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 Ca²⁺ 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 CADI 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).

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
protein that binds and stabilizes microtubules [19, 20], by these proteins result in the induction of long branched 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\textsuperscript{2+}-independent, volume-sensitive large conductance Cl\textsuperscript{−}-independent, volume-sensitive large conductance Cl\textsuperscript{−}-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 \(\alpha\) (x1.35), \(\alpha\)1 (x1.45) and \(\gamma\) (x2.5) isoforms, central to the regulation of glutamatergic synapses and of LTD/ 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\textsuperscript{2+} block [25, 26], CamKII is necessary for LTD induction, is persistently activated by stimuli that elicit LTD, and can, by itself, enhance the efficacy of synaptic transmission [27].

The auto-phosphorylation of CamKII \(\gamma\) 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 LTD 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 Gmr3/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 Gmr3 (reduction of glutamatergic neurotransmission) results in increased spontaneous synaptic activity in non-glutamatergic network members [31].

The over-representation of Cend1 (x1.4), Scrin1/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 underlining 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]. Scrin1 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\textsuperscript{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\textsuperscript{+} 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\textsuperscript{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\textsuperscript{+}-Cl\textsuperscript{−} co-transporter Scl2a5/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 Scl2a5, the K\textsuperscript{+}-Cl\textsuperscript{−} co-transporter which maintains chloride homeostasis in neurons, and Scl6a1/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. Thyh1, an integrin-associated membrane protein mainly found in invaginations of dendritic spines [47, 23] is over-represented in HAB CC synaptosomes (x1.9). Thyh1 expression silencing results in the swelling of synaptic spines, while Thyh1 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 proteins in 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 Sir2, 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 Sir2 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 Ca²⁺ 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 Ca²⁺ influx into post-synaptic structures. Rapid Ca²⁺ efflux from neurons occurs through two main systems: electrochemically driven Na⁺/Ca²⁺ exchangers (VGCCs) with a low Ca²⁺ affinity, and calmodulin-activated, plasmalemmal-specific Ca²⁺-ATPase (PMCA) extrusion pumps with a high Ca²⁺ 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 Ca²⁺ transients amplitude down-modulation following AMPARs activation (recovery of depolarization-mediated Ca²⁺ elevation) [68]. Atp2b2, which has high basal activity, fast Ca²⁺ activation kinetics and high Vmax, associates with rapid clearance of Ca²⁺ 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 ATP1x2 (catalytic subunit), β3 and β2 (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 α2 isoform deficiency display increased anxiety-related behaviour and impaired spatial learning [71]. Increased presence of β3 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 Alkalization and Perisynaptic ATP Hydrolysis

Following AMPAR activation, elevation of external K+ elicits a Ca2+-dependent acid transient followed by a large net alkaline shift. This depolarization-induced alkalization mechanism, which also improves neuronal excitability [72], is governed by Cl-dependent and Cl-independent HCO3- transporters [73], and in particular by Sclc4a8/Ndebe (x1.75) together with Sclc4a7/Nbecn1 (x1.9) or Slec4a10/Nbce2 (x2.0) and the electrogenic Slec4a4/Nbce1 (x1.35) transporters. The HCO3- 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 (x 2.85 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 FADH2 in the process; Sdhb (x2.0), the iron-sulfur protein (three Fe-S clusters) subunit of succinate dehydrogenase which accepts electrons from FADH2 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 HQ2 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 Uqcrfs1 (x2.15), the cytochromes b (x1.75) and C1 (x2.15), the two core proteins Uqrc1 and Uqrc2 (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-Co1 and mt-Co2 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
here, significant reactive oxygen species (ROS) generation could be expected. Strikingly however, none of the antioxidant enzymes protecting mitochondria against the effects of \( \text{H}_2\text{O}_2 \) generated by electrons transported from reduced substrates (NADH and FADH\(_2\)) to oxygen, or O\(_2^{-}\), resulting from complexes I and III increased activity, are over-represented in HAB CC synaptosomes. Furthermore, none of the mitochondrial uncoupling proteins (Slc25a7/UCP1; Slc25A8/UCP2; Slc25a9/UCP3; Slc25a27/UCP4 [neuron-specific]) nor Slc25a14/UCP5, the last two of which allow to maintain ATP levels in the face of impaired OXPHOS, are over-represented in HAB CC synaptosomes.

### 3.2.2. Mitochondrial Components Sustaining Oxidative Phosphorylation

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 [86], with Mpc2 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).

The Hk1-SA isoform of hexokinase-1, CA2, Gpd2, the Slc25a3 co-transporter and the mitochondrial Ckmt1 and 2 (creatine kinase 1 & 2 [inner membrane]) are all very significantly over-represented in HAB CC synaptosomes (x1.65, x1.9, x2.15, x1.9, x1.75 & x1.9, 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).

![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 (TPPP) 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]).](image-url)
phosphate (generated from fructose metabolism; [162]) in the cytosol followed by AST-mediated dual conversion of OAA into Asp and producing malate. The generated cytosolic malate is then converted to oxaloacetic acid (OAA) in a reaction coupled to the generation of NADH, which is then used for ATP production. In this reaction, NADH is generated by glycolysis and lactate dehydrogenation in the cytosol. 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 (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 carrier Slc25a12/AGC1 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 Slc25a12/AGC1 transporter, which performs the calcium-dependent exchange of cytoplasmic glutamate with mitochondrial aspartate across the mitochondrial inner membrane [92], and with the 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].

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, generates 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

![Fig. 3. The malate-aspartate shuttle. AGC1 (Slc25a12, x2.3), the only aspartate/glutamate carrier isozyme 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 NADH. 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]).](image)

![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]).](image)
slightly shuttle. Slc25a22, highly over-represented in HAB synaptosomes (x2.15), which co-transport glutamate with H\(^+\) across the inner mitochondrial membrane, fulfills 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].

### 3.2.5. 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 Mtch2 (Slc25a50), over-represented in HAB CC synaptosomes (x1.9), induces mitochondrial depolarization and is involved in the mitochondrial apoptotic program. Following apoptotic stimulation, Mtch2 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 synaptosomes, 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 –oxidation and ketone body production [112].

In HAB CC synaptosomes, practically all enzymes necessary for mitochondrial fatty acids –oxidation and ketone body production, namely Acsf6 (long-chain fatty-acid-CoA ligase 6; x1.35), Acox1 (acyl-coenzyme A oxidase 1; x1.65), Echs1 (enoyl-CoA hydratase; x1.65), Gadph (β-hydroxybutyrate dehydrogenase; x2.65) which catalyses the inter-conversion of acetocetate 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 Sle25a29 (mitochondrial carnitine/acylcarnitine carrier) and involving the intracellular lipid transfer protein Scep [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 CC 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 (Maao 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 b5 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 -oxidation, while pyruvate metabolism complemented by CA2-mediated increased lactate influx seems to be mainly utilized for the maintenance of NADH/FADH₂ 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, Ca²⁺-loading induces permeability transition pore (PTP) opening, sudden Ca²⁺ efflux from the mitochondrial matrix and membrane potential collapse, resulting in mitochondrial demise [126]. Synaptic Ca²⁺ influx into mitochondria is controlled through Ca²⁺-dependent transient K⁺ influx, mediated by mitochondrial ATP-sensitive K⁺ channels (Kₐ₅), into the matrix, causing activation of the K⁺/H⁺ exchanger and re-establishment of membrane potential [127, 128].

The high-affinity mitochondrial Ca²⁺ importer Letm1, which is highly over-represented (x2.15) in HAB CC synaptosomes, achieves mitochondrial Ca²⁺ sequestration at small Ca²⁺ increases. Knock-down of Letm1 strongly diminishes the transfer of entering Ca²⁺ into mitochondria [129]. Concurrently, connexin 43 (Cx43), over-represented in HAB CC synaptosomes (x1.55), stimulates mitochondrial Kₐ₅ channel opening [130] while counteracting Ca²⁺-induced PTP opening [126]. Genetic ablation of Cx43 reduces both ADP-stimulated complex I respiration and Kₐ₅-mediated mitochondrial potassium influx [131]. Hence, Letm1 and Cx43 over-representation may potentiate a protective mechanism against irreversible, Ca²⁺ 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, Ca²⁺ 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 (Gpr75, Pml, Acat1), nor NCLX (Slc24a6), which mediates sodium-dependent calcium efflux from mitochondria, are over-represented, suggesting an adaptation of synaptic mitochondria to enhanced Ca²⁺ uptake together with a possible dissociation between VDAC-mediated mitochondrial homeostasis and ER-associated Ca²⁺ flux regulation in HAB CC synapses.

Therefore, at least two synergistic protective mechanisms against different effects of Ca²⁺ 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 cytosplasmic 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 Opal, 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 Opal, 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 cristae 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-helix domain containing proteins 3 and 6 (Chchd3 and Chchd6), Disrupted-in-schizophrenia 1 (Disc1), the sorting and assembly machinery component 50 (Sam50), Metalin-1 and -2 (Mtx1 and Mtx2), DNAJC11/Hsp40 and ApoO [146-150].

In HAB CC synaptosomes, Immt, Opal1, 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 cristate architecture and protein import [147], Chchd6 is required for maintaining not only mitochondrial cristate 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, Tsp01), an outer mitochondrial membrane protein necessary for cholesterol import and steroid production [152].
3.2.9.2. The General Translocase of the Outer Membrane (TOM Complex)

Tomm22 (x1.75) is the central receptor component of the mitochondrial outer membrane translocase (TOM complex) responsible for the recognition and translocation of cytosolically synthesized mitochondrial pre-proteins as well as their insertion into the outer mitochondrial membrane [153].

3.2.9.3. The Translocase of the Inner Membrane (TIM22 Complex)

Timm22 (x1.9) is the essential core component of the TIM22 complex that mediates the import and insertion of multi-pass transmembrane proteins into the mitochondrial inner membrane. In the TIM complex, Timm22 constitutes the voltage-activated and signal-gated channel. It forms a twin-pore translocase that utilizes the membrane potential as external driving force in 2 voltage-dependent steps [154, 155].

3.2.9.4. The Matrix Translocase of the Inner Membrane (TIM23 Complex; Pre-Sequence Translocase)

Timm23 (x2.3) is an essential component of the TIM23 complex that mediates the translocation of transit peptide-containing proteins across the mitochondrial inner membrane [156].

Timm16 (Magmas/PAM16 [x2.3]) is an essential component of the PAM complex. Pam16 cooperates with Pam18 in mHsp70 negative regulation and seems to recruit Pam18 to the translocation machinery [157]. Timm16 over-expression has been suggested to be protective against apoptosis [158].

Taken together, the differential proteomics data pertaining to mitochondrial structural dynamics strongly suggest that in the CC synapses of HAB animals, mitochondria could have a tendency to form elongated structures with much higher protein contents, and possibly protein turnover, than LAB animals.

![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 membrane (OM) requires the small Tim chaperones of the IMS and the SAM. Proteins of the IMS that contain cysteine-rich signals (CxxC) are imported via the mitochondrial inter-membrane space import and assembly (MIA) pathway. Carrier proteins of the inner membrane (IM) are transported with the help of the small Tims and the translocase of the inner membrane 22 (TIM22 complex). Presequence-containing proteins are inserted into the inner membrane or imported into the matrix by the translocase of the inner membrane 23 (TIM23 complex; presequence translocase). Matrix translocation requires the activity of the presequence translocase-associated import motor (PAM). Presequences are proteolytically removed by the mitochondrial processing peptidase (MPP) upon import. Δψ: membrane potential across the inner mitochondrial membrane (adapted from [164]).](image)

4. Conclusion

Integrative analyses of the proteomics data reveals that in HAB mice, the CC is dominated by strong structural and functional reinforcement of existing glutamatergic post-synaptic densities, increased high-frequency stimulation of excitatory glutamatergic synapses together with enhanced control over the modulation of synaptic strength but relatively weakened GABAergic control. This appears concurrently with strikingly limited de-novo spine genesis, and deregulated pre-synaptic over-activation of excitatory synapses together with substantial LTP maintenance.

The over-represented mechanisms suggest the possibility that post-synaptic spines could now act as “signal amplifiers” attenuating signal versus noise discrimination within an overall situation dominated by high-frequency stimulation of excitatory synapses coupled with increased spontaneous synaptic activity in non-glutamatergic network members.

However, strong negative feedback regulatory mechanisms, tending to attenuate LTP, as well as mechanisms protective against excitotoxicity are clearly implemented. Prominent amongst the formers are CamKII γ-mediated accelerated decay of AMPA signaling, Na+/K+-ATPase and PMCA pumps-mediated increase in depolarization thresholds, and adenosine-mediated down-modulation of pre-synaptic glutamate release, while depolarization-induced alkalization, which preempts large, prolonged Ca2+-dependent acidosis together with very active glutamate uptake and metabolism mechanisms play major protective roles.

At the sub-cellular level, mitochondrial functions largely dominate the mechanisms implemented in the CC synapses of HAB animals. The amplified mitochondrial functions reveal a situation characterized by highly increased OXPHOS activities, enhanced substrate delivery and ATP export together with significantly strengthened compartmented pH control in a context of large and fluctuating energy demands. In view of the consistently increased expression in OXPHOS protein subunits together with glutamatergic-induced Ca2+ loading, significant mitochondrial ROS generation could be expected and the presence of pro-apoptotic and as well as pro-necrotic mechanisms is indeed a clear indication of increased mitochondrial metabolic and structural stress.
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 nitorgenous 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 serotoninergic 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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