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DNP, Mitochondrial Uncoupling and Neuroprotection: A Little Dab'll Do Ya

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Abstract

Recent findings have elucidated roles for mitochondrial uncoupling proteins (UCPs) in neuronal plasticity and resistance to metabolic and oxidative stress. UCPs are induced by bioenergetic challenges such as caloric restriction and exercise, and may protect neurons against dysfunction and degeneration. The pharmacological uncoupler 2,4-dinitrophenol (DNP), which was once prescribed to over 100,000 people as a treatment for obesity, stimulates several adaptive cellular stress response signaling pathways in neurons including those involving the neurotrophic factor BDNF, the transcription factor CREB, and autophagy. Preclinical data show that low doses of DNP can protect neurons and improve functional outcome in animal models of Alzheimer's and Parkinson's diseases, epilepsy and cerebral ischemic stroke. Repurposing of DNP and the development of novel uncoupling agents with hormetic mechanisms of action provide opportunities for new breakthrough therapeutic interventions in a range of acute and chronic insidious neurodegenerative/neuromuscular conditions, all paradoxically at body weight-preserving doses.

Bioenergetic Challenges, Hormesis and Neuroprotection

Three lifestyle factors that promote optimal brain function and neuronal resistance to injury and age-related neurodegenerative disorders are exercise, restriction of dietary energy intake, and regular engagement in social interactions and intellectual challenges [1–4]. For example, intermittent fasting and exercise can protect neurons against dysfunction and degeneration in animal models of Alzheimer's disease (AD), Parkinson's disease (PD) and stroke [5–11]. Conversely, individuals who are sedentary, possess the over-nourished metabolic phenotype and are not intellectually engaged (the so-called 'couch potato' lifestyle) are at increased risk of AD and stroke and a host of other comorbidities [4, 12, 13].

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Neurons regularly experience metabolic and oxidative stress, which results in part from increased activation of excitatory (glutamatergic) synapses. Emerging findings suggest that the cellular and molecular mechanisms by which dietary energy restriction, exercise and intellectual challenges bolster neuronal health and resilience involve activation of cellular signaling pathways that counteract metabolic, oxidative and proteotoxic stress [14]. The pathways include those involving transcription factors such as cyclic AMP response element-binding protein (CREB), NF-κB and Nrf-2 [15–17]. Gene targets of these transcription factors include neurotrophic factors such as brain-derived neurotrophic factor (BDNF), DNA repair enzymes, antioxidant enzymes, and lysine deacetylases [18–20]. In addition, mild bioenergetic challenges stimulate the removal of damaged proteins and mitochondria via autophagy/mitophagy [21, 22].

Cells, organ systems and organisms have evolved multiple integrated signaling mechanisms by which they respond adaptively to stress so as to enhance their ability to tolerate more severe stress. A major driving force for evolution of nervous systems has been the need to obtain food/energy to support survival and reproduction. Accordingly, brains and bodies have evolved so as to function well when the individual is hungry, thereby maximizing their chances of outwitting their competitors [4]. Studies of laboratory animals and human subjects support the notion that intermittent bioenergetic challenges, such as extended periods of time without food and vigorous physical exertion, bolster brain function and stress resistance [1]. This is a prominent example of the biological principle of hormesis, which is characterized by a biphasic dose response curve wherein low to moderate levels of a potentially damaging agent or condition trigger beneficial physiological responses [23] (Figure 1). It is well known that exposure of cultured cells and animals to conditions that cause mild metabolic stress can increase their resistance not only to more severe metabolic stress, but also to oxidative stress and other types of stress. For example, exposure of cultured neurons and mice to 2-deoxyglucose, a non-metabolizable analog of glucose that limits cellular glucose availability, protects neurons against oxidative stress, excitotoxicity and ischemic stroke [24, 25]. The neuroprotective action of 2-deoxyglucose involves induction of expression of the protein chaperones GRP-78 (glucose regulated protein 78) and HSP-70 (heat shock protein 70) [24].

Mitochondria not only generate ATP, but also play important roles in synaptic signaling and plasticity, and as mediators of adaptive responses to cellular stress [26]. Among the signaling pathways that affect mitochondrial function are those that play fundamental roles in synaptic plasticity and learning and memory, including glutamate and BDNF (both a neurotrophin and a myokine). For example, glutamate-induced Ca^{2+} influx results in mitochondrial Ca^{2+} uptake which in turn affects electron transport chain activity and can stimulate superoxide production [27, 28]. In addition, activation of transcription factors such as CREB and PGC-1α by glutamate and BDNF signaling can stimulate mitochondrial biogenesis and the expression of SIRT3, a lysine deacetylase that protects mitochondria against oxidative and metabolic stress [20, 29]. The latter studies provided evidence that mitochondrial biogenesis plays a critical role in the formation and maintenance of synapses, and that SIRT3 mediates beneficial effects of exercise (running) on neuronal resilience. Conditions which result in mild bioenergetic and oxidative stress in mitochondria can protect neurons against more severe stress. For example, inhibition of the mitochondrial ATP synthase (Complex V)

results in resistance of neurons to excitotoxic damage [30]. Neuroprotective effects of metabolic and oxidative preconditioning also involve enhanced mitochondrial robustness which is associated with increased expression of multiple mitochondrial stress resistance proteins including SOD2 and Bcl-2 [31]. These kinds of findings suggest that bolstering mitochondrial stress resistance may be an effective therapeutic avenue for preventing neuronal death and improving function outcome in a range of neurodegenerative disorders that involve metabolic and oxidative cellular stress.

Mitochondrial Uncoupling Proteins and Neuroprotection

Animals have evolved nuclear DNA-encoded mitochondrial uncoupling proteins (UCPs), which may serve multiple roles in regulating cellular metabolism and signaling processes. The first UCP discovered, UCP1, is expressed in brown fat cells where its function is to cause heat production in mammals living in cold environments [32]. However, humans have ~50-fold less brown fat than rodents, and UCP1 therefore plays a negligible role in thermogenesis in human [13]. However the UCP1 homologues, UCP2, UCP3, UCP4 and UCP5 (BMCP1), are more widely expressed and have roles in the mitochondria related to cellular stress adaptation [33]. Neurons express little or no UCP1 and, instead, express UCP2, UCP4 and UCP5 [34]. The latter UCPs are activated by fatty acid and free radicals, inhibited by purine nucleotides, and their expression can be induced by a variety of metabolic and oxidative challenges including exercise and dietary energy restriction [34, 35]. The transcription factors that regulate the expression of neuronal UCPs have not been established; cyclic AMP response element-binding protein (CREB) is a likely candidate as it mediates induction of UCP1 expression in adipocytes [36], and because it mediates adaptive responses of neurons to metabolic and excitatory challenges [37].

Cell culture and *in vivo* studies have provided evidence that UCP2, UCP4 and UCP5 play important roles in adaptive responses of neurons to bioenergetic and oxidative stress, and that these UCPs can prevent the death of neurons in experimental models relevant to both acute brain injuries and neurodegenerative disorders. Cultured mouse cortical neurons overexpressing UCP2 exhibited resistance to death induced by deprivation of oxygen and glucose, and transgenic mice overexpressing UCP2 exhibited less brain damage and improved functional recovery in models of ischemic stroke and traumatic brain and peripheral nerve injury [38, 39]. Ischemic preconditioning induced the expression of UCP2 in brain cells and this was associated with neuroprotection in an animal model of ischemia/reperfusion brain injury [40]. Data further suggest that UCP2 can protect neurons against excitotoxicity and traumatic injury [41–43]. A study of peripheral sensory neurons showed that hyperglycemia down-regulates UCP3 expression, and that overexpression of UCP3 or UCP1 protected the neurons against damage caused by chronic hyperglycemia, suggesting a potential role for UCPs in diabetic peripheral neuropathy [44]. Transgenic mice overexpressing UCP2 selectively in catecholaminergic neurons exhibited resistance of neurons in their substantia nigra to death induced by the neurotoxin MPTP/MPP^+ in a model of Parkinson's disease [45]. Similarly, overexpression of UCP4 [46] or UCP5 [47] protects dopaminergic neurons against MPP^+ toxicity by a mechanism involving reduced oxidative stress and preservation of mitochondrial membrane potential. Collectively these findings

document roles for UCPs in protecting neurons against metabolic, oxidative and excitotoxic stress.

The actions of UCPs in mitochondria can influence multiple signaling pathways. Dysregulation of cellular Ca^{2+} signaling and homeostasis is believed to play a major role in the early synaptic dysfunction and subsequent neuronal degeneration that occurs in both acute brain injuries (e.g., stroke and traumatic brain injury) and in Alzheimer's and Parkinson's diseases [48]. Neural cells overexpressing UCP4 exhibit reduced Ca^{2+} accumulation in the cytosol when challenged with thapsigargin, an agent that causes Ca^{2+} release from the endoplasmic reticulum [49]. The latter study showed that UCP4 attenuates mitochondrial Ca^{2+} accumulation and oxidative stress, which contributes to neuroprotection. One mechanism by which UCPs may regulate mitochondrial Ca^{2+} dynamics is by interacting with the mitochondrial Ca^{2+} uniporter, as suggested by experiments showing that UCP2 increases uniporter Ca^{2+} currents [50]. In addition to affects on cellular Ca^{2+} signaling, neuronal UCPs may also influence other prominent signaling pathways including the cyclic AMP pathway. Thus, overexpression of UCP2 protected cultured primary dopaminergic neurons against the mitochondrial Complex I inhibitor rotenone, by a mechanism involving cyclic AMP-dependent protein kinase [51].

Although the activation of UCPs can result in a decrease in the amount of ATP produced by the individual mitochondrion, overall cellular ATP pools may be maintained or even increased. Several factors explain this bioenergetic adaptation. First, UCPs are typically upregulated/activated by free fatty acids (e.g., docosohexanoid acid, palmitoleic acid and butyric) that are mobilized in response to fasting and related metabolic conditions in which ketones such as β-hydroxybutyrate are also elevated [52]. The metabolism of free fatty acids and β-hydroxybutyrate to acetyl CoA results in generation of ATP via the citric acid cycle.

Second, conditions that enhance mitochondrial uncoupling also stimulate mitochondrial biogenesis resulting in an increase in the number of mitochondria in the cell [53]. In neurons, increased mitochondrial biogenesis may enable the formation and maintenance of new synapses in response to bioenergetic challenges such as exercise and fasting [29]. Thus, by lessening activity of the mitochondrial electron transport chain, and increasing ATP generation via alternative pathways, UCPs can reduce mitochondrial free radical production will sustaining cellular bioenergetics. Interestingly, it was shown in a model of Alzheimer's disease that a rise of isoprostane levels, a biomarker of membrane-associated oxidative stress, was significantly elevated in blood/urine 4-months prior to that of plaque formation, suggesting that mitochondrial oxidative stress occurs early and upstream of amyloid pathology [54].

Chemical uncouplers and neuroprotection

Emerging findings have revealed the therapeutic potential of pharmacological agents that induce mild mitochondrial uncoupling (release of protons into the mitochondrial matrix) in a range of acute and chronic neurodegenerative conditions. The most widely studied and consistently effective uncoupling agent in experimental models of neurodegenerative conditions is 2,4-dinitrophenol (DNP). Treatment of cultured cortical neurons with low

levels of DNP (1 – 3 μM) protected them against oxygen and glucose deprivation [38]. Enhancing respiratory rates by mild uncoupling: 1) reduces the formation of superoxide radical anions (O_2^{-}) by decreasing O_2 tension in the microenvironment; 2) favors an oxidized state of respiratory chain intermediates, such those in Complexes I and III (the major sites of $O_2^{-\cdot}$ production);, 3) suppresses NADH levels and thereby prevents ROS formation by mitochondrial matrix flavoproteins; and 4) lowers membrane potential, which inhibits reverse flow of electrons from Complex II to I [55]. The reduction in mitochondrial membrane potential in response to DNP transiently increases cytosolic, Ca^{2+} levels but lowers intra-mitochondrial Ca^{2+} levels, thereby reducing levels of oxidative stress in cultured rat cerebral cortical neurons [56]. Increased intra-mitochondrial Ca^{2+} levels is associated with the loss of dystrophin in children with Duchenne's muscular dystrophy and diseases associated with the endoplasmic reticulum unfolded protein stress response, which can result in opening of the mitochondrial transition pore and consequent apoptosis [57, 58].

The ability of DNP to lower mitochondrial free radical generation and prevent mitochondrial Ca^{2+} accumulation could provide a therapeutic benefit in disorders involving cellular calcium overload. Preconditioning of the isolated perfused heart with DNP protected myocardial cells against ischemic injury [59, 60]. Administration of only one dose of DNP (5 mg/kg) to rats after 2 hours of middle cerebral artery occlusion and a subsequent 1 hour of reperfusion to mimic stroke in humans, resulted in a 40% reduction of infarct volume and bolstered indicators of mitochondrial health [61]. This is an example where mild pharmacological uncoupling protects threatened tissue in the ischemica penumbra by bolstering their stress resistance, and could also be beneficial for preserving tissue in burn victims [62]. When pretreated with DNP (100 nM), cultured rat substantia nigra dopaminergic neurons exhibited resistance to the toxicity of the mitochondrial Complex I inhibitor rotenone [63]. When mice were treated with DNP (5 mg/kg) once each day for 14 days, their performance on a learning and memory test was significantly enhanced compared to vehicle-treated control mice [56]. DNP induced a dose-dependent increase in levels of the mRNA encoding BDNF in the hippocampus of mice, with 1 and 5 mg/kg being the most effective doses (Figure 3A). Low concentrations of DNP stimulated cyclic AMP (cAMP) production, Tau expression and neurite outgrowth in cultured neural cells [64]. Analyses of gene expression in the cerebral cortex of mice treated with 5 mg/kg DNP demonstrated induction of the immediate early gene Arc, and genes involved in Ca^{2+}/calmodulin, cAMP and CREB signaling, BDNF signaling and autophagy [57]. In addition, data in the latter study suggest that DNP treatment reduces activities of the mTOR and insulin signaling pathways. Collectively, these findings are entirely consistent with a hormesis-based neuroprotective mechanism of action of DNP in which mild mitochondrial uncoupling stimulates a coordinated adaptive molecular response that bolsters neuronal resistance to metabolic, oxidative and excitotoxic stress (Figure 2).

Other uncoupling agents that have been reported to exhibit neuroprotective efficacy in one or more models include FCCP and diazoxide [65–67]. Treatment of cultured cerebellar granule neurons to a low concentration of FCCP (100 nM) increased glucose transport, activated AMPK and conferred resistance of the cells to excitotoxicity [68]. Low doses of FCCP were also reported to protect cardiac cells against ischemic injury [69]. Diazoxide, an agent that opens mitochondrial ATP-sensitive K^+ channels has been reported to be neuroprotective in

experimental models of Alzheimer's disease and stroke, by hormesis-based mechanisms [65, 70]. A component of diazoxide's neuroprotective action may involve mitochondrial uncoupling [71, 72].

The relative merits of interventions that up-regulate UCP expression versus treatment with pharmacological uncouples will be important to evaluate in future studies. Several interventions have been shown to upregulate the expression of one or more UCPs in brain cells. Running wheel exercise induced the expression of UCP2 in the hippocampus of rats [73]. Studies of UCP2 knockout mice showed that UCP2 is required for exercise to increase the number of synapses on dendrites of CA1 pyramidal neurons and dentate granule neurons in the hippocampus [74]. Caloric restriction and fasting also stimulate the expression of UCPs. For example, caloric restriction increased the expression of UCP4 in the cerebral cortex of adult rats [35]. Consistent with the general notion that UCP expression is increased in response to physiological metabolic challenges such as exercise and dietary energy restriction, exposure of animals to mild hypoxia increases the expression of UCP2 in the cerebral cortex and hippocampus [75]. A ketogenic diet has also been reported to increase UCP2 expression in the brain [76]. Interestingly, as is the case with UCP1 in brown fat cells, cold temperatures induce the expression of UCP4 in neurons [35]. There are certainly advantages of exercise and energy restriction as a means of promoting mild mitochondrial uncoupling in neurons. Exercise and energy restriction have far-reaching beneficial effects on organ systems that involve multiple highly integrated, evolutionarily-conserved cellular and molecular mechanisms [4]. With regards to brain function and neurological disorders, there is abundant evidence that exercise and energy restriction improve cognitive function and mood, and may reduce the risk of AD, PD and stroke [1]. However, many individuals live a sedentary overindulgent lifestyle and are unwilling or no longer physically able to commit to more healthy lifestyles. It is for such individuals that pharmacological approaches to inducing mild mitochondrial uncoupling, as with low doses of DNP, that treatment may be particularly beneficial.

The Phoenix of Uncoupling Agents: Development of DNP as a Neurohormetic Drug

Several commonly prescribed drugs and some dietary phytochemicals are toxic and even lethal when ingested in high doses. For example, prescription opioid overdoses caused nearly 15,000 deaths in the United States in 2008, and there has been an alarming increase in opioid overdose deaths in the past few years [77, 78]. Many patients also die from overdoses of other types of drugs including benzodiazepines [79]. Even widely consumed phytochemicals such as caffeine can be toxic and lethal when consumed in high amounts, an emerging health concern resulting from the proliferation of caffeine-laden "high energy" sports drinks and supplements [80]. It is therefore widely understood that most drugs and many natural products exhibit a 'therapeutic window' for clinical efficacy that, if exceeded, can result in adverse events, including death. Here we summarize the human experience with DNP, the notorious weight loss agent from the 1930's that acts as a protonophore that allows H^+ to leak across the inner mitochondrial membrane, and is therefore a "mitochondrial uncoupler" [13].

Before considering the past and potential future clinical applications of DNP, it is instructive to consider a different example of the development of a widely used and effective drug that exhibits serious adverse effects at high doses. The origin of warfarin as a possible anti-coagulant can be traced to the 1920's when entire farms lost their herds due to consuming spoiled silage called "sweet clover", which resulted in uncontrolled bleeding after common procedures such as dehorning [81]. In the 1930s the ingredient causing the bleeding, warfarin, was extracted and then used for rodent control. However, in the 1950s, warfarin was brought forward as a new treatment as a blood thinner, but it was not an easy path to the clinic due to the many years prior being used as a rat killer. Dr. Link writes "the transition to a substance originally promoted to exterminate rats and mice was a bit more than they (clinicians) could accept with real enthusiasm". The fortunate fate in 1951 of a newly enlisted soldier's failed attempt to commit suicide by ingesting a concentrated form of warfarin [82], provided the catalyst to finally move forward into the clinic using moderate doses of warfarin as a blood thinner [83]. Warfarin (Coumadin) became an approved drug and is still used today as the mainline therapy for protection against stroke. The Warfarin story provides a compelling example of the repositioning of an old drug with a tainted past just by reducing the dose to an amount that is within the disease-modifying hormetic range. Another example is the case of repositioning of the notorious drug, thalidomide. Thalidomide was provided as an unapproved "experimental" drug to completely uninformed pregnant women as a sleep/nausea aide, and caused horrible birth defects [84]. Now thalidomide and related analogs are effective disease-modifying treatments for cancer and other serious indications [85].

In an article published in 1891, Gibbs and Reichert reported that a high dose of DNP (300 mg/kg) was toxic to dogs, which was associated with pyrexia and rapid postmortem onset of rigor mortis (Figure 3). DNP was used in the manufacture of explosives during World War I and clinical observations and studies of animal models established that it induced heat production [86]. It was reported in 1933 by Cutting et al. that once daily oral doses of 3 or 5 mg/kg DNP increased the metabolic rate of overweight patients by 40%; all subjects lost weight (typically 2–3 pounds/week) with no evidence of adverse effects during a 3 month treatment period [87]. Within one year of that report, DNP had been used by more than 100,000 patients in the United States. At that time only two deaths attributable to DNP had been reported, with both cases involving individuals who consumed over 10 times the recommended dose (i.e., more than 50 mg/kg). No adverse effects of therapeutic doses of DNP on the cardiovascular, renal, gastrointestinal or hepatic systems were noted by Tainter et al. [88]. Evidence that DNP promotes cataract formation and rashes then emerged [89] which, together with the potential for lethal overdose, prompted the FDA to ban its use as a prescription drug. However, DNP continues to be used by bodybuilders and some athletes to enhance fat loss [90]. In support of potential health benefits of low doses of DNP, it was recently shown that DNP can protect mice against diet-induced obesity and hepatic steatosis, while improving glucose tolerance [91].

At the time of its development as a thermogenic weight loss-promoting drug in 1930s, it was not known that mitochondrial uncoupling is a normal physiological process that mediates cellular responses to environmental challenges or that it mimics the naturally occurring phenomenon of "proton leak" by which the body loses ~30% of its energy to heat [92].

Indeed, the first mitochondrial uncoupling protein UCP1 was not discovered until four decades later [93]. Only within the most recent 20 years have the UCPs expressed by neurons been established and their roles in neuroplasticity and neuroprotection have begun to be understood (see above). In addition to reports of neuroprotection and improved functional outcome in experimental models of stroke and Parkinson's disease [63, 65], DNP can protect mice against seizures induced by the excitotoxin kainic acid (Figure 3B). Moreover, data from a study of a mouse model of Alzheimer's disease (APP/PS1 double mutant transgenic mice) demonstrated that daily administration of a very low dose of DNP (0.5 mg/kg) for 4 months ameliorated spatial learning and memory deficits in a water maze task, with striking results on short-term memory (Figure 3C and D). These kinds of findings suggest that DNP may have potential applications for a host of insidious neurodegenerative diseases at weight neutral doses due to its hormesis-based effects found at low doses (Figures 1 and 2).

The development of uncoupling agents with improved safety profiles are being developed for obesity, diabetes and neurodegenerative disorders. For example, a controlled release oral form of DNP that produces mild uncoupling in hepatocytes reduced insulin resistance, hyperlipidemia and hepatic steatosis in a rat model of diabetes [94]. No evidence of toxicity was detected during chronic administration of the controlled release DNP. In another study, a DNP analog (DNP-methyl ether) targeted to liver was shown to reverse insulin resistance and fatty liver in rats fed a high-fat diabetogenic diet [95]. From a drug development perspective, perhaps it is time for a paradigm shift away from the lofty goals of weight loss *per se* with all the historic and recent hazards, to a "wellness program" using weight neutral steady state delivery of very low doses of uncoupling agents such as DNP. Since over-nutrition is not only associated in metabolic syndrome, but also with a significant increase in the incidence of neurodegenerative diseases, an uncoupling agent that mimics caloric restriction by lowering intra-hepatic, intra-muscular and circulating lipids may provide a therapeutic benefit. As proof-of-concept it was reported that mice chronically treated for ~80 weeks with DNP provided in their drinking water at a dose of ~100 μg/kg lived longer than control mice and had reduced levels of ROS in both liver and brain, lower amounts of oxidized proteins and DNA damage, and lower circulating glucose, lipid and insulin levels [87]. To put this into perspective, this is the equivalent to humans of ~0.5 mg per day or ~600-fold lower dose than what was used in the 1930s for weight loss (300 mg per day). The intermittent drinking of water containing DNP mimicked sustained delivery, and since DNP lacks a methyl-ether that would cause it to be sequestered in the liver, the drug distributes to cells in all organs. The findings in mice suggest that a very low sustained level of DNP can attenuate the age-related accumulation intra-hepatic and intra-muscular lipids, without reducing body weight [96]. It will therefore be of considerable interest to determine whether very low doses of a mitochondrial uncoupling agent such as DNP would ameliorates metabolic morbidities resulting from excessive calorie intake and a sedentary lifestyle in human subjects.

The theme that is building is that low doses of DNP provide broad neuroprotection, perhaps due to its unique mechanism of action initiated as an adaptive stress response, and its specificity to the mitochondria, the only cellular organelle with a basic pH environment. Indeed, the target of chemical uncouplers such as DNP is not a protein, but is instead the

mitochondrial membrane where they transfer protons to the matrix [13]. Although DNP's initial uncoupling action is non-genomic, several prominent signaling cascades involved in adaptive neuroplasticity and stress resistance are activated including those involving calcium and cyclic AMP, and downstream kinases and transcription factors (Figure 2) [56, 97].

During the 80 years after the initial use of DNP in humans at very high doses for inducing weight loss in obese subjects, the knowledge base of mitochondrial bioenergetics and chemical uncoupling has expanded greatly. It was not known until recently, however, that very low doses of uncoupling agents such as DNP are effective in ameliorating disease processes and improving functional outcome in preclinical models of a range of neurological disorders that involve metabolic and oxidative stress including Alzheimer's, Parkinson's diseases, epilepsy and ischemic stroke [61, 65, 98] (Figure 4). The emerging findings described in this article suggest that, similar to the broadly beneficial effects of caloric restriction, even very low levels of mitochondrial uncoupling can protect multiple organ systems against dysfunction and degeneration in preclinical models of a wide range of disorders that involve dysregulation of energy metabolism metabolic and oxidative stress. Translation to humans is the next critical step.

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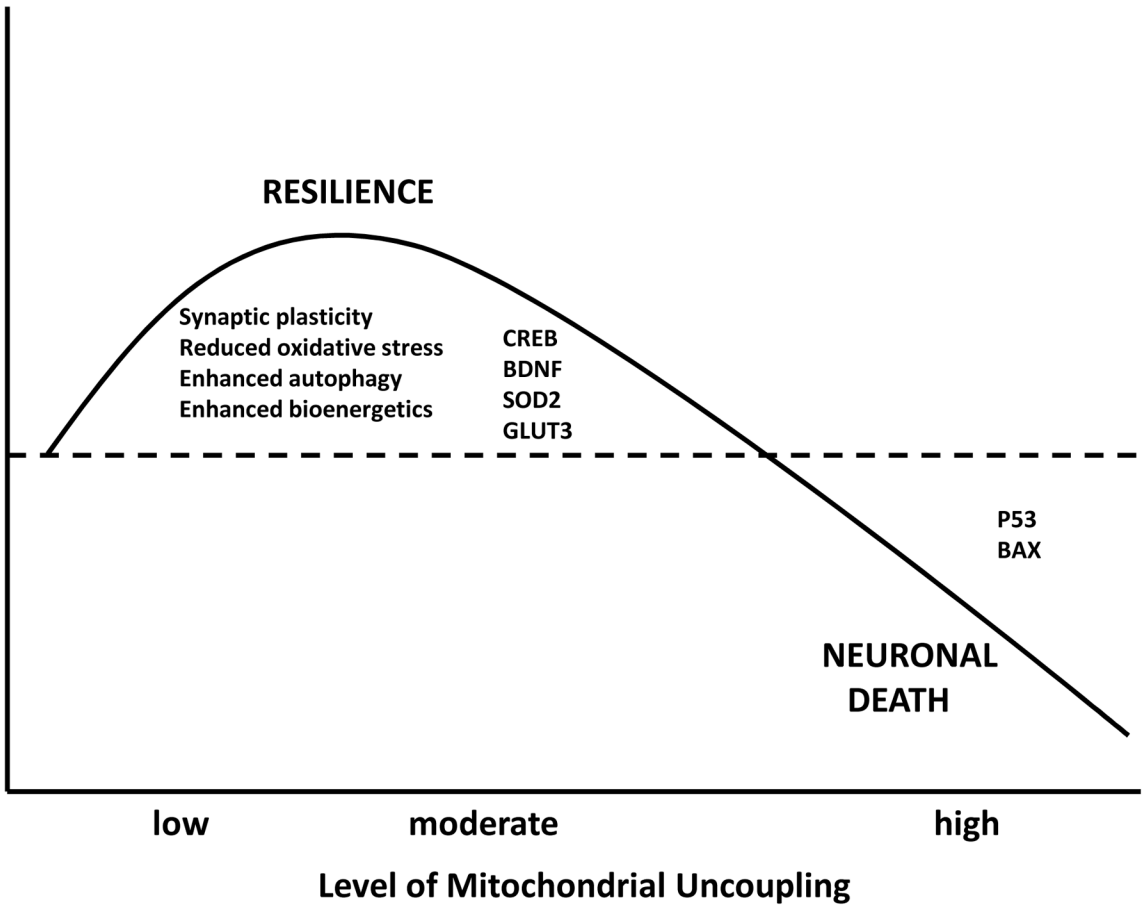

Figure 1.

Neuronal responses to mitochondrial uncoupling are consistent with a hormesis-based mechanism of action. As is true with many processes in biology and medicine, the dose – response curve for mitochondrial uncoupling is biphasic, with mild uncoupling eliciting beneficial adaptive responses and high levels of uncoupling causing cell damage and death. Mild uncoupling may enhance neuronal resilience by activating signaling pathways that promote synaptic plasticity, reduce oxidative damage, increase autophagy and bolster bioenergetics. Some of the proteins that may mediate the hormetic effects of mild uncoupling are brain derived neurotrophic factor (BDNF), cyclic AMP response element-binding protein (CREB), superoxide dismutase 2 (SOD2) and glucose transporter 3 (GLUT3). Excessive sustained uncoupling can trigger cell death which is mediated by pro-apoptotic proteins such as p53 and BAX.

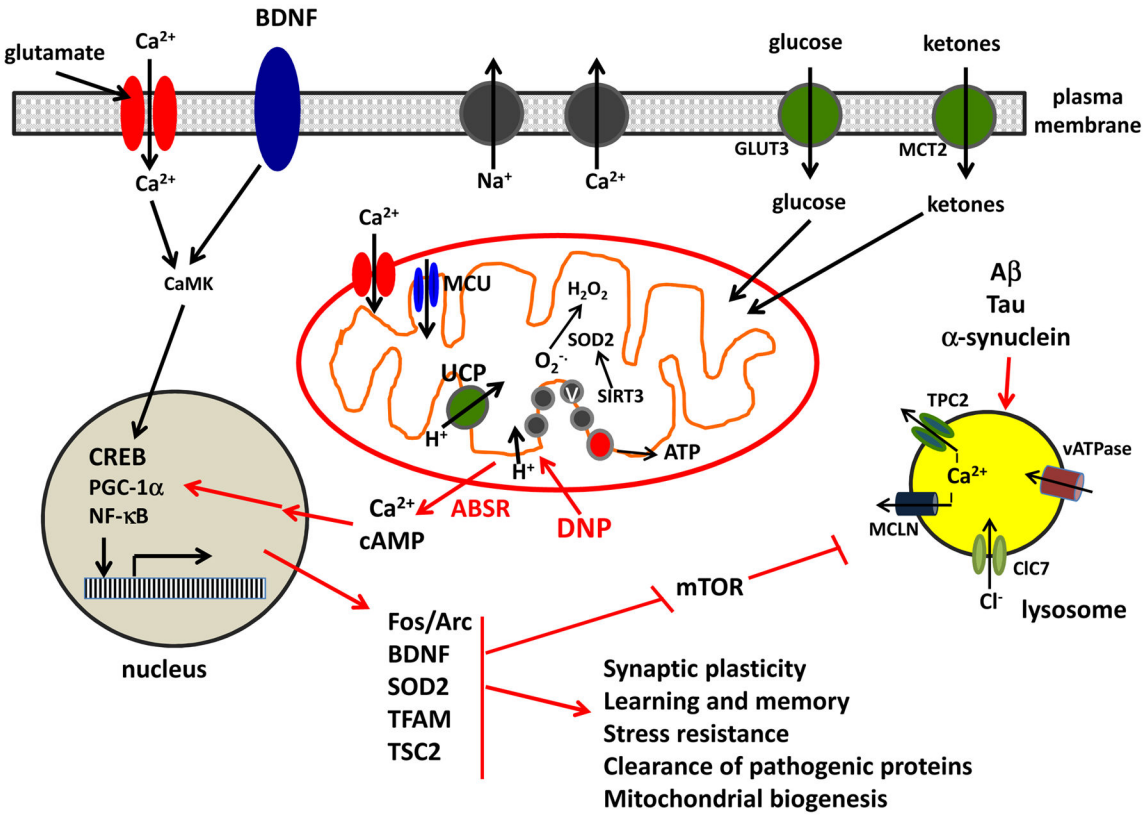

Figure 2.

Mechanisms by which mild mitochondrial uncoupling protects neurons against oxidative stress, excitotoxicity and the accumulation of disease-related self-aggregating proteins such as amyloid β-peptide, Tau and α-synuclein. Mild uncoupling resulting from activation of endogenous uncoupling proteins (UCP) or pharmacological agents such as 2,4-dinitrophenol (DNP) triggers an adaptive bioenergetic stress response (ABSR) involving multiple signaling pathways and organelles. The ABSR involves activation of kinases, and transcription factors such as CREB, PGC-1α and NF-kB which, in turn, induce the expression of genes encoding proteins that enhance stress resistance and neuroplasticity including: the immediate early gene products Fos and Arc; the neurotrophic factor BDNF; the antioxidant enzyme SOD2; the regulator of mitochondrial biogenesis TFAM; an inhibitor of the mTOR pathway (TSC2). CamK, calcium/calmodulin-dependent kinase; ClC7, chloride channel 7; GLUT3, glucose transporter 3; MCLN, mucolipin; TPC2, two pore channel 2; MCT2, monocarboxylic acid transporter 2; vATPase, vesicular ATPase.

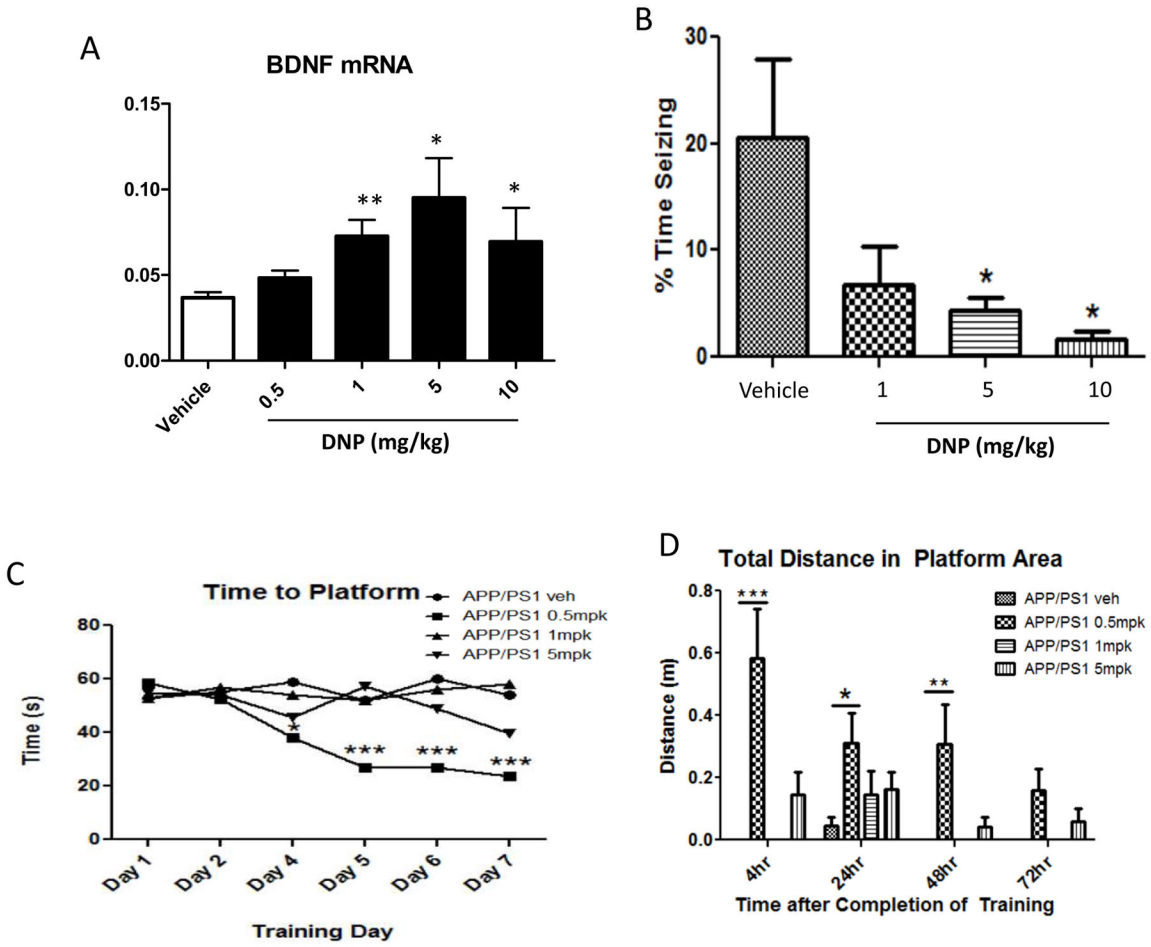

Figure 3.

Treatment of mice with low doses of the uncoupler DNP results in increased expression of BDNF in the brain and improves functional outcomes in models of epileptic seizures and Alzheimer's disease (AD). **A.** Mice were administered the indicated doses of DNP or vehicle by oral gavage once daily for 14-days. BDNF mRNA levels in cortex tissue samples were measured by semi-quantitative PCR (qPCR). Values are the mean and SEM of measurements made on samples from 8 mice/group. *p<0.05, **p<0.01 compared to the value for vehicle-treated mice. **B.** Mice were administered the indicated doses of DNP or vehicle by oral gavage once daily for 7-days. Mice were then administered the seizure-inducing excitotoxin kainic acid by direct injection into the dorsal hippocampus [99]. Seizures were evaluated during the ensuing 4 hours using a semi-quantitative rating scale as described previously [100]. Values are the mean and SEM (6 mice/group). *p<0.01 compared to the value for vehicle-treated mice. **C and D.** APP/PS1 double mutant transgenic mice (an animal model of AD) were administered the indicated doses of DNP (mpk, mg/kg) or vehicle by oral gavage once daily for 4 months. Hippocampus-dependent spatial learning and memory were then evaluated in a water maze test in which the time taken to locate a submerged platform in the pool (goal latency; memory acquisition) was measured daily for 7 days of training (C). The platform was then removed from the pool and the total distance the mouse swam in the specific area where the platform had been was

determined at 4, 24, 48 and 72 hours (an indicator of memory retention) (D). See ref. 101 for methods. Values are the mean and SEM of measurements made on samples from 5 or 6 mice/group. *p<0.01, **p<0.01, ***p<0.001.

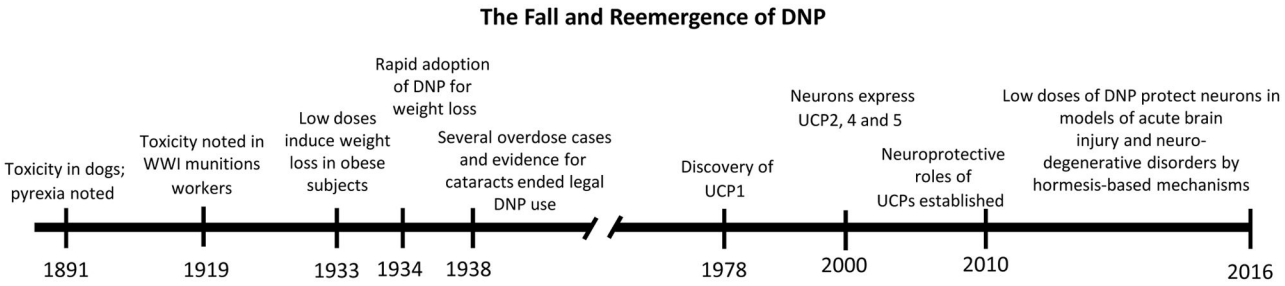

Figure 4.

Historical timeline of studies of DNP dose-dependent clinical efficacy and toxicity, mechanisms of action, and potential applications to acute and chronic neurodegenerative conditions.