



Published in final edited form as:

Prog Neurobiol. 2017 October ; 157: 273–291. doi:10.1016/j.pneurobio.2015.10.005.

Alternative Mitochondrial Electron Transfer for the Treatment of Neurodegenerative Diseases and Cancers: Methylene Blue Connects the Dots

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Abstract

Brain has exceptional high requirement for energy metabolism with glucose as the exclusive energy source. Decrease of brain energy metabolism and glucose uptake has been found in patients of Alzheimer's, Parkinson's and other neurodegenerative diseases, providing a clear link between neurodegenerative disorders and energy metabolism. On the other hand, cancers, including glioblastoma, have increased glucose uptake and rely on aerobic glycolysis for energy metabolism. The switch of high efficient oxidative phosphorylation to low efficient aerobic glycolysis pathway (Warburg effect) provides macromolecule for biosynthesis and proliferation. Current research indicate that methylene blue, a century old drug, can receive electron from NADH in the presence of complex I and donates it to cytochrome C, providing an alternative electron transfer pathway. Methylene blue increases oxygen consumption, decrease glycolysis, and increases glucose uptake *in vitro*. Methylene blue enhances glucose uptake and regional cerebral blood flow in rats upon acute treatment. In addition, methylene blue provides protective effect in neuron and astrocyte against various insults *in vitro* and in rodent models of Alzheimer's, Parkinson's, and Huntington's disease. In glioblastoma cells, methylene blue reverses Warburg effect by enhancing mitochondrial oxidative phosphorylation, arrests glioma cell cycle at s-phase, and inhibits glioma cell proliferation. Accordingly, methylene blue activates AMP-activated protein kinase, inhibits downstream acetyl-coA carboxylase and cyclin-dependent kinases. In summary, there is accumulating evidence providing a proof of concept that enhancement of mitochondrial oxidative phosphorylation via alternative mitochondrial electron transfer may offer protective action against neurodegenerative diseases and inhibit cancers proliferation.

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Introduction

Cancer and neurodegeneration are often believed to be two distinct pathological disorders of opposite etiology and therapeutic intervention. While cancers are characterized by the enhanced resistance to cell death, neurodegenerative diseases are featured by the progressive premature neuron death (Plun-Favreau *et al.*, 2010). Indeed, neurodegenerative diseases such as Alzheimer's disease (AD) have been found to be inversely associated with cancers (Bajaj *et al.*, 2010; Driver *et al.*, 2012). On the other hand, there is increasing evidence that cancers and neurodegenerative diseases might share common etiologic mechanisms and therapeutic targets. Age is the single most important risk factor for both neurodegeneration and cancers (Niccoli and Partridge, 2012). Dietary restriction has been found to be one of the most effective interventions to extend lifespan and retard age-related diseases including cancers and neurodegenerative diseases (Hursting *et al.*, 2013)(Graff *et al.*, 2013; Hursting *et al.*, 2010; Prolla and Mattson, 2001). There are many drugs that may be effective for the treatment of both cancers and neurodegenerative diseases. For example, Bexarotene, a retinoid X receptor agonist for the treatment of T cell lymphoma, has been shown to reduce A β plaques and attenuates cognitive deficits in murine models of AD (Aicardi, 2013; Cramer *et al.*, 2012; Tai *et al.*, 2014).

From the discoveries of Krebs cycle (Krebs and Johnson, 1937) to mitochondrial oxidative phosphorylation (Boyer *et al.*, 1973; Gresser *et al.*, 1982), biology has experienced great advance of our knowledge in cellular metabolism from 1920s to 1980s. For the past 3 decades, molecular biology approaches have been dominating biological research without paying much attention to the metabolic state of cells. Despite the dramatic advantage of molecular biology and modern medicine, neurodegenerative disorders and cancers remain the most devastating diseases and effective therapeutic interventions are desperately needed. Recently, it is beginning to be accepted that transcription participates in dictating cellular metabolism (McKnight, 2010). The integrative molecular, cellular, and metabolic approaches might provide a better understanding of neurodegenerative disorders and cancers, thus, leads to discover novel therapeutics for those incurables.

Abnormal metabolism is the key feature of many disorders. Neurodegenerative diseases are a heterogeneous group of disorders which presume to primarily affect different subset of neurons at the CNS, mainly including AD, Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). Despite the heterogeneity, both experimental and clinical studies have indicated that many neurodegenerative disorders often coexist metabolic dysfunction (Cai *et al.*, 2012). Similarly, cancer cells are genetically and phenotypically heterogeneous given the different origination and the inter-tumor genomic instability (Burrell *et al.*, 2013). Nonetheless, cancer cells have long been known to have characteristic alterations in their metabolism since 1920s (Warburg, 1956a, b).

Reprogramming of energy metabolism has recently been proposed as an emerging cancer hallmark (Hanahan and Weinberg, 2011). Accordingly, novel therapeutic targets on metabolic pathway have been exploring for the treatment of cancers and neurodegeneration. In this review, we recapitulate the findings that highlight the metabolic alteration in neurodegenerative diseases and cancers, summarize the novel function of methylene blue, a century old drug, as an alternative mitochondrial electron transfer carrier, and propose that

alternative mitochondrial electron transfer as a common therapeutic mechanism for the treatment of both neurodegenerative diseases and cancers.

Brain Energy Metabolism

Brain bioenergetics

Einstein's famous equation, $E=MC^2$, simplifies the relationship of two fundamental physics entities, energy and mass, and linked them with the speed of light. Thus, mass and energy, used to be thought as separate entities, are known to be interchangeable. The significance of Einstein's formula is even beyond physics. In biology, energy is an attribute of all living organisms from bacteria to human being. The conversion between mass and energy are fundamental to the biological processes defined as metabolism by which living organisms cycle energy through different mechanisms to produce the necessary molecules and perform the essential functions of life. Through anabolism, complex compounds are biosynthesized from simpler molecules with the energy expense provided by ATP hydrolysis. Through catabolism, complex nutrients are broken down to simpler oxidized molecules with an energy releasing process coupled to ATP production. Life is the interplay between energy and structure (Wallace, 2005). As the metabolism goes on, the life goes on (Figure 1).

Mammalian brain is characterized by high metabolic activity with fine regulatory mechanisms to ensure adequate energy substrates supply in register with neuronal activity. The human brain constitutes only 2% of the body weight, but receives 15% of cardiac output, accounts for almost 20% of the total oxygen consumption, and consumes approximately 25% of total body glucose utilization. The human brain is by far the most expensive organ in term of energy expenditure in the whole body. Maintenance and restoration of transmembrane resting potential dissipated by postsynaptic and action potential and neurotransmitters recycling represent the main energetic cost at the brain (Alle *et al.*, 2009; Attwell and Laughlin, 2001; Howarth *et al.*, 2012). An updated computation study has indicated that postsynaptic glutamate receptors, action potentials, resting potentials, presynaptic transmitter release, and transmitter recycling consume 50, 21, 20, 5, and 4% of energy budget at cerebral cortex, respectively (Howarth *et al.*, 2012).

In addition to the extreme high energy consumption, brain energy supply and expenditure are tightly coupled by neurovascular and neurometabolic mechanisms. As the brain has very limited energy storage, local brain activity has to be complied with a coincidental increase of cerebral blood flow (CBF), referred as neurovascular coupling. Brain vasculature has many unique structural and functional features that are distinct from the peripheral characterized by the intimate relationships between endothelium, pericyte, astrocyte, and neuron, termed together as the neurovascular unit (Del Zoppo, 2013; Iadecola, 2004). Anatomically, the neurovascular coupling has to be orchestrated by the synergistic action of neurovascular unit. Therefore, brain activity evoked increase of CBF, functional hyperemia, is likely mediated through the concerted action of numerous vasoactive agents derived from the neurovascular unit components (Girouard and Iadecola, 2006; Petzold and Murthy, 2011).

Under normal physiological condition, adult brain almost exclusively uses glucose as substrate for energy metabolism in which glucose is delivered from the circulation to the

brain through facilitated diffusion predominantly mediated by glucose transporter 1 (GLUT1) and GLUT3. GLUT1 was the first GLUT to be cloned with two isoforms been detected in the brain (Gerhart *et al.*, 1989; McCall *et al.*, 1996). The 45 kDa isoform has been found to be localized in glial cells and the higher molecular weight (55 kDa) isoform, the glycosylated GLUT1, present in both the luminal and abluminal membranes of endothelium (Birnbaum *et al.*, 1986). In addition, the 55 kDa GLUT1 reside in endothelial cytoplasm as an intracellular pool (Duelli and Kuschinsky, 2001). On the other hand, GLUT3 has been found to be almost exclusively expressed in neurons (Simpson *et al.*, 2007). Brain is considered to be an insulin insensitive organ as GLUT1 and GLUT3 are less insulin sensitive as comparing to GLUT4 (Heidenrich *et al.*, 1989; Mueckler *et al.*, 1985). The heterogeneous metabolism rate at the brain is accompanied with a heterogeneous distribution of GLUTs in the brain. The correlations between the metabolic rate and GLUTs distribution suggest that the densities of GLUT1 and GLUT3 might contribute to the local metabolic demand of the different brain structures (Duelli and Kuschinsky, 2001). Upon transported into the brain, as in other organs, glucose is phosphorylated by hexokinase to produce glucose-6-phosphate which can be further processed via different metabolic pathways: glycolysis, pentose phosphate pathway, and glycogenesis. Under normal condition, glucose is almost entirely metabolized to CO₂ and water through glycolysis, tricarboxylic acid (TCA) cycle, and mitochondrial oxidative phosphorylation (Belanger *et al.*, 2011).

Glycolysis is an evolutionary preserved and nearly universal energy production pathway whereby glucose is anaerobic fermented to lactic acid with the production of 3 ATP. In the presence of oxygen, glycolysis is tightly coupled with TCA cycle and mitochondrial oxidative phosphorylation and serves as a molecular interconversion system. Lactate, the product of glycolysis, is shuttled into mitochondria via the mitochondrial monocarboxylate transporter (MCT) where it is oxidized to pyruvate. Then, pyruvate is converted into a series of organic acid through TCA cycle with the liberation of carbon dioxide (Schurr, 2014). In addition, the TCA cycle reduces NAD⁺ to NADH, which is fed into the mitochondrial oxidative phosphorylation to generate ultimate biochemical energy, ATP.

Mitochondrial oxidative phosphorylation encompasses electron transfer through the mitochondrial respiratory chain, trans-inner mitochondrial membrane proton pumping, generating of mitochondrial membrane potential, and the ultimate ATP synthesis. The mitochondria produce energy in the form of ATP by oxidizing carbohydrates and fats-derived hydrogen. Electrons derived from NADH or succinate are passed sequentially through electron transfer chain (ETC) complexes and the released energy is used to pump protons into the intermembrane space through complex I, III, and IV to create mitochondrial membrane potential which is coupled to ATP synthesis. As protons flow cross mitochondrial inner membrane back into the mitochondrial matrix through complex V, P_i is bound to ADP to produce ATP (Wallace, 2005). As the by-product of mitochondrial oxidative phosphorylation, electrons leaked from the ETC complexes, mainly at complex I and III, can be directly donated to O₂ to produce superoxide anion and other reactive oxygen species (ROS) (Birch-Machin, 2006; Murphy, 2009).

Although mitochondrial structure and function are highly conserved, there are many qualitative and quantitative differences in mitochondria between different cells and tissues. In addition, mitochondria dynamically undergo shape and number changes through regulated processes of fusion and fission. There are studies indicated that brain mitochondria were complicated by the cellular and regional heterogeneity and aging and that mitochondria in the brain can generate ATP at a faster pace than peripheral (Battino *et al.*, 1991; Kwong and Sohal, 2000; Tesco *et al.*, 2007). Perturbation of mitochondrial dynamics has been indicated to be involved in a wide spectrum of human disorders, including neurodegenerative diseases and cancers (Chen and Chan, 2009; Grandemange *et al.*, 2009).

Beyond Bioenergetics

Glucose derived from carbohydrates provides not only the major fuel for ATP production through mitochondrial oxidative phosphorylation but also the essential substrate for *de novo* synthesis of proteins, lipids, and nuclei acids (Bauer *et al.*, 2005). Similarly, in addition to its role as cellular bioenergetics center, mitochondria function as biosynthetic hub and play critical roles in synthesis of macromolecules to meet the biosynthetic demand for cell proliferation (Jones and Thompson, 2009). For bioenergetic pathway, pyruvate, generated from glycolysis, is converted to acetyl-CoA which feeds into the TCA cycle to be oxidized for energy production. For biosynthetic pathway, carbon atoms derived from glucose enter mitochondria and are then extruded from the TCA cycle at various steps to provide precursors for biosynthesis of lipids, nucleotides, and amino acids (Jones and Thompson, 2009).

In TCA cycle, 2-carbon acetyl group derived from acetyl-CoA is transferred to 4-carbon oxaloacetate to form 6-carbon citrate which is further converted to isocitrate. As an alternative, citrate is exported to the cytosol through pyruvate-citrate shuttle for lipid biosynthesis and protein acetylation. In the cytosol, citrate is cleaved back to form oxaloacetate and acetyl-CoA. The citrate is further converted into malonyl-CoA by acetyl-CoA carboxylase and feed into fatty acid synthesis pathway (Ahn and Metallo, 2015). Thus, in addition to its critical role in bioenergetics, metabolic intermediate acetyl-CoA functions as a central biosynthetic precursor for fatty acid synthesis. Consistently, eukaryote mitochondria also harbor a highly conserved fatty acid synthetic machinery independent of the cytosolic fatty acid synthesis apparatus although its end products and functions are largely unclear (Hiltunen *et al.*, 2009).

Glucose metabolites also provide substrates for *de novo* biosynthesis of amino acids. Pyruvate, α -ketoglutarate, and oxaloacetate can be converted into amino acids through the addition of an amino group. α -ketoglutarate can be converted into glutamate by reductive amination. Then the amino group from glutamate can be transferred to pyruvate and oxaloacetate to produce alanine and aspartate, respectively. Glutamate is also the precursor of proline and arginine. Glucose metabolism through the pentose phosphate pathway produces a key intermediate in nucleotide biosynthesis, ribose-5-phosphate (Munoz-Pinedo *et al.*, 2012). In addition, pentose phosphate pathway produces NADPH, which supplies reducing equivalents for the biosynthesis of fatty acid and nucleotide (Jones and Thompson, 2009).

Mitochondrial oxidative phosphorylation is the dominant metabolic pathway in the brain. The central dogma of brain energy metabolism assumed that brain glucose utilization fuels neuronal activity through mitochondrial oxidative phosphorylation in both basal and activated state (Simpson *et al.*, 2007; Sokoloff *et al.*, 1977). However, there is increasing evidence suggesting an uncoupling between the brain glucose and oxygen utilization. The measurement of glucose utilization has been found to be up to 31 mmol/100g weight/min calculated from the oxygen consumption with a respiratory quotient of 1 (Kety and Schmidt, 1948) (Pellerin and Magistretti, 2012). Therefore, in addition to bioenergetic production of ATP through mitochondrial oxidative phosphorylation, biosynthetic pathways involved in both glycogenesis and lipogenesis may contribute to the glucose metabolism by the brain in excess of oxygen consumption. Indeed, aerobic glycolysis has been found to account for ~10% of the glucose consumed by the adult human brain (Goyal *et al.*, 2014).

Although brain is believed to have very limited energy storage, glycogen, the single largest glucose reserve, has been found in the brain which predominately localize in astrocytic end-feet, bodies, and processes (DiNuzzo *et al.*, 2011; Oz *et al.*, 2007). Theoretically, given their intimate relationship with neurons both anatomically and functionally, astrocytes can mobilize glycogen for neuronal metabolism during normal circumstance and in case of increased energy demand or failure of neurons. Given the relatively low content of glycogen in the brain as comparing to liver and skeletal muscle, the astrocytic glycogen is unlikely accounted for as an alternative energy supply in the brain during hypoglycemia. Instead, astrocytic glycogen more likely serve as an integral factor for brain metabolism under normal condition (DiNuzzo *et al.*, 2011; Obel *et al.*, 2012). The glycogenesis and glycogen degradation is fine-tuned by glycogen synthase and glycogen phosphorylase (Obel *et al.*, 2012). There is much controversy regarding the function of astrocytic glycogen in term of neuron-glia metabolic coupling which is beyond the scope of this review. Importantly, astrocytic glycogen seems play a role in learning process (Hertz and Gibbs, 2009). Furthermore, disturbance of glycogen homeostasis between astrocyte and neuron has been found to result neurodegeneration. Recent studies have indicated that neurons have the enzymatic machinery for glycogenesis and that Lafora disease (progressive myoclonus epilepsy) related gene mutations activated glycogen synthesis in neurons and caused apoptosis (Duran *et al.*, 2012; Magistretti and Allaman, 2007; Vilchez *et al.*, 2007).

Lipid metabolism is of particular interest due to its high concentration in the brains and that mutation in lipid metabolizing enzymes result in debilitating neurological diseases. Lipids could potentially serve two principle functions as substrate for energy production through β oxidation and as structural components of cell membranes. It was conjectured that the selection of glucose, the predominant substrate for brain energy metabolism, over fatty acids for energy metabolism in the brain may be evolutionarily beneficial to avoid the disadvantages of β -oxidation (Schonfeld and Reiser, 2013). While fatty acids are used by the brain as well (Ebert *et al.*, 2003; Kuge *et al.*, 1995; Panov *et al.*, 2014), neurons have low level rate-setting fatty acid catabolism enzymes and are generally not thought to rely on fatty acid β -oxidation for bioenergetics (Cahoy *et al.*, 2008; Lee and Wolfgang, 2012) (Schonfeld and Reiser, 2013).

Beside potential energy substrate, lipids are structural components of cell membranes and have been found to be involved in intracellular architecture, membrane trafficking, and regulation of proteins and sub-compartments in the membranes (Adibhatla and Hatcher, 2007; Liu and Zhang, 2014). Cholesterol is an indispensable biological membrane component. Brain is a highly cholesterol-enriched organ resided with ~25% of total body cholesterol (Dietschy and Turley, 2001). The majority of brain cholesterol resides in the myelin sheaths and plasma membranes required for brain development, synapse and dendrite formation, and axonal guidance (Orth and Bellosta, 2012). Cholesterol synthesis is at the highest in oligodendrocytes during myelination at the developmental stage (Dietschy and Turley, 2004). In the adult brain, BBB prevents the cholesterol uptake from the blood, thus, *de novo* cholesterol synthesis continues in astrocytes at a low rate (Nieweg *et al.*, 2009) (Vance, 2012). Consistently, lipid synthetic pathways are highly enriched in astrocytes (Cahoy *et al.*, 2008).

In addition as the pivotal structural component, brain lipids have critical cell signaling function mainly through three mechanisms. First, bioactive lipids as lysophospholipids, eicosanoids, and endocannabinoids are known to interact with G-protein coupled membrane receptors that activate the downstream signaling (Bieberich, 2012). Secondly, brain bioactive lipids as phosphatidylinositols and ceramide bind to protein kinases, phosphatases, and other signaling proteins that affect intracellular cascades (Bieberich, 2012). In addition, brain bioactive lipids regulate cell signaling pathways through cholesterol- and sphingolipid-enriched lipid rafts (Lingwood and Simons, 2010). Lipid rafts have been shown to be involved in many brain functions both physiologically and pathologically. For example, lipid rafts have been shown to influence the potency and efficacy of neurotransmitter receptors and transporters (Allen *et al.*, 2007). Lipid L-carnitine, its primary role appears to facilitate the transport of long chain fatty acids into mitochondria for β -oxidation cycle, has been indicated to be neuroprotective (Rau *et al.*, 2012; Wang *et al.*, 2007). Lipogenesis has been shown to regulate adult neural stem proliferation (Folmes *et al.*, 2013; Knobloch *et al.*, 2013).

Astrocyte-Neuron Metabolic Coupling and Astrocyte Neuron Lactate Shuttling

In the last decade, our knowledge in neuroenergetics has been rapidly evolving from the “neurocentric” view to an integrated picture of neuron-astrocyte coupling (Allaman *et al.*, 2011; Belanger *et al.*, 2011). Anatomically, neurons are separated from circulation by BBB and do not have direct access to blood-born fuel. Glucose from the circulation has to cross 4 plasma membrane to reach neurons, 2 endothelial and 2 astrocytic. Thus, neurons might not be able to directly control their own fuel supply. On the other hand, astrocytes, with their end feet cover 99.7% of the brain capillary, have an anatomical advantage to control over glucose flux into the brain (Mathiisen *et al.*, 2010). Therefore, astrocytes may server as an intermediary between neuron and vasculature for neurovascular coupling.

Consistent to the anatomical evidence, the knockout mice studies have indicated that glia/ endothelial GLUT1 play a major role in glucose transport at the mammalian brain as comparing with neuronal GLUT3. Heterogeneous GLUT1 knockout mice have decreased brain GLUT1 expression and display many features similar to the human GLUT1 deficiency

syndrome with the decreased brain glucose uptake, seizures, impaired motor activity, hypoglycorrhachia, and disturbances of microencephaly incoordination and learning (Wang *et al.*, 2006). On the other hand, GLUT3 haploinsufficiency is not associated with any brain dysfunction and impairment of brain glucose uptake (Stuart *et al.*, 2011). Thus, astrocytes may play an even more important role in glucose metabolism compared with neurons in the brain. Indeed, at the hippocampus and cerebellum, glucose transport and metabolism has been found to be faster in the glial compartment than in the neuronal compartment (Jakoby *et al.*, 2014). *In vivo* imaging of brain slices have indicated that stimulation of astrocytes triggers an intra-astrocytic calcium surge which subsequent dilate or constrict adjacent arterioles (Girouard *et al.*, 2010).

Besides glucose, a wide range of metabolic intermediates can be oxidized for energy production in the brain, including lactate, pyruvate, acetate, glutamate, and glutamine (Belanger *et al.*, 2011; Zielke *et al.*, 2009). Importantly, neurons and astrocytes present different metabolic profiles in physiological condition. Beside GLUT1 and GLUT3, glycolytic products are transported into and out of neurons by monocarboxylate transporters (MCT) with MCT1 been found in the BBB and astrocytes and MCT2 in neurons (Simpson *et al.*, 2007). In addition, astrocyte expresses MCT4 (Pierre and Pellerin, 2005). MCT1-4 have been shown to transport endogenously generated monocarboxylate such as pyruvate, lactate, and ketone bodies (Pierre and Pellerin, 2005). Glycolytic product, lactate, has been shown to have a role in long-term memory formation. Antisense oligonucleotides targeting either astrocytic MCT4 or neuronal MCT2 cause memory defects. Interestingly, addition of exogenous lactate only rescued the memory consolidation defects caused by MCT4 knockout, but not MCT2 knockout, suggesting lactate shuttling from astrocytes into neurons (Bezzi and Volterra, 2011; Suzuki *et al.*, 2011). Although there are substantial evidence that pyruvate derived from neuronal glucose is the major oxidative fuel for neurons (Patel *et al.*, 2014), there is increasing evidence suggested that astrocytes play critical roles in both bioenergetic and biosynthetic metabolism at the brain. Furthermore, dysfunction of astrocyte metabolism has been found in many neurodegenerative diseases (Stobart and Anderson, 2013).

AMPK, a Master Sensor and Regulator of Energy Metabolism

Matching energy supply with expenditure is essential for brain function. ATP, produced from ADP and inorganic phosphate, is the universal energy currency in the cells. Reversely, energy-required cellular processes are powered by the hydrolysis of ATP to ADP and phosphate. Thus ADP/ATP ratio provides an important parameter for both cellular ATP consumption and synthesis. In addition, the ubiquitously expressed adenylate kinase catalyze the inter conversion of adenine nucleotides which make AMP/ATP ratio another important indicator for energy status (Hardie, 2011). At the cellular level, maintenance of the energy homeostasis as reflected in relative level of ATP, ADP, and AMP is of critical importance for energy metabolism. Recent studies demonstrated that the relative level of ATP, ADP, and AMP, in term of ADP/ATP and AMP/ATP ratios, play a critical role in the regulation of energy metabolism through AMP-activated protein kinase (AMPK) signaling (Gowans *et al.*, 2013; Oakhill *et al.*, 2011).

AMPK, a Ser/Thr kinase, is an evolutionarily conserved energy sensor and regulator for energy metabolism (Kahn *et al.*, 2005). AMPK is a heterotrimeric protein comprised of catalytic α subunits ($\alpha 1$, $\alpha 2$) and regulatory β and γ subunits ($\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$, $\gamma 3$) (Hardie, 2007). It is activated by events that either increase ATP consumption or compromise cellular ATP production (Hardie, 2007; Long and Zierath, 2006). The glycogen-binding domain on the β subunit also allows AMPK to function as a glycogen sensor (McBride *et al.*, 2009; McBride and Hardie, 2009). AMPK elicits diverse effects on glucose and lipid metabolism, cell cycle, and protein synthesis. Activated AMPK stimulates catabolic pathways that generate ATP through increasing glucose and fatty acid uptake, enhancing glycolysis and fatty acid oxidation, and stimulating mitochondrial biogenesis. Concomitantly, AMPK activation inhibits the rate of anabolic pathways that consume ATP, thus, restores the correct adenylate energy charge (Hardie, 2011). AMPK activation inhibits glycogen and fatty acid synthesis by phosphorylates and inactivates glycogen synthase and Acetyl-CoA carboxylase (ACC), respectively (Hardie, 2007). In addition, AMPK inhibits protein synthesis and cell proliferation (Horman *et al.*, 2002; Motoshima *et al.*, 2006). When nutrients are limited, AMPK inhibits cell growth via the suppression of mammalian target of rapamycin complex I (mTORC1) (Gowans *et al.*, 2013). Furthermore, AMPK activation promotes autophagy to maintain essential cellular activity in response to nutrient scarcity (Kim *et al.*, 2011).

In the mammalian adult brain, AMPK predominately expresses in neurons characterized by high catabolic activity (Culmsee *et al.*, 2001; Turnley *et al.*, 1999; Vingtdoux *et al.*, 2011). Different AMPK expression levels have been identified in different neuronal subtypes of the adult brain which higher glucose metabolism have higher AMPK expression (Spasic *et al.*, 2009). At the hypothalamus, AMPK is expressed in energy sensing neurons and functions as a master regulator of organismal energy balance (Ronnett *et al.*, 2009). Energy deficit signals such as hypoglycemia (McCrimmon *et al.*, 2004), thyroid hormones (Ishii *et al.*, 2008), glucocorticoids (Shimizu *et al.*, 2008), cannabinoids (Kola *et al.*, 2005), and adiponectin (Kubota *et al.*, 2007) enhance hypothalamic AMPK activity to increase feeding. On the other hand, energy surplus signals induced by high glucose, leptin, insulin, and resistin inhibit feeding behavior via decreasing hypothalamic AMPK activity (Minokoshi *et al.*, 2004). AMPK activation is mediated through phosphorylation by tumor suppressor LKB-1 complex and calcium/calmodulin-dependent protein kinase kinase β (CaMKK β). LKB-1 has been found to be ubiquitously expressed which enable basal constitutive AMPK activation, while, CaMKK β is expressed predominantly in neurons (Spasic *et al.*, 2009). On the other hand, consistent with its pro-anabolic phenotype, astrocytes only express minimal AMPK under physiological condition (McCullough *et al.*, 2005).

Energy Metabolism in Neurodegenerative Diseases

Neurodegenerative diseases are heterogeneous in term of clinical symptom and pathophysiology due to the progressive loss of different subsets of neurons at the CNS (Przedborski *et al.*, 2003). Most of neurodegenerative diseases are aging-related and present progressive motor and/or cognitive dysfunction that causes reduced life quality, increase medical costs, and eventual death. As the aging population increases worldwide, neurodegenerative diseases are becoming an enormous social and economic burden in the world. Unfortunately, efforts towards discovering effective treatment for neurodegeneration

have been fruitless and no cure has been developed so far for any of the neurodegenerative disorders.

For decades, research in neurodegenerative diseases has been dominated by the neurocentric view holds that neurodegeneration is primarily caused by neuronal defects. However, there is increasing evidence indicates that vasculature and astroglia might also contribute the onset and progression of many neurodegenerative disorders (Maragakis and Rothstein, 2006; Zacchigna *et al.*, 2008). The heterogeneity of the neurodegenerative diseases does not forestall common biochemical features. An integrative view of the neurovascular unit and their common biochemical feature in neurodegenerative diseases might shed light on our future research in neurodegeneration that ultimately lead to the discovery of effective therapies. There is accumulating evidence indicating a causative role of mitochondrial dysfunction in both aging and age-related neurodegenerative disorders (Bratic and Larsson, 2013; Chen and Chan, 2009). In fact, the causative factor is likely beyond the mitochondria dysfunction and involved in energy metabolism.

Metabolic Alteration in AD

AD is the most common neurodegenerative disease hallmarked pathologically by the amyloid- β plaque in neuropil and cerebral vessels, and by the hyperphosphorylated neurofibrillary tangles in neurons (Girouard and Iadecola, 2006). Accordingly, AD research has been dominated by the “amyloid cascade hypothesis” and “tau and tangle hypothesis” (Korczyn, 2008; Mudher and Lovestone, 2002). However, decades of research focusing on A β and tau has not lead to the identification of any effective treatment for AD. All therapeutics purported to decrease amyloid- β plaque have failed to demonstrate any efficacy in clinical trials (Karran and Hardy, 2014; Karran *et al.*, 2011). The fact that amyloid deposition is not strongly correlated with cognition argues against amyloid hypothesis. Furthermore, it has been clear that amyloid cascade and tau hyperphosphorylation can be induced by a plethora of brain damages. In experimental models, ischemic stroke induces overexpression of β -amyloid precursor protein (Nihashi *et al.*, 2001; Shi *et al.*, 2000), increases beta-secretase expression and activity (Tesco *et al.*, 2007; Wen *et al.*, 2004a), induces site-specific hyperphosphorylation (Wen *et al.*, 2004c) and AD like tauopathy (Wen *et al.*, 2004b). Consistently, accumulation of A β 1-40 and A β 1-42 were found in human hippocampus after ischemic stroke (Qi *et al.*, 2007). Amyloid- β and tau pathology have also been observed after traumatic brain injury in human (Chen *et al.*, 2009; Johnson *et al.*, 2012b). These evidence suggests that neither amyloid nor tau cascade may be the cause of neurodegeneration in AD. Thus, alternative hypotheses are needed to be explored beyond the amyloid- β and tau cascade.

Metabolic syndrome has been found to contribute to cognitive impairment in elderly persons and be associated with AD (Razay *et al.*, 2007; Yaffe *et al.*, 2004). Hypometabolism has emerged as a robust biomarker of neurodegeneration (Johnson *et al.*, 2012a). Reduction of glucose metabolism has long been implicated in AD brain (Iadecola, 2015). Impairments in brain energy metabolism and glucose utilization have been found to be among the very early abnormalities that accompany or even precede the early stage of cognitive dysfunction (Hoyer, 2004; Nordberg *et al.*, 2010; Sims *et al.*, 1980). The reductions in brain glucose

transport and utilization were found in cognitively normal people at genetic risk for AD and at the early state in AD patients (Iadecola, 2015; Nordberg *et al.*, 2010). There are many underlying mechanisms that may contribute to the impairment of glucose metabolism in AD brain. Endothelial and astrocyte localized GLUT1 plays a major role in glucose transport at the mammalian brain. Reduction of GLUT1 has been found in AD brain (Kalaria and Harik, 1989; Mooradian *et al.*, 1997). A very recent study indicated that GLUT1 deficiency exacerbated the pathophysiology in the brains of AD mice and may act synergistically with AD pathology to promote the progression of dementia (Winkler *et al.*, 2015). Reciprocally, restoration of GLUT1 reduced A β levels in AD mice brains (Winkler *et al.*, 2015). In addition, alternative energy substrates such as ketogenic diet and enhancement glucose uptake by intranasal insulin have demonstrated beneficial effects (Mamelak, 2012; Stafstrom and Rho, 2012; Yarchoan and Arnold, 2014).

Vascular mechanisms might also contribute to the metabolic alteration in AD. Cerebral amyloid angiopathy characterized by amyloid deposition within brain vasculature is common in aged individuals and even more common in AD patients (Castellani *et al.*, 2004). Thus, it is no surprise that cerebrovascular function is profoundly altered in AD. Ironically, the presence of cerebrovascular disease is considered an exclusive criterion for AD diagnosis and the contribution of vascular factor to the onset and progression of AD has been long debated (Iadecola, 2004; McKhann *et al.*, 2011). There is increasing evidence from both autopsy and long-term observational studies indicated that AD are highly pathologically heterogeneous with many exhibiting mixed pathologies, including infarcts (Schneider *et al.*, 2009). Indeed, the coexistence of AD and vascular dementia, termed mixed dementia, is the most common dementia (Langa *et al.*, 2004). In the brain, neurons, astrocyte, and vasculature constitute the neurovascular unit to maintain the brain homeostasis. Alteration of the neurovascular coupling has been found not only in cerebrovascular disease but also neurodegenerative diseases including AD. Cerebral amyloid angiopathy induces progressive neurovascular unit dysfunction featured by the failure of vascular reactivity, smooth muscle cell loss, and breakdown of vessel integrity (Zipfel *et al.*, 2009). Amyloid- β , the main component of amyloid plaques in AD brain, is vasoactive both *in vitro* and *in vivo* (Crawford *et al.*, 1998; Niwa *et al.*, 2000). Functional hyperemia has been found to be perturbed in both animal models and AD patients (Iadecola, 2004; Petzold and Murthy, 2011). Thus, the impairment of neurovascular coupling, together with other cellular mechanisms, might trigger and promote neurodegeneration in AD.

Besides the amyloid β plaque and neurofibrillary tangles, AD brain also display high occurrence of adipose inclusions (Foley, 2010). The risk of AD is affected by inheritance of different isoforms of Apolipoprotein E (APOE), a pivotal regulator for cholesterol metabolism, with the ϵ 4 allele as the strongest genetic risk factor for AD (Bertram and Tanzi, 2008; Corder *et al.*, 1993), providing strong evidence that lipid metabolism dysfunction may be involved in AD pathophysiology (Smith, 2000). It is well established that many lipids are implicated in AD pathogenesis (Di Paolo and Kim, 2011). Studies have indicated that change of brain cholesterol level influence APP processing and A β production (Lim *et al.*, 2014). There is also evidence that lipid rafts might function as platform for the production of neurotoxic proteins, thus, involve in the AD pathogenesis (Allen *et al.*, 2007; Eehalt *et al.*, 2003).

Mitochondria play pivotal roles in cellular functions including energy production, cell proliferation, and apoptosis (Birch-Machin, 2006; Wallace, 2005). At the brain, mitochondria regulate synaptic transmission, brain function, and cognition (Picard and McEwen, 2014). Given the identified metabolic dysfunction in AD, it might not be surprising that mitochondria deficit is involved in the onset and progression of AD. Abnormalities of mitochondrial morphology, biochemistry, and genetics have been found in AD brains although its causative action in AD is still debatable (Mancuso *et al.*, 2008; Swerdlow *et al.*, 2014; Swerdlow and Khan, 2004). AD related oxidative stress has been associated with alteration of mitochondrial ETC complexes (Morais and De Strooper, 2010). A β has been shown to alter the assembly and enzyme activities of ETC complexes (Moran *et al.*, 2012). Accordingly, studies have indicated that A β could gain access into mitochondria in both AD patients and transgenic AD mice (Chen and Yan, 2006). In addition, neurofibrillary tangles detected in AD brains have been shown to inhibit ETC complexes activity, increase ROS production, and decrease mitochondrial membrane potential (Moran *et al.*, 2012). Reciprocally, AD transmitochondrial cybrids, cell depleted endogenous mtDNA repopulated with mtDNA from AD patients, showed overproduction of A β although no causative mtDNA mutation has been discovered in AD patients (Mancuso *et al.*, 2009). Therefore, A β , neurofibrillary tangles, and mitochondrial deficit may synergistically initiate the AD pathophysiology and promote AD progression in a vicious cycle.

Considering AMPK as a master energy sensor and regulator, functional defects in AMPK signaling might be potentially involved in the deficiencies of energy metabolism in the AD brains. Consistently, increased AMPK phosphorylation has been found in the brains of AD patients and AD mouse models (Ma *et al.*, 2014; Mairret-Coello *et al.*, 2013; Vingtdeux *et al.*, 2011). There is evidence that AMPK regulates tau phosphorylation at numerous sites (Thornton *et al.*, 2011). In the normal brains, phosphorylated AMPK is predominately localized in the nuclei of neurons. On the other hand, cytoplasmic accumulation of activated AMPK have been found in neurons with hyperphosphorylated tau in AD brains, indicating that the cytoplasmic translocation and activation of AMPK may precede the tau hyperphosphorylation and tangle formation (Vingtdeux *et al.*, 2011). Paradoxically, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), an AMPK activator, has been shown to inhibit tau phosphorylation. In addition, it has been suggested that leptin decreased tau phosphorylation through AMPK activation in primary neurons (Greco *et al.*, 2009a; Greco *et al.*, 2009b). Similarly, the paradoxical action of AMPK in amyloidogenesis has also been indicated. A β 42 oligomers have been shown to activate AMPK (Mairret-Coello *et al.*, 2013). On the other hand, studies have demonstrated that AMPK can inhibit amyloidogenesis (Salminen *et al.*, 2011). Genetic and pharmacological activation of AMPK decreased A β accumulation both *in vitro* and *in vivo* (Vingtdeux *et al.*, 2010; Won *et al.*, 2010). Taken together, the effect of AMPK on tauopathy and amyloidogenesis may be context-dependent. While amyloid β can activate AMPK which further directly phosphorylate tau protein under certain conditions, activation of AMPK can inhibit amyloidogenesis and tau aggregation in other instances (Salminen *et al.*, 2011).

Metabolic Alteration in PD

PD is the second most common neurodegenerative disorder featured pathologically by the progressive death of dopaminergic neurons in the substantia nigra. The typical symptoms of PD include slowness of movements (bradykinesia), muscle stiffness (rigidity), tremor, and balance disturbance. Etiopathologically, PD is due to the significant loss of dopaminergic neurons in the substantia nigra and the subsequent dopamine depletion at the striatum. To date, there are only symptomatic treatments available for PD patients, particularly at the early stages. No therapy has been discovered that can cure or halt the progression of PD.

Dysregulation of glucose metabolism has been found as an early event in sporadic PD patients. FDG-PET studies have indicated significant reduction in glucose metabolism in PD brains (Borghammer, 2012; Borghammer *et al.*, 2010; Borghammer *et al.*, 2009; Dunn *et al.*, 2014). Interestingly, glucose hypometabolism was found extensively in cerebral cortex in PD patients with and without dementia (Edison *et al.*, 2013).

The pathophysiology underlying the degeneration of dopaminergic neurons at the substantia nigra is still unclear. Mitochondrial dysfunction and oxidative stress have been consistently observed in PD brains. There is increasing pharmacological and genetic evidence sustain a link between PD and mitochondrial respiratory chain dysfunction, particular a deficit in mitochondrial ETC complex I (Franco-Iborra *et al.*, 2015). Accidental exposure to 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), a mitochondrial complex I inhibitor, has been known to result in acute and irreversible PD syndrome (Calne and Langston, 1983; Langston and Ballard, 1983). Later on, mitochondrial complex I inhibition has been identified in the brains of sporadic PD patients (Schapira *et al.*, 1990). In addition, chronic systemic inhibition of mitochondrial ETC complex I by pesticide rotenone has been found to link to sporadic PD (Betarbet *et al.*, 2000). Interestingly, complex I deficiency has been found not only in the postmortem substantia nigra but also in cerebral cortex (Schapira *et al.*, 1990), which is consistent to the cortical glucose hypometabolism observed in PD patients. Indeed, the pathology of PD has been found to involve several brain regions other than the SNc and many neurotransmitters other than dopamine (Lang and Obeso, 2004a, b). PD models using MPTP and rotenone have now been used extensively in PD research (Beal, 2010).

Although majority of PD cases are sporadic, mutations of many genes have been found to be related directly or indirectly to mitochondrial dysfunction identified in familial PD. Parkin and PINK1 mutations have been identified in autosomal recessive Parkinsonism (Pickrell and Youle, 2015). The functions of PINK1 and Parkin have been indicated in the maintenance of healthy mitochondria through regulating mitochondrial dynamics and autophagy that eliminate dysfunctional mitochondria (Moran *et al.*, 2012). Recently, the DJ-1 protein, an antioxidant and transcriptional modulator, has been implicated to work with PINKs and Parkin to regulate mitochondrial function (Irrcher *et al.*, 2010).

The deficiencies of energy metabolism in PD pathophysiology might be associated with potential functional defects in AMPK signaling. Activation of AMPK signaling has been demonstrated in PD models using MPTP, MPP⁺, and 6-OHDA, both *in vitro* and *in vivo* (Choi *et al.*, 2010; Kim *et al.*, 2013). In addition, inhibition of AMPK by compound C

resulted in MPP⁺ induced cell death (Choi *et al.*, 2010). On the other hand, both pharmacological and genetic activation of AMPK have been shown to provide protective action in PD models (Choi *et al.*, 2010; Ng *et al.*, 2012).

Metabolic Alteration in HD and FRDA

Beside AD and PD, dysfunctions of mitochondria and key energy metabolism signaling have been observed in other neurodegenerative diseases. HD is known as the result of mutant Huntingtin gene and featured by the preferential loss of medium spiny GABAergic neurons in the striatum (Damiano *et al.*, 2010). There is increasing evidence indicated that mutant huntingtin binds directly to mitochondria and alter mitochondrial functions (Choo *et al.*, 2004; Orr *et al.*, 2008). Reduction of mitochondrial ETC complex II, III, and IV were found at the caudate putamen in HD patients (Damiano *et al.*, 2010). In addition, hyper-activation of AMPK has been found in the brains of HD patients and mice HD models (Ju *et al.*, 2011). Consistently, AICAR, an AMPK activator, induced neuronal death and decreased the lifespan of HD mice (Ju *et al.*, 2011).

Friedreich's ataxia (FRDA) is the most common early-onset inherited ataxia due to the frataxin deficiency. The GAA trinucleotide expansion of the frataxin gene (FXN) results in defective frataxin transcription and reduces the amount of frataxin expression (Gonzalez-Cabo and Palau, 2013; Kaplan, 1999). Frataxin is known to be associated with iron metabolism and play an important role in heme biogenesis and the formation of iron-sulfur clusters (Gonzalez-Cabo *et al.*, 2005; Karthikeyan *et al.*, 2003). Deficiency of the iron-sulfur cluster-containing subunits of mitochondrial ETC complexes I, II, and III as well as aconitase have been identified in FRDA patients. In addition, severe deficit of mitochondrial oxidative phosphorylation and elevated intramitochondrial iron have been observed in FRDA patients. Consistently, reduction of maximum rate of mitochondrial ATP production was found in muscle of FRDA patients (Lodi *et al.*, 1999). Accordingly, mitochondrial respiration has been the main target for discovery novel therapy for FRDA.

Energy Metabolism and Cancers

Revisit Warburg's Effect

A century ago, in 1924, Otto Warburg postulated that cancers may be caused by the increased glycolysis and impaired respiration based on his observations that tumor tissue actively metabolizes glucose in oxygen-rich environment and produces excessive lactic acid while exhibiting a comparably low respiratory rate (Koppenol *et al.*, 2011; Warburg, 1956b). This unique cancer metabolism was later known as Warburg effect which was subsequently substantiated by many cancer researchers. The Warburg effect presented a paradox phenomenon that highly proliferating cancer cells in a great need for ATP use a ~18-fold lower efficient ATP production pathway. Without knowing the underlying mechanisms and significance of the Warburg effect, Warburg's hypothesis on the origin of cancers had limited impact in cancer research and the metabolic signature had not been recognized as a cancer hallmark even toward the end of last millennium (Hanahan and Weinberg, 2000).

In the beginning of the new millennium the Warburg effect re-catches the attention of cancer researchers. The development of biology and the accumulating knowledge in energy metabolism and genetics have enabled us to further elaborate the cellular and molecular mechanisms underlying metabolic switch of cancer cells. We now know that the lower efficiency of ATP production through aerobic glycolysis in cancer cells is compensated by up-regulating glucose transporters. In addition, highly proliferative cancer cells do not necessarily have defects in mitochondrial oxidative phosphorylation (DeBerardinis *et al.*, 2008a; Moreno-Sanchez *et al.*, 2007). Given that energy metabolism pathway can serve both bioenergetic and biosynthetic function, the gaining function of aerobic glycolysis may mainly support the unusually high proliferation and growth rate of cancer cells (DeBerardinis, 2008; DeBerardinis *et al.*, 2008a; Jones and Thompson, 2009; Vander Heiden *et al.*, 2009).

The insensitivity to antigrowth signals and self-sufficiency in growth signals, two of the cancer hallmarks (Hanahan and Weinberg, 2000), render the cancer cells not only high proliferation rate but also huge demands of protein, lipids, and nucleic acids for self-replication. To meet the challenge, cancer cells have to gain ability to capture sufficient nutrients and establish appropriate metabolism to produce macromolecules to build up the cancer biomass. Lipid and nucleotide biosynthesis use glucose as a carbon source, consume TCA cycle intermediates, and requires NADPH as reductive power (Deberardinis *et al.*, 2008b). Cancer cells redirect glucose metabolism away from TCA cycle and mitochondrial oxidative phosphorylation to generate acetyl CoA through pyruvate-citrate shuttle for fatty acid synthesis and protein acetylation (Kamphorst *et al.*, 2013; Metallo *et al.*, 2012). In addition, cancer cells divert carbon from glycolysis into pentose phosphate pathway to generate ribose-5-phosphate for nucleotide biosynthesis (Deberardinis *et al.*, 2008b). Thus, the tradeoff between the efficient energy production through mitochondrial oxidative phosphorylation and aerobic glycolysis ultimately benefit the biomass increase of cancers (Deberardinis *et al.*, 2008b; Jones and Thompson, 2009). Now, switch of energy metabolism has been proving to be widespread in cancers and reprogramming of energy metabolism has been recognized as an emerging cancer hallmark (Hanahan and Weinberg, 2011).

Oncogenesis and Aerobic Glycolysis of Cancer Cells

No long after the Warburg proposed his theory of cancer origin, his contention of mitochondrial defects in cancers was negated by others (Chance and Castor, 1952; Weinhouse, 1956). Indeed, mitochondria oxidative phosphorylation capacity is not defective in most cancers (DeBerardinis *et al.*, 2008a; Moreno-Sanchez *et al.*, 2007; Ward and Thompson, 2012a). Consistently, we observed a dramatic increase in glycolytic flux which was accompanied with a less increase of oxidative phosphorylation in glioblastoma cells (Poteet *et al.*, 2013). Thus, cancer cells still produce significant fraction of ATP through mitochondrial oxidative phosphorylation and the Warburg effect in cancer is not due to mitochondrial damage. The technological improvements in genetics and molecular biology have enabled us to reconcile the mechanisms underlying the metabolic switch of cancer cells and re-evaluate the connection of Warburg effect and tumorigenesis.

Through the advance of molecular biology in the last 30 more years, we now understand that tumor suppressor genes and oncogenes encode many cellular signal pathways that not only sustain cancer proliferation but also reprogram cancer energy metabolism to meet the biosynthetic challenge associated with the cancer cells growth and proliferation (Hanahan and Weinberg, 2011; Vander Heiden *et al.*, 2009; Ward and Thompson, 2012a). Many signaling pathways including PI3K/Akt/mTORC1 and HIF are involved in cancer metabolic reprogramming for cellular biosynthesis (Jones and Thompson, 2009; Ward and Thompson, 2012a, b). Furthermore, oncogenes and tumor suppressors have been found to be critical components of these metabolic signaling networks. PI3K/Akt activation is tightly controlled by PTEN, a key tumor suppressor. PTEN loss and/or PI3K mutations compose the most common genotype in human cancers that lead to biosynthetic reprogramming (Jones and Thompson, 2009). Many oncogenes such as H-Ras, c-Myc, and src are known to enhance anabolic metabolism in cancer cells (Dang, 2011; Jones and Thompson, 2009). Taken together, mutations of oncogenes, loss of tumor suppressor genes, and many aberrant signaling are involved in the metabolic reprogramming for cancers to arise and proliferate. In addition, the relationship between cellular signaling and metabolism could be bidirectional (Metallo and Vander Heiden, 2010; Ward and Thompson, 2012b). The feedback control of cellular metabolites on biosynthetic activities has been found at the level of both posttranslational modification and gene expression (Metallo and Vander Heiden, 2010; Ward and Thompson, 2012b).

MB, New Application for a Century Old Drug

A Century Old Drug with Broad Medical Application Against an Ever-expanding Spectrum of Diseases—In the late 1800s, the prospering textile industry dramatically increased the dye demands and brought rapid development of the synthetic dye research (Oz *et al.*, 2011). In 1876, methylene blue (MB), an aniline-based dye, was synthesized for cotton staining by Heinrich Caro (Schirmer *et al.*, 2011). Although MB failed to meet the standards of the textile industry, its biological application was soon discovered. Given the ever-expanding drugs derived from MB structure for over 100 years and its broad medical applications for the treatment of an ever-expanding spectrum of diseases, MB is probably by far the most important pharmaceutical lead structure (Ohlow and Moosmann, 2011).

MB is the first lead chemical structure of phenothiazine and its derivatives. In 1883, German chemist Heinrich August Bernthsen synthesized phenothiazine. In 1891, the staining of MB for plasmodia and its effect on malaria were developed by Paul Guttman and Paul Ehrlich which provided the foundation of modern chemotherapy (Kaufmann, 2008; Parascandola, 1981). In 1890s, MB was administered in psychiatric patients to monitor their compliance, which led to the discovery its antipsychotic effects and the later discovery of chlorpromazine, the first synthetic antipsychotic drug, in 1951 (Ohlow and Moosmann, 2011; Schirmer *et al.*, 2011). In the early 20th century, the antifungal, insecticidal, and anthelmintic activities of phenothiazine, a methylene blue derivative, were discovered (Ohlow and Moosmann, 2011). Since 1930s, intravenous MB has been used as the first-line antidotal agent for methemoglobinemia (Wendel, 1939) and cyanide poisoning (Alston, 2014). Since 1990s, MB has been introduced for the treatment of ifosfamide-induced

encephalopathy (Kupfer *et al.*, 1994; Pelgrims *et al.*, 2000; Zulian *et al.*, 1995). In addition, MB has also been used as a tracer for cancer diagnosis and as a photosensitizer for cancer treatment (Chen *et al.*, 2007).

Since 1940s, phenothiazine has been used as an antioxidant for preventing oxidative changes in polyethylene oils (Murphy *et al.*, 1950). The neuroprotective effect of phenothiazine and some of its derivatives have been explored since 1990s (Yu *et al.*, 1992a; Yu *et al.*, 1992b). The protective action of phenothiazine derivatives have been demonstrated in models of ischemic stroke (Yu *et al.*, 1992a) and PD (Mocko *et al.*, 2010). On the other hand, the protective action of MB was discovered more recently.

MB has been known as an electron carrier for more than half century, manifested by its action to accelerate cytochrome c reduction in isolated mitochondria (Weinstein *et al.*, 1964). There is increasing evidence indicated that MB may exist neuroprotective action through mechanisms different from other phenothiazine derivatives (Poteet *et al.*, 2012). Our recent structure-activity relationship study indicated that the cytoprotective effects of MB are distinctive to other phenothiazine derivatives in its action as an alternative mitochondrial electron carrier and as a re-generable anti-oxidant in the mitochondria (Poteet *et al.*, 2012).

5.2. MB as an Alternative Mitochondrial Electron Transfer Carrier

MB has very unique redox property that exists in equilibrium between oxidized state in dark blue (MB) and colorless reduced state (leucomethylene blue), making it both prooxidant and antioxidant under different conditions. The unique redox property of MB might directly contribute to its therapeutic effect on several disorders such as ifosfamide-induced encephalopathy. Although the exact pathophysiological mechanisms underlying the ifosfamide-induced encephalopathy are not known, there is evidence indicated that it might due to the inhibition of mitochondrial respiratory chain by ifosfamide metabolite, chloroethylamine (Kupfer *et al.*, 1996; Pelgrims *et al.*, 2000). For the treatment of methemoglobinemia, MB is reduced to leucomethylene blue by erythrocyte's methemoglobin reductase, thus, reducing methemoglobin to hemoglobin (Clifton and Leikin, 2003).

The capability of MB as an electron carrier has long been recognized. MB directly accepts electrons from NADH, NADPH, and FADH₂ (Atamna *et al.*, 2008; Buchholz *et al.*, 2008; Dixon, 1971; May *et al.*, 2004; Wen *et al.*, 2011b). MB is able to mediate the electron flow from certain enzymes to cytochrome c under both anaerobic and aerobic conditions (McCord and Fridovich, 1970). Thus, MB might be able to act as an alternative electron transfer carrier that replaces the damaged mitochondrial respiratory complexes. This notion is supported by the fact that MB attenuates rotenone-induced neuropathy in the retina and striatum (Rojas *et al.*, 2009a; Rojas *et al.*, 2009b; Zhang *et al.*, 2006). Consistently, using isolated mitochondria, MB has been demonstrated to enhance mitochondrial ETC complex I, I–III activities, but not complex II–III activities (Atamna *et al.*, 2008; Poteet *et al.*, 2012; Wen *et al.*, 2011a). More importantly, the MB-induced increase of mitochondrial complex I and I–III activities is insensitive to complex I and III inhibition (Atamna *et al.*, 2008; Poteet *et al.*, 2012; Wen *et al.*, 2011b). Furthermore, reduced form MB (MBH₂) has been found to be able to deliver the electrons to cytochrome c in the presence of oxygen in mitochondria

(Atamna *et al.*, 2008; Wen *et al.*, 2011b). Taken together, there is growing consensus that MB could function as a mitochondrial alternative electron carrier. Upon its redox cycle (MB- MBH₂-MB), electrons from NADH are delivered to cytochrome c in an alternate route despite the inhibition of complex I and III (Atamna *et al.*, 2008; Bruchey and Gonzalez-Lima, 2008; Wen *et al.*, 2011b) (Poteet *et al.*, 2012; Rojas *et al.*, 2012) (Figure 2).

Cytochrome c oxidase, the mitochondrial ETC complex IV, is the terminal enzyme of mitochondrial respiration that reduces oxygen to water coupled pumping proton across the inner mitochondrial membrane (Wallace, 2005). MB has been demonstrated to enhance cytochrome c oxidase activity both *in vitro* and *in vivo* (Atamna *et al.*, 2008; Callaway *et al.*, 2004; Gonzalez-Lima and Bruchey, 2004; Poteet *et al.*, 2012; Rojas *et al.*, 2012). It was proposed that a direct interaction between MB and cytochrome c oxidase may underlie the memory-enhancing action of MB (Rojas *et al.*, 2012). On the other hand, the MB-induced increase of cytochrome c oxidase activity might be secondary, at least in part, to the action of MB as an alternative electron carrier that enhances electron transport and increase cytochrome c reduction (Atamna *et al.*, 2008; Poteet *et al.*, 2012). Our recent study demonstrated that 2-chlorophenothiazine, a phenothiazine derivative, has similar neuroprotective action against glutamate-induced oxidative stress in HT-22 cells without increase of oxygen consumption rate. Interestingly, 2-chlorophenothiazine has no effect on cytochrome c oxidase expression and activity suggesting that the action of MB as an alternative mitochondrial electron transport might directly contribute to its effect on cytochrome c oxidase (Poteet *et al.*, 2012).

The action of MB on mitochondrial electron transport is consistent to its action on mitochondrial oxidative phosphorylation. The effect of MB on the oxygen consumption was demonstrated back to 1930s in which MB was found to increase oxygen consumption of normal tissues having aerobic glycolysis and of tumors (Barron, 1930). Using fiber optic oxygen sensor, Riha *et al.* have found that leucomethylene blue decreased oxygen concentration in rat brain homogenates *in vitro* (Riha *et al.*, 2005). In addition, oxygen concentration was found to be significantly lower in brain homogenates obtained from rats at 24 hours after MB treatment *in vivo* (Riha *et al.*, 2005). Using Clark oxygen electrode, Atamna *et al.* have discovered that MB increased oxygen consumption in fibroblast cells (Atamna *et al.*, 2008). Using Seahorse extracellular flux analyzer and ruthenium fluorescence-lifetime imaging microscopy, we have discovered that MB increased oxygen consumption rate and decreased extracellular acidification rate in both neuronal cells and astrocytes (Poteet *et al.*, 2012; Roy Choudhury *et al.*, 2015). Using multimetric neuroimaging and vascular blood oxygenation measurement, we further observed that acute treatment of MB increased cerebral metabolic rate of oxygen (CMRO₂) in rat brains (Lin *et al.*, 2012). The stimulating effect of MB on mitochondrial electron transport is in line with its action on glucose metabolism. In fibroblast cells, MB has been shown to stimulate 2-deoxyglucose uptake (Louters *et al.*, 2006; Roelofs *et al.*, 2006). Using MRI and PET, we demonstrated that acute treatment of MB significantly enhance glucose uptake and increase regional CBF in rats (Lin *et al.*, 2012).

Mitochondrial oxidative phosphorylation is the dominant metabolic pathway in the brain with tight coupling between brain energy supply and expenditure. Thus, MB, as a brain

metabolic and hemodynamic enhancer, may improve brain function in physiological condition. The effect of MB on nerve system was explored in late 1880s. Paul Ehrlich found that MB had a selectively affinity to living nerve tissue and was expected to be neurotropic, in Ehrlich's word (Parascandola, 1981). Indeed, pharmacokinetic study in rat has shown that MB can cross BBB and reach brain at concentrations 10 times higher than that in the circulation (Peter *et al.*, 2000). Since 1970s, MB has been found to improve various experimental memory tasks in rodents (see review (Rojas *et al.*, 2012)). In addition, the potential causative role of brain metabolic dysfunction in both aging and aging-related neurodegenerative disorders indicated that MB might be an ideal candidate for future investigations for the treatment of neurodegenerative diseases.

MB is different from the traditional antioxidants which have failed in all clinical trials for the treatment of neurological disorders. First, the action of MB provides an alternative mitochondrial electron transport route bypass complex I and III inhibition not only preserves mitochondrial oxidative phosphorylation but also attenuates the production of superoxide and reactive oxygen species (Atamna *et al.*, 2008; Poteet *et al.*, 2012; Wen *et al.*, 2011b). Secondly, the distinct redox property enables MB as a regenerable anti-oxidant in mitochondria that distinct from the traditional free radical scavengers (Poteet *et al.*, 2012). In addition, the alternative mitochondrial electron transfer action might not only enable MB to enhance brain metabolism but also to switch the Warburg's effect, hence, inhibit cancer proliferation.

MB for the Treatment of Neurodegenerative Diseases

MB and AD—Despite decades of drug development for AD, only memantine and 4 cholinesterase inhibitors have been approved for clinical application on AD treatment. However, these approved drugs have shown very limited effect and there is controversy whether they are therapeutically useful (Schneider *et al.*, 2014). MB has recently attracted increasing scientific attention after more than one century of its synthesis. At the 2008 annual meeting of the Alzheimer's Association, promising results were emerged from a clinical Phase II trial testing MB treatment on the cognitive dysfunction in 332 probable AD patients. Over the course of a year, MB treatment significantly improved cognitive function with an 81% reduction in the rate of cognitive decline as compared with those receiving placebo (Gura, 2008; Oz *et al.*, 2009). Concerns have been raised regarding the drug formulation, the potential underlying tau-dissolving mechanism, the problem with blinding due to that MB turns the urine in blue, and lack of peer-review publications derived from the clinical trial (Gravitz, 2011; Oz *et al.*, 2009). Nonetheless, the MB clinical trial triggered global interests in exploring the effect of MB on AD in both experimental models as well as clinical studies. MB is currently in on global phase III trials for AD and frontotemporal dementia.

The Phase II clinical trial of MB treatment in AD was based on the early identified tau-dissolving properties of MB *in vitro* without any publication testing of MB in preclinical animal models. MB, at a higher concentration that may not be able to achieve clinically, blocks tau-tau binding interaction through the repeat domain (Wischnik *et al.*, 1996). Similarly, MB, at the concentration of 50 μ M, has been demonstrated to reduce tau

phosphorylation in organotypic slices derived from transgenic AD mice (Jinwal *et al.*, 2009). Late on, chronic MB administration has been shown to decrease amyloid deposits and neurofibrillary tangles *in vivo* (Hochgrafe *et al.*, 2015; Hosokawa *et al.*, 2012; Melis *et al.*, 2015). Consistently, we found that 8 months feeding of MB containing diet significantly reduced A β plaque and improved water maze and bridge walking performance in 5 X FAD mice (Figure 3).

There is increasing evidence that the beneficial effects of MB on cognition decline in AD might be independent of the action on tauopathy. In fact, MB enhances memory function in normal rodents potentially through neurometabolic mechanisms (Rojas *et al.*, 2012). The anti-tau aggregation action of MB requires higher concentrations that may not be able to reach *in vivo*. MB failed to reduce tau phosphorylation and aggregation in zebrafish expressing mutant human tau (van Bebber *et al.*, 2010). Chronic MB treatment decreases brain A β levels and improves learning and memory in an AD mouse model (3 x Tg-AD) without affecting tau pathology (Medina *et al.*, 2011). These studies argue for additional mechanisms underlying the beneficial effect of MB beyond its inhibitory action on the tau aggregation and A β plaques. Interestingly, the reduction of tauopathy and amyloid plaque upon chronic MB treatment in AD mice were found to be accompanied by an upregulation of autophagy (Hochgrafe *et al.*, 2015). It has been indicated that elevated autophagic activity prevented protein aggregates in neurons, including tauopathy (Lee *et al.*, 2013). Indeed, MB has been demonstrated to induce autophagy and attenuate tauopathy through inhibition of mTOR signaling both *in vitro* and *in vivo* (Congdon *et al.*, 2012). AMPK, a master regulator of cellular energy homeostasis, plays an important role in autophagy (Hardie, 2011). MB increases mitochondrial respiration and enhances AMPK signaling which may further activate autophagy (Xie *et al.*, 2013). Taken together, all these studies demonstrated that MB, as an alternative electron carrier, maybe effective for AD therapy.

MB and PD—Levodopa, a dopamine precursor to restore intracerebral dopamine, remains the single most effective treatment for PD. However, levodopa does not affect the disease progression and dyskinesia complicates its treatment in most patients within 5 to 10 years after treatment initiation (Lang and Obeso, 2004a). No treatment so far has been developed that change the progressive course of PD.

There is substantial evidence that indicates the involvement of the dysfunction of mitochondrial ETC complexes and oxidative stress in the etiopathogenesis of PD (Abou-Sleiman *et al.*, 2006; Sayre *et al.*, 2008). Consistently, as discussed above, mitochondrial complex I inhibitors such as rotenone and MPTP have been indicated to be involved in the etiology of sporadic PD. The unique action of MB as an alternative mitochondrial electron carrier bypasses the ETC complex I and III, hence, renders it protection against rotenone and MPTP toxicity. In isolated mitochondria, MB preserves respiration despite mitochondrial complex I and III inhibition by rotenone and antimycin, respectively (Poteet *et al.*, 2012; Wen *et al.*, 2011b). In cell cultures, MB protects neuronal HT-22 cells and primary rat retinal ganglion cells against rotenone insult (Daudt *et al.*, 2012; Poteet *et al.*, 2012; Wen *et al.*, 2011b). In rodents, MB has been shown to prevent neurodegeneration induced by intravitreal or intracerebral administration of rotenone in retina and striatum, respectively (Rojas *et al.*, 2009a; Rojas *et al.*, 2009b; Zhang *et al.*, 2006). Experimental animal models of PD using

MPTP and rotenone are valuable tools for the development of novel therapeutics. Systemic administration of MPTP or rotenone induces dopaminergic neurodegeneration in *Caenorhabditis elegans* (Braungart *et al.*, 2004), mouse, rat, and primates (Beal, 2001). MB has been shown to be protective in *caenorhabditis elegans* models of PD (Mocko *et al.*, 2010). Importantly, our study demonstrated that MB attenuated rotenone-induced loss of nigral dopaminergic neurons and resulted in a better locomotor function in a rat PD model (Wen *et al.*, 2011b). On the other hand, dopamine agonists only rescue locomotor deficit in PD. Therefore, MB might be a superior therapy compared to levodopa that not only ameliorate symptoms but also prevent the dopaminergic neurons degeneration, thus, slow down the disease progression.

MB and HD—HD is a devastating neurodegenerative disease manifested with a typical sporadic, rapid, involuntary limb movement, limbs' stiffness, psychiatric disturbance, and cognitive impairment (Kumar *et al.*, 2015). The current therapeutics in HD is mainly designed to improve the life quality of the patients by symptom controlling and no disease-modifying treatment is available. It has been indicated that mutant huntingtin impairs mitochondrial respiration, leading to reduction of ATP production, thus, promoting oxidative stress, excitotoxicity, and apoptosis (Kumar *et al.*, 2015). Consequently, mitochondrial respiration represents a potential therapeutic target for HD. Co-enzyme Q10, a mitochondrial ETC complex component, has been shown to enhance mitochondrial activity, delay motor deficits development, decrease weight loss, prevent cerebral atrophy, and extend survival in HD mouse model (Ferrante *et al.*, 2002). Now Co-enzyme Q10 is in phase II clinical trial for HD (Kumar *et al.*, 2015).

The unique function of MB as an alternative mitochondrial electron carrier that enhances mitochondrial respiration may render its therapeutic effect in HD. MB treatment has been found to reduce behavioral phenotypes and delay disease progression in both *Drosophila* and R6/2 mouse models of HD (Sontag *et al.*, 2012). The aggregation of misfolded Huntingtin has been considered as a main cause of HD pathogenesis (Kim and Kim, 2014). And the protective action of MB on HD was proposed to be secondary to its action on the aggregation of huntingtin variant (Sontag *et al.*, 2012). MB indeed prevented aggregation and deposition of poly-Q-expanded huntingtin in zebrafish at a dose of 10 and 100 μM . However, no protective effect of MB was observed even it almost completely reduced huntingtin aggregates at these concentrations (van Bebber *et al.*, 2010). Similarly, MB blocked aggregation of mutant huntingtin in primary neuron culture at the concentrations of 1, 10, and 100 μM upon acute treatment (Sontag *et al.*, 2012). On the other hand, it took up to 10 day for low dose of MB (100 nM) treatment to decrease oligomer formation of mutant huntingtin. Interestingly, 100 nM MB was found to increase the survival of mutant huntingtin expressing primary neurons by 25% over a time period of 3 days (van Bebber *et al.*, 2010). Therefore, the anti-aggregation effect of MB is likely secondary to the protective action, potentially through its function as an alternative mitochondrial electron carrier.

6.4 MB and FRAD

FRAD is debilitating progressive neurodegenerative disease featured by the progressive ataxia and dysarthria due to the degeneration of the sensory neurons at the dorsal root

ganglia, the corticospinal and spinocerebellar tracts of the spinal cord, and the dentate nucleus. FRAD also is associated other non-neurological features as muscle weakness, diabetes, cardiomyopathy and ultimate cardiac failure, which is the most common cause of death in FRAD. The current therapy is limited to the standard treatment for heart failure, arrhythmias, and diabetes mellitus (Pandolfo, 2009). Over the last decade, co-enzyme Q10 and its analogue, idebenone, have been extensively studied for FRAD treatment. So far, the efficacy of co-enzyme Q10 and idebenone on FRAD remains elusive (Parkinson *et al.*, 2013). Co-enzyme Q10 is an essential cofactor of the mitochondrial ETC and its deficiency has been implicated in several neurodegenerative disorders including FRAD (Molyneux *et al.*, 2008). Co-enzyme Q10 transfers electrons from complex I or II to complex III. In addition, the reduced form of co-enzyme Q10 is an antioxidant (Sohal, 2004). As an endogenous mitochondrial ETC component, the effect of co-enzyme Q10 on mitochondrial respiration is greatly limited by the activities of complex II and III. Indeed, co-enzyme Q10 had no effect on basal oxygen consumption rate in isolated mitochondria and neuroblastoma cells (Fink *et al.*, 2009; Sadli *et al.*, 2013).

MB differs from co-enzyme Q10 in its unique action that bypassing mitochondrial ETC complex I to III inhibition and increasing mitochondrial respiration. MB has been shown to be protective against L-buthionine (S,R)-sulfoximine (BSO)-induced oxidative stress in FRAD fibroblast cultures (Richardson *et al.*, 2013). A very recent study demonstrated that MB rescues heart defect in a frataxin-depleted drosophila model of FRDA. Further analysis of MB derivatives indicated that only compounds with electron carrier properties, but not idebenone, were able to prevent the heart dysfunction (Tricoire *et al.*, 2014). Therefore, the alternative mitochondrial electron transferring mechanism may render MB a better therapeutic strategy for the treatment of FRAD.

MB for Cancer Treatment

Targeting Metabolic Signaling and Enzymes for Cancer Therapy

The renewed interest in Warburg effect makes metabolism an emerging hot target for anti-cancer drug discovery (Vander Heiden *et al.*, 2009). Oncogenes and tumor suppressor genes orchestrate many cellular signal pathways that are involved in the metabolic reprogramming of cancer cells. Therapeutic targets on the identified metabolic signaling pathways for anticancer therapies have been exploring. The PI3K/Akt/mTOR signaling has long been recognized as a key pathway downstream of growth factor receptor tyrosine kinase that is involved in cancer cells survival, growth, and metabolism (Cantley, 2002; Shaw and Cantley, 2006). Correspondingly, inhibitors of PI3K/Akt/mTOR signaling pathway have been extensively exploited for cancer therapies (Engelman, 2009). There are concerns that cancer cells may take advantage of signaling redundancy and cross-talk to tackle the anabolic challenge (Logue and Morrison, 2012). Indeed, despite the advance of our understanding of cancer metabolism, our knowledge in the metabolic signaling regulation is incomplete. Further studies are warranted for determine the high plasticity of the metabolic network in cancer. Nonetheless, the complexity nature of the signaling network argues against targeting a single signal component for cancer therapy.

The signature catabolic metabolism and metabolic dependencies of cancer cells have raised interest in targeting metabolic enzymes for cancer treatments (Vander Heiden, 2011). Many inhibitors against key metabolic enzymes have been developed as potential anticancer therapies. The targeted enzymes are distributed across the wide spectrum of glucose uptake, pentose pathway, glycolysis, TCA cycle, and lipid synthesis including GLUTs, hexokinase, phosphofructose kinase 2, pyruvate kinase M2 (PKM2), pyruvate dehydrogenase, pyruvate dehydrogenase kinase, lactate dehydrogenase, MCT, glutaminase, ATP citrate lyase, monoacylglycerol lipase, and fatty acid synthase (Jones and Schulze, 2012; Vander Heiden, 2011). It is uncertain whether targeting these enzymes provides a sufficient therapeutic window for cancer treatment (Vander Heiden, 2011). And the clinical efficacy of these inhibitors are to be further determined. There are two major obstacles that challenge the anticancer drug development through targeting metabolic enzymes. First, the redundancy of synthetic pathway may impair the efficacy of a drug inhibiting a specific metabolic enzyme for cancer treatment. Luckily, there is increasing evidence that genetic mutations render cancer cells specific metabolic pathways with less redundancy (Vander Heiden, 2011). Nonetheless, inter-tumor genomic instability leads to an increased mutation rate of cancer cells that may further develop drug resistance (Burrell *et al.*, 2013). Second, metabolism, including both catabolic and anabolic pathways, is a key function of normal cells. Identification of specific metabolic enzymes in cancer cells is crucial for drug development that has minimal side effects on normal tissue. Thus, the unacceptable side effect of inhibiting metabolic enzymes in normal proliferating cells will likely be another major challenge for the cancer drug development (Vander Heiden, 2011).

MB Functions as an Alternative Mitochondrial Electron Transfer Carrier for Cancer Therapy

The effect of MB on cell metabolism was studied as early as 1920s. MB was found to increase sugar degradation and diminish lactic acid formation in blood cells, indicating that MB treatment shifts cellular carbohydrate metabolism from anaerobic toward oxidative path (Harrop and Barron, 1928). MB was further found to increase oxygen consumption rate of 19.2 to 116% in human and animal tumors (Barron, 1930). However, without understanding the mechanisms and significance of the Warburg effect, the dramatic effect of MB on cancer metabolism has not caught much attention of cancer researchers.

MB has been found to be selectively accumulated in certain tumors *in vivo* (Fukui *et al.*, 1983; Gill *et al.*, 1984; Gill and Strauss, 1984). Interestingly, MB also has high affinity to brain and nerve tissues when administered systemically or supravivally (Kristiansen, 1989; Oz *et al.*, 2011; Parascandola, 1981; Peter *et al.*, 2000). Although the mechanism underlying the selectivity of MB to neuronal and cancer tissues is not clear, the high metabolic rate of both tissues indicates that cellular metabolism may play a role in the accumulation of MB in these tissues. Consistently, MB has been found to actively bind to mitochondria and enters the mitochondrial matrix in a manner enhanced by the mitochondrial membrane potential (Gabielli *et al.*, 2004). The high affinity of MB towards nerve and cancer tissues lead to its application for staining neuronal structures and cancer tissues in both clinical and histochemistry studies (Oz *et al.*, 2011). MB-assisted lymph node dissection has also been introduced in breast cancer and rectal cancer patients (Chen *et al.*, 2007; Markl *et al.*, 2007; Resch and Langner, 2013; Thevarajah *et al.*, 2005).

The electron transfer reaction has been indicated to involve in photosensitizer to initiate radical-induced damage in biomolecules (Cappugi *et al.*, 2001). The unique redox action of MB for reversible electron transfer may confer its action as a photosensitizer. Indeed, MB has been used as a photosensitizer for various cancers treatment, including basal cell carcinoma, melanoma, and Kaposi's sarcoma (Tardivo *et al.*, 2005).

Mitochondrial ETC sits at the very downstream of anabolic pathway which couples electron transferring with proton pumping cross inner mitochondrial membrane and provides driving force for ATP production. Targeting ETC complexes has been proposed as a possible strategy for cancer therapy (Rohlena *et al.*, 2011). Indeed, some anti-cancer drugs do inhibit components of ETC complexes. For example, tamoxifen, a first-line anti-breast cancer drug, has inhibitory action on mitochondrial complex I (He *et al.*, 2015). The role of mitochondrial ETC complex I inhibition in the anti-cancer effect of tamoxifen is till debatable. Nonetheless, the strategy of inhibiting mitochondrial ETC for cancer therapy may bear unacceptable side effect, given the indispensable role of mitochondrial ETC in ATP production in normal cells. On the other hand, our recent study indicated that enhancement of mitochondrial respiration through alternative mitochondrial electron transfer approach may reverse Warburg effect and, thus, inhibit cancer proliferation (Poteet *et al.*, 2013).

The Warburg effect characterizes the metabolic signature of cancer that cancer cells exhibit a predominate anabolism with a comparably low mitochondrial respiration rate. Interestingly, increase in oxidative phosphorylation has been shown to suppress cancer growth *in vitro* and *in vivo* (Schulz *et al.*, 2006). Our recent study provided the proof-of-concept that MB, as an alternative mitochondrial electron carrier, reverses the Warburg effect, attenuates anabolism, and inhibits glioblastoma cells proliferation (Poteet *et al.*, 2013). In glioblastoma cells, MB increase oxygen consumption rate, decrease lactic acid production and extracellular acidification rate, reduce NADPH, and inhibit proliferation (Poteet *et al.*, 2013). These results indicated that MB, as an alternative mitochondrial electron carrier, enhances mitochondrial electron transfer, increases oxidative phosphorylation, decreases glycolytic flux and metabolic intermediates, hence, exhausts the building brick for cancer cell proliferation. Consistently, MB arrests cancer cells at S phase corresponding to a reduction of cyclin A2, B1, and D1 expression (Poteet *et al.*, 2013). We further demonstrated that MB is capable of activating AMPK signal pathway, likely secondary to its bioenergetic action (Poteet *et al.*, 2013). Thus, the direct targeting mitochondria electron transfer may provide further advantage given the potential feedback of cellular metabolites on biosynthetic activities (Metallo and Vander Heiden, 2010; Ward and Thompson, 2012b).

The alternative mitochondrial electron transfer provides a novel mechanism for anti-cancer drug development with many advantage compared with other metabolism targeting strategies. First, targeting metabolic adaptations could be applied to many cancers given that catabolic metabolism as a signature of all cancers. Second, inhibiting cellular signaling and/or metabolic enzymes to attenuate cancer anabolism might not be effective due to the redundancy of metabolic signaling and anabolic pathways. On the other hand, mitochondrial ETC is indispensable for mitochondrial oxidative phosphorylation. Thus, the alternative mitochondrial electron transfer approach enhances mitochondrial respiration may provide a more effective way for cancer therapy. Third, the redundancy of metabolic signaling and

anabolic pathways make request targeting multiple metabolic signaling pathways and/or enzymes to increase cancer treatment efficacy, which will likely induce severe side effects as no metabolic signaling and enzyme are unique to cancer cells. The alternative mitochondrial electron transfer strategy differs from the traditional chemotherapeutic agents in that it is less cytotoxic. The increase of mitochondrial electron transfer may reciprocally decrease glycolysis and other anabolic pathways, switch the role of mitochondria from biosynthetic hub to bioenergetic hub, thus, inhibit cancer growth and proliferation. Thus, alternative mitochondrial electron transfer strategy might be a safer anti-cancer therapy approach with less serious side effects.

Conclusion

Neurodegenerative diseases and cancers are among the most leading causes of death and pose the worst threat to public health worldwide (Fitzmaurice *et al.*, 2015; Jin *et al.*, 2015). Due to the declining fertility and increasing life expectancies, the speed of global population aging will accelerate over the coming decades (Lutz *et al.*, 2008). Without a doubt the global burden of neurodegenerative disorders and cancers, both as major aging-related diseases, will continue to increase significantly over the next century. As reiterated in this review, neurodegenerative diseases and cancers may share common mechanistic foundations such as metabolic reprogramming. Accordingly, targeting energy metabolism may provide a one stone two birds strategy for the treatment of both neurodegenerative diseases and cancers. While enforcing brain bioenergetics may enhance brain function hence slow down or prevent the progression of neurodegenerative diseases, switching cancer metabolic phenotype from biosynthetic back to bioenergetic might exhaust building bricks for cancer biomass, thus, inhibit cancers proliferation (Figure 4). As recapitulated in the review, MB functions as an alternative mitochondrial electron transfer carrier and is effective for the treatment of both neurodegenerative diseases and glioblastoma in various experimental models, providing a proof-of-concept that alternative mitochondrial electron transfer might be a common novel therapeutic mechanism for cancers and many neurodegenerative disorders.

As a more than one century old drug, MB has many desirable properties for the treatment of neurodegenerative disorders and cancers, including its well-known pharmacokinetics, the ability to cross the BBB and accumulate in the brain with low toxicity, and the high affinity to both neuronal and cancer tissues (Kupfer *et al.*, 1994; Peter *et al.*, 2000). The effect of MB on neurodegenerative diseases and cancers require further investigations. However, MB is unlikely a magic bullet for all neurodegenerative diseases given the heterogeneous etiopathology of the neurodegenerative disorders. Indeed, MB treatment failed to confer neuroprotection in both SOD1 G93A and TDP-43 mouse models of amyotrophic lateral sclerosis (ALS) (Audet *et al.*, 2012; Loughheed and Turnbull, 2011). Similarly, alternative mitochondrial electron transfer strategy may also have its limitation for cancer treatment. AMPK can exert both anti- and pro-tumorigenic roles in cancer depending on context. Thus, AMPK activation, induced by MB, may provide a survival advantage to cancer cells during chemotherapy by providing them the flexibility to cope the metabolic stress (Faubert *et al.*, 2015).

Acknowledgments

This work was partly supported by National Institutes of Health grants R01NS054651 (SY), R01NS088596 (SY), American Heart Association grant SDG16960084 (RL).

Abbreviation list

MB	methylene blue
AD	Alzheimer's disease
PD	Parkinson's disease
ALS	amyotrophic lateral sclerosis
HD	Huntington's disease
CBF	cerebral blood flow
GLUT	glucose transporter
TCA	tricarboxylic acid
ETC	electron transfer chain
NADH	dihyronicotinamide adenine dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
MCT	monocarboxylate transporter
AMPK	AMP-activated protein kinase
ACC	Acetyl-CoA carboxylase
CaMKKβ	calcium/calmodulin-dependent protein kinase kinase β
APOE	Apolipoprotein E
MPTP	1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine
FRDA	Friedreich's ataxia
FXN	frataxin
mTORC1	mammalian target of rapamycin complex I

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Highlights

- Reprogramming energetic metabolism is a common feature for neurodegenerative diseases and cancers.
- Methylene blue functions as an alternative mitochondrial electron transfer carrier, enhancing bioenergetics and inhibiting biosynthetics.
- Methylene blue provides protective effect in rodent models of Parkinson disease, Alzheimer's disease, Huntington's disease, and Friedreich's ataxia.
- Methylene blue reverses Warburg's effect and inhibits cancers proliferation.
- Alternative mitochondrial electron transfer may provide a common novel therapeutic mechanism for cancers and neurodegenerative diseases.

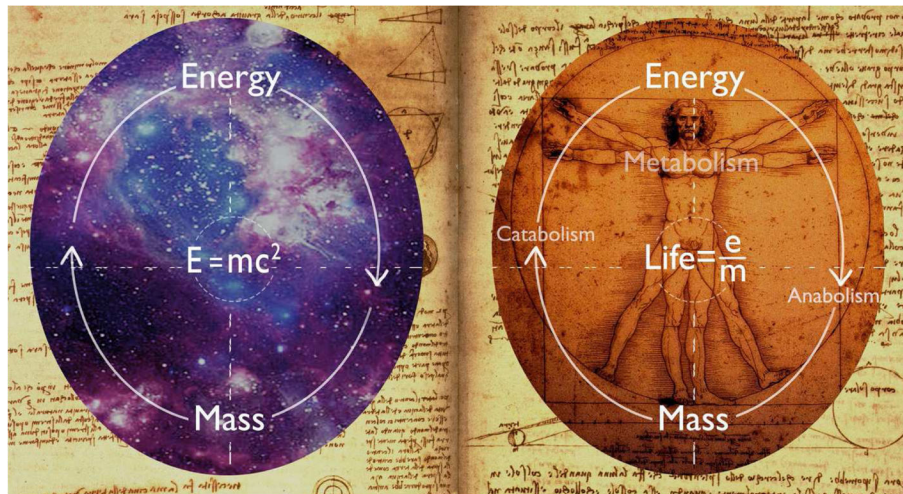


Figure 1.

Energy and mass: from Einstein equation to energy metabolism. Einstein's famous equation reveals the interchangeable relationship of mass and energy. The significance of Einstein's formula is even beyond physics. In biology, conversion between mass and energy are fundamental processes defined as metabolism. Through anabolism, complex compounds are biosynthesized from simpler molecules with the energy expense provided by ATP hydrolysis. Through catabolism, complex nutrients are broken down to simpler oxidized compounds with an energy releasing process coupled to ATP production. Life is the interplay between energy and structure. As the metabolism goes on, the life goes on. E: energy. M: mass. C: speed of light.

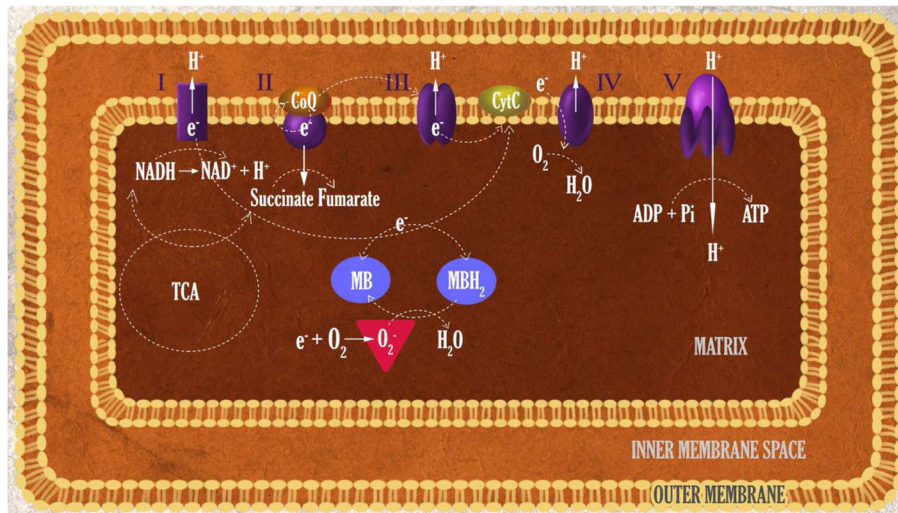


Figure 2. Function of methylene blue (MB) as an alternative mitochondrial electron transfer carrier and regenerable antioxidant. MB accepts electron from NADH in the presence of complex I. Upon the redox cycle (MB- MBH₂-MB), electrons are delivered to cytochrome c in an alternate route despite the inhibition of complex I and III. The distinct redox property enables MB as a regenerable anti-oxidant in mitochondria that distinct from the traditional free radical scavenges.

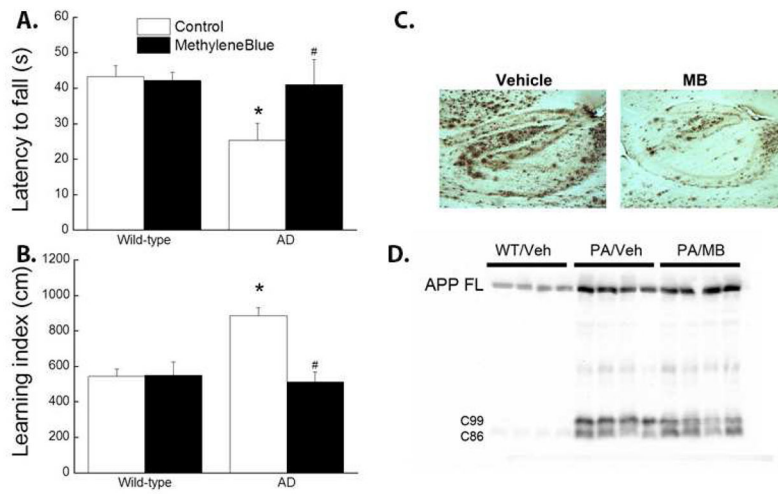


Figure 3. Methylene blue feeding improved water-maze and bridge walking performance in 5 X FAD mice. Wild type and 5 X FAD female mice were switched to diets containing MB or vehicle at the age of 8 weeks and continually fed for 8 months. At the age of 10 months, the mice were subjected to batteries of behavioral testing of water-maze and bridge walking as described previously (Shetty *et al.*, 2014). MB diet group had significantly reduced average latency to fall in the bridge walking test as compared to control diet group (A). In water-maze test, MB feeding significantly improved learning index (B). All mice were sacrificed and the effect of MB on brain A β plaques were determined. MB feeding decreases A β plaque evidenced by 6E10 immunohistochemistry (C) and Western blot analysis (D). # p<0.05 vs Control AD. * p<0.05 vs Control wild-type.

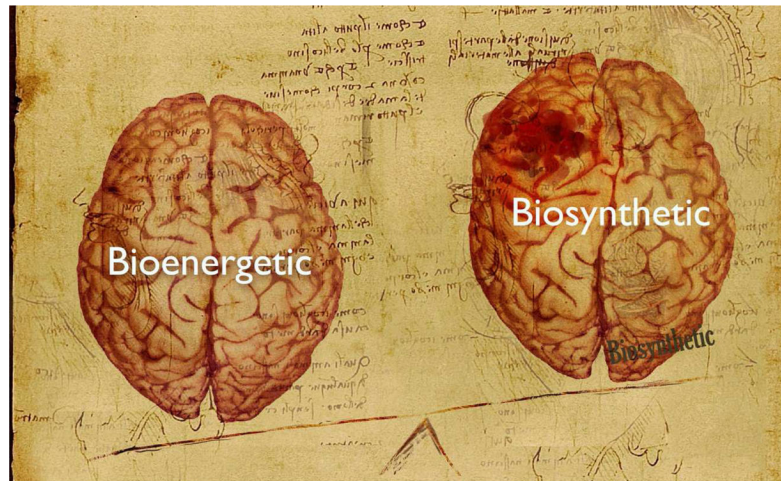


Figure 4. Reprogramming energetic metabolism for the treatment of neurodegenerative diseases and brain tumor. Metabolic reprogramming may serve as a common mechanistic foundation for neurodegenerative diseases and cancers. Accordingly, targeting energy metabolism may provide a one stone two birds strategy for the treatment both neurodegenerative diseases and cancers. While enforcing brain bioenergetics may enhance brain function hence slow down or prevent the progression of neurodegenerative diseases, switching cancer metabolic phenotype from biosynthetic back to bioenergetic might exhaust building brick for cancer biomass, thus, inhibit cancers proliferation.