Most major components of the necroptosis mechanism are over-represented in HAB CC synaptosomes (Cst3: x1.55; Glud1: x1.75; Pgam5: x2.65; Mtp18/MTFP: x1.75 and Opa1: x1.55).

Furthermore, there are also indications of mitochondrial apoptotic program induction. The mitochondrial carrier Mth2 (Slc25a50), over-represented in HAB CC synaptosomes (x1.9), induces mitochondrial depolarization and is involved in the mitochondrial apoptotic program. Following apoptotic stimulation, Mth2 mediates the recruitment of activated BH3 interacting domain death agonist (tBID) which, in turn, leads to oligomerization of BAX/BAK and cytochrome c release [107]. However, in HAB CC synaptosomes, these deleterious mechanisms, while clearly activated, appear to be held in check by the active implementation of the protective mechanisms described below.

3.2.6. Mechanisms Protecting Against Mitochondrial Oxidative Stress

Various forms of stress (restrain, sleep deprivation, anxiety, etc.) result in significantly decreased activity of mitochondrial antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase) in the brain [108-110]. Furthermore, oxidative stress-induced cognitive deficit apparently results from the failure to maintain dendritic spines [111]. Despite the fact that OXPHOS protein subunit levels are consistently increased in HAB CC synapses, none of these antioxidant enzymes is over-represented in HAB CC synaptosomes. However, a metabolic means whereby high ATP production could be significantly increased while attenuating ROS generation could be achieved through fatty acids α -oxidation and ketone body production [112].

In HAB CC synaptosomes, practically all enzymes necessary for mitochondrial fatty acids α -oxidation and ketone body production, namely Acs16 (long-chain fatty-acid-CoA ligase 6; x1.35), Acox1 (acyl-coenzyme A oxidase 1; x1.65), Echs1 (enoyl-CoA hydratase; x1.65), Hadha (x2.15), which catalyses the last three steps of mitochondrial β -oxidation of long chain fatty acids [112], and Bdh1 (D- β -hydroxybutyrate dehydrogenase; x2.65) which catalyses the inter-conversion of acetoacetate and β -hydroxybutyrate, the two major ketone bodies produced during fatty acid catabolism [113], are over-represented

(Fig.2). However, an alternative mechanism for fatty acyl-CoAs transfer to mitochondria, independent of Slc25a29 (mitochondrial carnitine/acylcarnitine carrier) and involving the intracellular lipid transfer protein Scp2 [114], which is over-represented in HAB CC synaptosomes (x1.75), is utilized.

Numerous mitochondrial proteins directly involved in OXPHOS are critically dependent upon functional iron-containing prosthetic groups (Fe-S clusters and haem groups) [115] and mitochondrially-produced superoxide releases iron from these protein complexes, leading to their functional failure [116].

Cisd1/MitoNEET, a negative regulator of the β -oxidation pathway which inhibits mitochondrial iron transport into the matrix, thus limiting maximal capacity for electron transport and lowering the rate of β -oxidation [117], is also over-represented in HAB synaptosomes (x1.75). Increased Cisd1 levels result in lower mitochondrial membrane potential and lower levels of ROS production [118] together with increased potential for the transfer of [2Fe-2S] clusters to cytosolic or mitochondrial acceptors [119]. Concurrently, three sideroflexins, namely Sfxn1 (x1.75), Sfxn3 isoform 1 (x1.75) and most particularly Sfxn5 (x2.15), which facilitate iron utilization into mitochondria [120, 121] are over-represented in HAB CC synaptosomes.

The over-representation of Cisd1 together with Sfxn1, 3 and 5 suggest a mechanism protecting mitochondria from superoxide-mediated functional loss of iron sulfur domain proteins and toxic accumulation of iron [122].

Free nitrogen radicals may be generated by the activity of the outer mitochondrial membrane monoamine oxidases (Maoa and Maob), involved in the metabolism of amines such as serotonin, norepinephrine and dopamine. Maob is over-represented (x1.9) in HAB CC synaptosomes. Free nitrogen radicals can induce nitration of mitochondrial proteins at tyrosine residues, with consequent loss of function [123]. The mitochondrial amidoxime reducing component mARC (Mosc1 and Mosc2) functions together with the ETC protein NADH-cytochrome b5b reductase (Cyb5b) to catalyse the reduction of N-hydroxylated molecules [124]. Both Mosc2 and Cyb5b are over-represented in HAB CC synaptosomes (x1.75 and x1.4, respectively), suggesting a mechanism that protects HAB CC neurons from the accumulation of deleterious Lewy bodies [125] while contributing to the activation of a cytochrome b5-dependent mechanism that regenerates both cytosolic NAD^+ and mitochondrial FAD^+ .

Hence, in HAB CC synapses, it appears that most energy requirements are met through fatty acids β -oxidation, while pyruvate metabolism complemented by CA2-mediated increased lactate influx seems to be mainly utilized for the maintenance of NADH/FADH_2 homeostasis.

These metabolic adaptations, which attenuate ROS generation, together with the mechanisms controlling the effects of ROS and nitrogenous free radical species detailed

above, could be expected to significantly limit mitochondrial oxidative stress under conditions of high energy demands.

3.2.7. Mechanisms Protecting Against Mitochondrial Ionic Stress

In synaptic mitochondria, Ca^{2+} -loading induces permeability transition pore (PTP) opening, sudden Ca^{2+} efflux from the mitochondrial matrix and membrane potential collapse, resulting in mitochondrial demise [126]. Synaptic Ca^{2+} influx into mitochondria is controlled through Ca^{2+} -dependent transient K^+ influx, mediated by mitochondrial ATP-sensitive K^+ channels (K_{ATP}), into the matrix, causing activation of the K^+/H^+ exchanger and re-establishment of membrane potential [127, 128].

The high-affinity mitochondrial Ca^{2+} importer Letm1, which is highly over-represented (x2.15) in HAB CC synaptosomes, achieves mitochondrial Ca^{2+} sequestration at small Ca^{2+} increases. Knock-down of Letm1 strongly diminishes the transfer of entering Ca^{2+} into mitochondria [129]. Concurrently, connexin 43 (Cx43), over-represented in HAB CC synaptosomes (x1.55), stimulates mitochondrial K_{ATP} channel opening [130] while counteracting Ca^{2+} -induced PTP opening [126]. Genetic ablation of Cx43 reduces both ADP-stimulated complex I respiration and K_{ATP} -mediated mitochondrial potassium influx [131]. Hence, Letm1 and Cx43 over-representation may potentiate a protective mechanism against irreversible, Ca^{2+} influx-dependent mitochondrial membrane potential collapse.

The mitochondrial voltage-dependent anion channels (Vdac1, 2 and 3) facilitate metabolite flux by enabling transport of ATP, ADP, NADH, phosphocreatine, Ca^{2+} and other small ions across the outer mitochondrial membrane [132], anchoring hexokinases during glycolysis [133] and aiding release of cytochrome c from the inter-membrane space [134, 135]. VDACs protect against, rather than contribute to, PTP opening, mitochondrial membrane permeabilization and neuronal cell death [136-138]. Consistent with this, pharmacological closure of VDAC increases the steady-state level of intra-mitochondrial ROS in isolated mitochondria [139]. All three VDAC isoforms are over-represented in HAB CC synaptosomes (x1.65 each). However, none of the proteins tethering mitochondria to the endoplasmic reticulum (Mfn1, Mfn2, Pacs2, Atad3) or linking VDACs to the ER (Grp75, Pml, Acat1), nor NCLX (Slc24a6), which mediates sodium-dependent calcium efflux from mitochondria, are over-represented, suggesting an adaptation of synaptic mitochondria to enhanced Ca^{2+} uptake together with a possible dissociation between VDAC-mediated mitochondrial homeostasis and ER-associated Ca^{2+} flux regulation in HAB CC synapses.

Therefore, at least two synergistic protective mechanisms against different effects of Ca^{2+} loading are potentially implemented in CC synaptic mitochondria of HAB animals, namely: (1) High-affinity mitochondrial

Ca^{2+} importer Letm1-mediated localised sequestration of incoming mitochondrial Ca^{2+} and Cx43-mediated K_{ATP} channels potentiation acting together to down-modulate PTP opening potential, and (2) VDAC-mediated efflux of excess ROS together with regulation of the intra-mitochondrial metabolic environment.

However, this is likely to increase cytoplasmic ROS levels which could be expected to result in selective dopamine release inhibition [140], thereby contributing to neurotransmitter signaling imbalance.

3.2.8. Mechanisms Maintaining Mitochondrial Structural Integrity

At least 19 proteins, closely involved with mitochondrial morphological dynamics are over-represented in HAB CC synaptosomes. Mitochondrial morphology is partly dependent on an equilibrium balance between fusion and fission processes and in part dependent upon Ca^{2+} buffering mechanisms at the synaptic termini.

Mitochondrial fusion is mediated mainly by Mfn1, Mfn2 and Opa1, the latter of which is over-represented (x1.55). Reduction in the activity of these proteins by repression or by the presence of loss-of-function mutations or ablation causes mitochondrial fragmentation or reduction in the extent of mitochondrial filaments [141]. Furthermore, the processing and stabilisation of the dynamin-like GTPase Opa1, which regulates mitochondrial fusion and crista morphogenesis, is regulated by the prohibitins Phb1 and Phb2 [142], both highly over-represented in HAB CC synaptosomes (x2.0 & x2.15, respectively).

Mitochondrial fission is mediated by Drp1, Fis1 and Mtp18 which is also over-represented (x1.75). While reduction in the activity of these proteins by repression or by mutations, or ablation causes elongation of mitochondrial network, their overexpression causes mitochondrial fragmentation [143]. Hence, these data suggest that, in HAB CC, synaptic mitochondria are in a state of equilibrium where fusion is slightly favored over fission.

The mitochondrial inner membrane contains a large protein complex crucial for membrane architecture, the mitochondrial inner membrane organizing system (MINOS). MINOS is required for keeping crista membranes attached to the inner boundary membrane via crista junctions and interacts with protein complexes of the mitochondrial outer membrane [144].

Only a few proteins have been linked with the control of mitochondrial cristae morphology. Mitofilin (Immt) is a core component of MINOS [145] and is highly over-represented in HAB CC synaptosomes (x2.3). Several interaction partners of mammalian Immt (Fig.5) are now known and include the coiled-coil-helix-coiled-coil-helix domain containing proteins 3 and 6 (Chchd3 and Chchd6), Disrupted-in-schizophrenia 1 (Disc1), the sorting and assembly machinery component 50 (Samm50), Metaxin-1 and -2 (Mtx1 and Mtx2), DNAJC11/Hsp40 and ApoO [146-150].

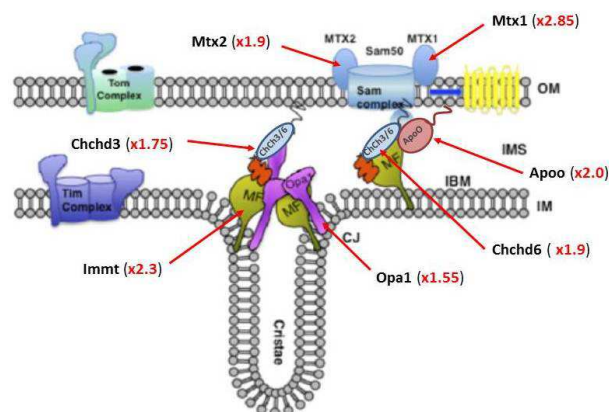


Fig. 5. Chchd3/6 interacts with Mitofilin, Sam50, OPA1 and ApoO to maintain crista morphology and regulate protein import. ChChd3 and 6 are synthesized in the cytoplasm and kept in a reduced and soluble form with the help of HSP70, before being imported and folded in the mitochondria. In mitochondria, ChChd3 and 6 appear to exist at two discrete foci: 1) at crista junctions (CJ) and 2) at contact sites between outer and inner membranes. At the CJs, ChChd3 and 6 form a complex with mitofilin (MF/Immt) and Opa1, and at the contact sites they associate with mitofilin, ApoO and Sam50 thereby influencing the regulation of crista biogenesis and mitochondrial protein import, respectively. Here, the motor protein MF/Immt acts as organizer of the MINOS complex (mitochondrial inner membrane organizing complex) at mitochondrial crista junctions and promotes protein import into the inter-membrane space. In addition, MF/Immt interacts with the protein-conducting channel of the TOM complex and the inner membrane protein Mia40 which mediates the mitochondrial disulfide relay system. It probably thereby brings both outer and inner membranes of mitochondria in close contact to improve the translocation of inter-membrane space proteins. MF: mitofilin/Immt; IBM: inner boundary membrane; IMS: inter-membrane space (modified from [147]).

In HAB CC synaptosomes, Immt, Opa1, Chchd3, Chchd6, ApoO, Mtx1 and Mtx2 are all over-represented (x2.3, x1.55, x1.75, x1.9, x2.0, x2.85 and x1.9, respectively). It is important to note that while Chchd3 acts as a scaffolding protein that stabilizes protein complexes involved in maintaining crista architecture and protein import [147], Chchd6 is required for maintaining not only mitochondrial crista morphology, but also ATP production and oxygen consumption [151], which are intimately dependent upon protein imports and integrations into the inner mitochondrial membrane.

3.2.9. Protein Import into Mitochondria

The sorting and assembly pathway of outer membrane proteins (Fig.6) involves three machineries: the translocase of the outer membrane (TOM) complex, the sorting and assembly machinery (SAM) complex and the mitochondrial distribution and morphology (MDM) complex.

3.2.9.1. The Sorting and Assembly Machinery (SAM)

Mtx1 (x2.85) and Mtx2 (x1.9) are outer membrane proteins components of the outer mitochondrial membrane β -barrel protein transport complex (SAM). Mtx1 is necessary for the formation and import of the translocator protein (18-kDa, Tspol1), an outer mitochondrial membrane protein necessary for cholesterol import and steroid production [152].

Fig. 6. Different targeting signals direct nuclear encoded precursor proteins on specific transport routes to their final localization within mitochondria. After translocation of precursors through the general TOM complex, distinct downstream import pathway diverge in the inter-membrane space (IMS): Biogenesis of β -barrel proteins of the outer

Interestingly, none of the antioxidant enzymes classically protecting mitochondria against the effects of increased ROS generation are over-represented in HAB CC synaptosomes. Instead, the mitochondrial effects of oxidative and ionic stress appear to be simultaneously and synergistically controlled through at least seven different mechanisms. (1) Most of the energy requirements are met through fatty acids β -oxidation, while pyruvate metabolism complemented by CA2-mediated increased lactate influx seem mainly utilized for the maintenance of NADH/FADH₂ homeostasis through increased activity of the malate-aspartate and the glycerol-3-phosphate shuttles. The protective effects of these metabolic mechanisms are further enhanced and supplemented by (2) Cisd1/MitoNEET-mediated negative feedback regulation of β -oxidation and ROS production rates; (3) sideroflexins-mediated protection of proteins containing Fe-S domains, (4) mARC-mediated reduction of N-hydroxylated molecules, thus preventing the accumulation of Lewy bodies, (5) K_{ATP}-mediated prevention of irreversible Ca²⁺ influx-dependent mitochondrial membrane potential collapse and down-modulation of ETC efficacy, (6) LETM1-mediated localized sequestration of mitochondrial Ca²⁺ and down-modulating PTP opening potential, and (7) VADC-mediated efflux of excess ROS together with regulation of the intra-mitochondrial metabolic environment.

These mechanisms suggest metabolic adaptations to persistent mitochondrial Ca²⁺ loading that also attenuate intra-mitochondrial generation and accumulation of oxygen free radicals. Together with the mechanisms controlling the production of nitrogenous free radical species, these adaptations could be expected to significantly limit mitochondrial functional stress under conditions of high energy demands.

However, they can also be expected to result in higher levels of intra-synaptic ROS, with possible negative effects upon dopamine release. Since proteomics data integration also reveals the implementation of mechanisms suggestive of a possible dominance of serotonergic over dopaminergic activity, the above metabolic adaptations might exacerbate the effects of an already existing neurotransmitter imbalance which appears to underlie and sustain the HAB phenotype.

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