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TRPM7 and *TRPM2* – Candidate Susceptibility Genes for Western Pacific ALS and PD?

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Abstract

Recent findings implicating TRPM7 and TRPM2 in oxidative stress-induced neuronal death thrust these channels into the spotlight as possible therapeutic targets for neurodegenerative diseases. In this review, we describe how the functional properties of TRPM7 and TRPM2 are interconnected with calcium (Ca^{2+}) and magnesium (Mg^{2+}) homeostasis, oxidative stress, mitochondrial dysfunction, and immune mechanisms, all principal suspects in neurodegeneration. We focus our discussion on Western Pacific Amyotrophic Lateral Sclerosis (ALS) and Parkinsonism Dementia (PD) because extensive studies conducted over the years strongly suggest that these diseases are ideal candidates for a gene-environment model of etiology. The unique mineral environment identified in connection with Western Pacific ALS and PD - low Mg^{2+} and Ca^{2+}, yet high in transition metals, creates a condition that could affect the proper function of these two channels.

Keywords

TRPM7; TRPM2; Western Pacific ALS; Parkinsonism Dementia; oxidative stress; neurodegeneration; Ca^{2+} and Mg^{2+} homeostasis; mitochondrial dysfunction; microglial activation

Western Pacific ALS and PD

Amyotrophic lateral sclerosis (ALS), a fatal disease characterized by the progressive degeneration of motor neurons, is among the most common of adult onset neurodegenerative diseases [1]. The underlying pathogenic processes are not known but are believed to be due to a multitude of contributory factors including genetic predisposition, environmental toxins, aberrant cellular calcium and metal ion homeostasis, oxidative stress, mitochondrial dysfunction, and inflammatory reactions [2,3]. More than fifty years ago, an unusually high incidence of ALS was reported in three geographically separate areas in the Western Pacific: the islands of Guam and Rota, the Kii Peninsula of Japan, and southern West New Guinea [4-6]. On Guam, the incidence rate of ALS in 1954 was estimated to be 50-100 times higher than the worldwide incidence [7]. A related but distinct neurodegenerative disorder, parkinsonism dementia (PD), characterized by the clinical manifestation of both parkinsonism

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and dementia in the same patient, was also prevalent in the same three Western Pacific ALS foci [5-9]. These two disorders can have overlapping clinical symptoms, frequently occur together in the same families and even in the same individual, have neurofibrillary tangle-bearing neurons in the brain and spinal cord, and both are progressive, and eventually fatal [10-12]. While two opposing views exist as to whether ALS and PD are separate diseases or clinical variants of the same disease, there is general agreement that the basic pathogenic mechanisms underlying these disorders are similar. Since the topic of this review is mechanistic in nature, Western Pacific ALS and PD will be considered as one (ALS/PD), except where it is absolutely necessary to consider them separately.

The hyperendemic ALS/PD foci in the Western Pacific attracted considerable interest among medical researchers and scientists for a variety of reasons including the fact that these foci of ALS/PD are ideal for studying the relative contributions of genes and environment in disease etiology because each disease focus occurs in a defined, restricted environment among the three genetically different homogeneous groups of people. ALS/PD is a tragic disease and a serious public health problem and deserve to be investigated for those reasons alone. In addition, ALS/PD foci in these areas are extremely informative disease clusters that could provide valuable leads to the pathologic mechanisms underlying the more common, sporadic forms of ALS, Parkinson's Disease (PD), and Alzheimer's Disease (AD) worldwide.

In 1956, the National Institute of Neurological Diseases and Blindness (NINDB) as it was then called, of the National Institutes of Health (NIH), established a research center on Guam to investigate the clinical, epidemiologic, neuropathological, and genetic aspects of ALS/PD. The intensive research that followed did not yield a clear understanding of disease etiology, but nevertheless suggested that a complex interplay between genetic susceptibility and exposure to specific environmental factors were involved. Extensive epidemiological studies subsequently identified two candidate environmental triggers: (1) altered mineral content of the soil and drinking water coupled with abnormal mineral metabolism [13,14]; and (2) toxins of the cycad plant, a traditional food source in Guam [15,16].

The Environmental Factors

Altered mineral content in the environment

All three high incidence foci in the Western Pacific were reported to have severely low levels of calcium (Ca^{2+}) and magnesium (Mg^{2+}), coupled with high levels of bioavailable transition metals such as manganese (Mn^{2+}), aluminum (Al^{3+}), and iron (Fe^{3+}). Soil and rivers in areas of high incidence in the Kii Peninsula were found to contain high levels of Mn^{2+} but very low levels of Ca^{2+} and Mg^{2+} [13]. On Guam, the highest incidence of ALS/PD occurred in the southern part of the island, the same area where Ca^{2+} levels in the soil and traditional spring drinking water were found to be 10- to 100-fold lower compared with other regions of the island. Mg^{2+} in the water supply is similarly low, about 10-fold lower in the South than elsewhere in Guam [14]. The levels of Ca^{2+} and Mg^{2+} in soil and drinking water in the high incidence villages in West New Guinea were even lower than those seen in southern Guam [5]. The unusual similarity of the mineral composition in these three ALS/PD foci led to the proposal that *prolonged* exposure to such an environment could be involved in the pathogenesis of ALS/PD [13]. This hypothesis is supported by findings of altered Ca^{2+} and Vitamin D metabolism [17]; hypocalcemia and reduction of critical bone mass [18]; and the accumulation of Ca^{2+}, and transition metals such as Al^{3+}, Fe^{2+}, Mn^{2+} and Zn^{2+} in the brain and spinal cord of patients afflicted with ALS/PD in Guam and Kii peninsula [19-23]. Animal models fed altered mineral diets mimicking the environmentally observed Ca^{2+}, Mg^{2+}, Mn^{2+} and Al^{3+} levels, showed evidence of altered Ca^{2+} homeostasis and deposition of Ca^{2+}, Mn^{2+}, Al^{3+} in the brain and spinal cord tissues [24-26]. These models also showed signs of neuronal damage including neurofibrillary pathology and mitochondrial degeneration. In a recent study, rats

were exposed to low Ca^{2+} and/or Mg^{2+} intake over two generations in order to simulate human conditions in Guam more closely [27]. Of the various combinations of Ca^{2+} and Mg^{2+} contents tested, exposure to low Mg^{2+} (one-fifth of the normal level), was more deleterious, causing significant loss of dopaminergic neurons in the substantia nigra. We will offer a possible mechanistic explanation of these observations later in this review.

L-beta-N-methylamino-L-alanine (L-BMAA) is a putative neurotoxin from the cycad plant

Traditionally used as a food source in Guam and for medicinal applications in Kii Peninsula and West New Guinea, cycad and its toxin, L-BMAA, is the second environmental factor that was proposed to be involved in the pathogenesis of ALS/PD [15,16]. It is also one of the most controversial topics in ALS/PD research. L-BMAA is a non-protein amino acid present in cycad seeds that has been shown to possess neurotoxic properties in cell culture models [28, 29]. Importantly, macaque (*Cynomolgus*) monkeys fed large doses of BMAA exhibited clinical and neuropathological ALS-like symptoms [15]. The massive dose used, however, was considered unrealistic in terms of human consumption because very low amounts of L-BMAA remain in washed cycad flour, considered the primary source of human exposure [30,31]. Weak neurotoxic properties detected in cell culture studies further substantiated the view that L-BMAA may not significantly contribute to ALS/PD pathogenesis. High (millimolar) concentrations of L-BMAA were needed to induce cytotoxic effects in cultured neuronal cells [28]. NMDA receptor (NMDAR) antagonists largely blocked these effects suggesting that L-BMAA acts via NMDARs [32,33]. It was further shown that the addition of bicarbonate ions considerably increased the potency of L-BMAA due to the formation of β-carbamate, a structurally more potent agonist of NMDARs [32]. While nuclear magnetic spectroscopy (NMR) could indeed demonstrate that carbamate compounds form when BMAA and bicarbonate are mixed, it must be stressed that, as far as is known, no study has ever reported the isolation of carbamates from brain or spinal cord tissues of ALS/PD patients.

Subsequent studies indicate that NMDAR activation may not be the dominant pathway through which L-BMAA exerts its neurotoxic effects. L-BMAA was reported to preferentially interact with metabotropic receptors [34-36], and non-NMDA receptors in the presence of bicarbonate [37,38]. Preferential injury to NADPH-diaphorase neurons induced by lower BMAA exposures was found to be predominantly mediated by AMPA/kainate receptors [28,32]. Exposure to L-BMAA (and bicarbonate) increased intracellular Ca^{2+}, $[Ca^{2+}]_i$, levels suggesting Ca^{2+} involvement [39]. Since AMPA/kainate receptors are Ca^{2+}-impermeable, the involvement of these receptors was not easy to integrate into the prevailing hypothesis of L-BMAA toxicity. However, it was soon discovered that a special class of AMPA/kainate receptors, Ca-A/K, which are Ca^{2+}-permeable, exists [40,41]. Of particular relevance to ALS, motor neurons (MNs) were shown to possess large numbers of Ca-A/K channels [42].

Prompted by a report that protein-bound forms of L-BMAA could potentially serve as an endogenous neurotoxic reservoir of this toxin [43], the issue of L-BMAA neurotoxicity was recently reexamined by Rao, et al [44]. Spinal neurons plated on an astrocytic monolayer were exposed to controlled doses of L-BMAA, cycad toxin extracts, and the antagonists NBQX (specific for AMPA/kainate receptors) and Mk801 (an NMDAR antagonist). Cell death, $[Ca^{2+}]_i$ changes, and reactive oxygen species (ROS) production were monitored. Results showed L-BMAA to be more potently toxic to MNs than to all other spinal neurons. Moreover, L-BMAA caused substantially greater $[Ca^{2+}]_i$, rises and ROS generation in MNs than in the other spinal neurons. The addition of NBQX, but not Mk801, conferred substantial protection, suggesting that L-BMAA toxicity is predominantly mediated through Ca-A/K channels. Nevertheless, NBQX was not completely protective. In motor neurons exposed to 100 μM L-BMAA, NBQX or a combination of NBQX and Mk801 reduced L-BMAA-induced cell death by two-thirds, suggesting the involvement of other pathways. Interestingly, an earlier study

using cultured hippocampal neurons showed that exposure to L-BMAA activated an unidentified linear current [38]. It is possible that this current is carried by TRPM2, one of the channels covered in this review, because increased $[Ca^{2+}]_i$ and ROS promote TRPM2 activation.

The Rao, et al. study reported three important findings: (1) motor neurons are selectively more vulnerable to L-BMAA toxicity; (2) these toxic effects are mediated through $[Ca^{2+}]_i$ rises via Ca-A/K channels and other pathways, and subsequent ROS generation; and (3) L-BMAA, at concentrations found in cycad extracts, exert neurotoxic effects. The demonstration that relatively low (tens of micromolar) levels of L-BMAA could damage motor neurons firmly puts L-BMAA as a valid candidate in the etiology of ALS/PD. Nevertheless, it must be stressed that L-BMAA was not found in *all* ALS/PD autopsy specimens and that another study conducted by a separate research group tested for, but did not detect, free L-BMAA in Alzheimer's Disease patients from the US Pacific Northwest nor in Guam PD patients or Chamorro controls [45]. For the purposes of this review, the important point is that L-BMAA causes $[Ca^{2+}]_i$ rises and ROS generation in motor neurons, creating conditions conducive to the activation of TRPM2, an ion channel that has been linked to oxidative stress-mediated neuronal death [46,47].

The Question of Genetic Predisposition

Epidemiological studies have implicated genetic factors in the development of ALS/PD because cases cluster in families - siblings and parents of afflicted patients were found to be at increased risk of developing these diseases [48]. An intensive effort was launched to identify the genetic cause of ALS/PD, including pedigree analyses and calculation of inbreeding coefficients in high incidence villages, identification of selected gene markers such as HLA antigens, blood group systems, red cell enzymes, immunoglobulin allotypes, and serum proteins [49]. None yielded satisfactory answers.

A prospective case-control registry was established in 1958 (completed in 1963) to determine if first-degree relatives and spouses of patients with ALS/PD have a higher risk of developing the disease than relatives of unaffected controls (matched for age, sex, and village). A 25-year follow-up study revealed significantly increased risk of developing ALS and PD among siblings, and to a lesser extent, among spouses of patients [50]. Offsprings, however, were not found to have increased risk in the 25-year follow-up study probably because most were below the age of risk for both diseases. Siblings of controls did not have increased risk and have, in fact, lower risk than the general Chamorro population. A more recent 40-year follow-up showed similar results: first-degree relatives of ALS or PD patients have a significantly higher risk of developing either disease than the Guamanian population whereas relatives of controls have significantly lower risks [51]. There is, however, a notable difference because this time, offsprings of PD patients were found to have increased risk of developing either ALS or PD. While these data suggest a genetic contribution to etiology, simple Mendelian rules do not apply. For one, both 25-year and 40-year follow-up studies demonstrated increased risk for spouses of patients. Additionally, the 40-year study found an increase in age-of-onset and a dramatic decline in incidence for both diseases since the 1950's when the first systematic epidemiological studies commenced. The decline was coincident with increasing modernization of Guam that changed lifestyles, changed access to locally grown foods and changed or improved local drinking water supplies. These findings, especially the rapid decline over three decades in southern Guam, where the highest incidence of disease is found, strongly suggest that environmental factors contribute to the etiology [52,53]. In support of this conclusion, segregation analysis performed on the registry data that were used in the 25-year follow-up study, including all patients from 1950-1983, rejected a purely environmental hypothesis and a Mendelian dominant or a Mendelian recessive hypothesis, but could not reject

a two-allele additive major locus hypothesis [54]. This segregation study therefore proposed that a major gene in combination with environmental factors could be involved in the development of ALS/PD.

A search for candidate genes

In an effort to identify the genes that are involved in the etiology of ALS/PD, those genes that have previously been linked to familial ALS, PD, or AD were sequenced. No disease-associated variant or mutation was found after sequence analyses of Cu-Zn superoxide dismutase, *SOD1* (familial ALS), the microtubule-associated protein tau, *TAU* (PD), apolipoprotein E, *ApoE Σ4* (early-onset AD) and *CYP2D6*, which encodes a detoxifying enzyme (PD) [55-58].

It is likely that what we should be looking for are susceptibility genes that do not directly cause disease but contribute predisposition when functioning in a conducive environment. Importantly, the genetic causation of complex diseases like ALS/PD is likely polygenic in nature, that is, minor functional variants of several genes could together contribute to pathology. To identify functional candidate susceptibility genes for ALS/PD, we decided that an effective approach would be to use the environmental risk factors found in all three hyperendemic Western Pacific foci as a screening tool. *What gene product's function will most likely be affected by low Mg^{2+}, low Ca^{2+}, and high transition metals*? Foremost among several functional candidates are ion channel genes. In order to narrow the field further, we imposed two additional limitations: the candidate channel/s must be expressed in the central nervous system (CNS) and be affected by oxidative stress. Oxidative stress is the price that a eukaryotic cell pays for being able to use molecular oxygen (O_2) as an energy source. Oxidative phosphorylation, the process of converting energy from O_2 to a form that can be used by the cell, produces high- energy by-products, ROS, which are normally managed by the cellular antioxidant defense system. Oxidative stress results when the balance tilts heavily in favor of ROS production such as occurs in conditions of high metabolic rates, weakened cellular defense, and presence of environmental toxins such as transition metals. Such imbalance leads to molecular damage, failure of cellular functions, and ultimately cell death. Increased oxidative stress happens as a consequence of aging, the universal risk factor for all neurodegenerative diseases. In the hyperendemic ALS/PD foci in the Western Pacific, the unique mineral composition of the environment creates a condition of increased oxidative stress because low Mg^{2+} levels cause lipid peroxidation [59] while transition metals act as redox catalysts causing increased ROS production [60].

We found two candidate genes that meet our criteria - ion channels that are expressed in the brain and affected by oxidative stress: *TRPM7* and *TRPM2*. Both belong to the transient receptor potential melastatin (TRPM) family of channels. *TRPM7* resides in chromosome 15q21, within a locus that has been linked to a form of autosomal recessive familial ALS [61]. *TRPM2* is located in disease-rich chromosome 21 (21q22.3), near the SOD1 locus (21q22.1-22.2) that has been associated with familial ALS [62].

TRPM7

TRPM7 is a ubiquitously expressed plasma membrane protein that conducts virtually all divalent ions – from the physiologically abundant Ca^{2+} and Mg^{2+}, to essential trace metals such as Mn^{2+} and Zn^{2+} and non-essential, even toxic transition metals such as Cd^{2+} [63,64]. It was proposed that TRPM7 plays a central role in regulating cellular homeostatic levels of Ca^{2+}, Mg^{2+} and trace metals [63-65]. TRPM7 is required for cell viability as evidenced by the resulting lethal phenotype when the channel is knocked out in DT40 B lymphocytes [63]. Studies have implicated TRPM7 involvement in various cell functions including cell growth, proliferation, embryonic development, anoxic neuronal death, pathological response to vessel wall injury, actomyosin contractility, and neurotransmitter release in sympathetic neurons

[65-72]. Perhaps of particular relevance to the topic of this review is the recent report implicating TRPM7 in familial Alzheimer's disease [73].

TRPM7 is a dual-function protein, one of only three known channels possessing both a channel and an enzyme domain, the other two being TRPM6 and TRPM2. TRPM7 and TRPM6 show approximately 50% sequence homology and both possess a C-terminal serine/threonine alpha (α)-kinase [74]. α-kinases are characterized by their unique ability to phosphorylate substrates within α-helices, as opposed to conventional protein kinases which phosphorylate their substrates within loops, β-turns and irregular structures [75,76]. Biochemical studies have shown kinase activity in both TRPM6 and TRPM7 [65,77,78]. The relationship between a channel and a kinase moiety coexisting in the same molecule has justifiably been the subject of considerable interest. It was initially thought that TRPM7 kinase activity regulates channel function [77] but subsequent studies have demonstrated that phosphotransferase activity is not required for channel activity [78,79]. TRPM7 was recently implicated in the regulation of cell adhesion and podosome formation by modulating actomyosin contractility through phosphorylation of the myosin IIA heavy chain [72]. Annexin 1, a Ca^{2+}-regulated phospholipid binding protein, has also been identified as a substrate for TRPM7 kinase [80]. Phosphorylation of annexin 1 and the myosin IIA heavy chain by TRPM7 kinase requires Ca^{2+} influx through the channel domain, indicating an intricate association between channel and kinase functions [72,80]. It has thus been suggested that ions permeating the pore regulate kinase function and the subsequent recruitment or activation of downstream signaling components [72]. This is an attractive hypothesis that definitely merits further investigation.

TRPM7 regulation

TRPM7 channels are constitutively open but inhibited intracellularly by free Mg^{2+} [63,81], a fact reflected in one of the names accorded to endogenous TRPM7 currents, *MIC* for ***M***g^{2+}-***i***nhibited ***c***urrent [82]. The other terminology, MagNuM (***Mag***nesium ***Nu***cleotides ***M***etal) takes into account block by Mg^{2+} and Mg^{2+}-nucleotides (primarily MgATP) and metal permeation [81]. TRPM7 also responds to changes in pH [83,84], a feature that may be relevant during certain pathological conditions such as when acidosis occurs during ischemia. In addition to intracellular free Mg^{2+}, MgATP and pH, other modulators of channel activity have been suggested in various experimental systems: phospholipase C-coupled receptors acting through phosphatidylinositol 4,5- bisphosphate (PIP_2), cyclic adenosine 3,5-monophosphate (cAMP), polyvalent cations, and phosphorylation by TRPM6 [78,85-89].

Mg^{2+} and Ca^{2+} are permeant blockers

In addition to inhibiting TRPM7 from the cytoplasmic side, Mg^{2+} also plays a crucial role in regulating external ion permeation through these channels. This effect of Mg^{2+} is of paramount physiological relevance because external Mg^{2+} affects the permeation of other ions. This channel is cation nonselective, and yet, the TRPM7 I/V curve under physiological ionic conditions is characterized by pronounced outward rectification, with very small inward currents at negative potentials, and increasingly large outward currents at positive voltages. The reason for this is external divalent block – divalents present in the extracellular medium (physiologically Mg^{2+} and Ca^{2+}) produce a voltage-dependent block that is progressively removed by depolarization. Divalent block is also relieved at very negative voltages. Kerschbaum et al. [89] conducted a detailed study of the external divalent (and polyvalent) block and presented a model for ion permeation through MIC/TRPM7 channels. The model successfully predicted experimentally observed effects of Mg^{2+} on the permeation of monovalent cations. It proposed the presence of two low-affinity binding sites and a single high-affinity site within the conducting region of the channel. According to this model, when present at low μM concentrations, Mg^{2+} and Ca^{2+} displace Na^{+} from the high-affinity binding site thereby blocking the monovalent current. Mg^{2+} binds to this site more strongly than

Ca^{2+} ($K_{1/2}$ at −40 mV is 1 μM for Mg^{2+} vs. 4 μM for Ca^{2+}) indicating that it is the stronger blocker. Permeation of Mg^{2+} and Ca^{2+} occurs when either is present at sufficiently high concentrations, possibly by a 'knock off' mechanism involving the high-affinity binding site, and 'punch-through' of the blocking ion to the inside.

Whether there is one binding site or more, it is important to bear in mind that Mg^{2+}, Ca^{2+}, and Na^{+} likely interact in the pore, competing for binding sites, each influencing the others. While Mg^{2+} and Ca^{2+} impose strong block of Na^{+} permeation, the block is not total because some degree of Na^{+} permeation have been observed [67,82]. The experimentally determined binding affinities show that Mg^{2+} is the stronger blocker, suggesting that it will be harder for a Ca^{2+} ion to displace a bound Mg^{2+} ion than vice versa. It can thus be envisioned that as the extracellular Mg^{2+} concentration is reduced, the ability of Ca^{2+} (and Na^{+}) to enter the channel will correspondingly increase. Moreover, removal of Mg^{2+} from the external solution altogether (as is done in some experiments, particularly excitotoxicity assays) will favor the increased influx of Ca^{2+} through TRPM7. Indeed, we observed increased Ca^{2+} influx through overexpressed murine TRPM7 as external Mg^{2+} concentration is reduced (M. Hermosura, et al., unpublished data). Following this line of reasoning, removal of both Mg^{2+} and Ca^{2+} from the external solution will remove divalent block, allowing monovalent (Na^{+}) influx. This has been demonstrated experimentally [67,81,89].

TRPM7 and neuronal death

TRPM7 has recently been implicated in anoxic neuronal death by mediating I_{OGD}, a cation current activated during prolonged oxygen-glucose deprivation, OGD [68]. In thismodel of hypoxia using mouse cortical neurons, it was proposed that ROS produced during oxidative stress activated TRPM7 channels, permitting the unregulated influx of Ca^{2+} and triggering a vicious loop because high intracellular Ca^{2+} caused the production of more ROS, increasing I_{OGD}/TRPM7 activation. Downregulating TRPM7 expression by small interfering RNA (siRNA) specific to TRPM7 protected the cells from oxygen and glucose deprivation-induced stress. However, that same TRPM7-specific siRNA also downregulated TRPM2 channels. This result was explained as suggestive of interdependent protein expression between these two channels and/or that they form heteromers in cortical neurons and that the observed anoxia-induced current is carried by TRPM2/TRPM7 heteromers. The existence of the latter remains to be established and to the best of our knowledge, interaction between TRPM2 and TRPM7 at a protein level has not yet been demonstrated.

The fact that oxidative and nitrative stressors activate TRPM2 is clearly established [90,91] and will be discussed in more detail later. Unfortunately, we have not seen H_2O_2- mediated activation of human TRPM7 expressed in HEK-293 cells in our whole-cell patch clamp experiments, in the presence of physiological concentrations of Ca^{2+} and Mg^{2+}. We offer an alternative explanation for the results observed in this OGD study. As far as we can ascertain, these experiments were conducted in the absence of external Mg^{2+}. As we discussed in the preceding section, this condition favors Ca^{2+} entry through the constitutively open TRPM7 channels. The degree of Ca^{2+} influx will be determined by the number of TRPM7 channels available for permeation that is in turn dependent on existing levels of intracellular Mg^{2+} and MgATP. The experimental induction of OGD causes significant reduction of cellular MgATP [92] thereby lessening intracellular TRPM7 block. As more TRPM7 channels become available for permeation, intracellular Ca^{2+}, $[Ca^{2+}]_i$, will rise. One of the consequences of this $[Ca^{2+}]_i$ rise will be increased ROS production in the mitochondria. The combined $[Ca^{2+}]_i$ rise and increased oxidative stress is conducive to TRPM2 activation. In this scenario, the activation of TRPM2 causes the massive influx of Ca^{2+} and Na^{2+} that precedes cell death. This explanation is not in conflict with the protective effect observed following siRNA inhibition of TRPM7 because the early $[Ca^{2+}]_i$ rise needed to trigger ROS/RNS production will not occur

when the number of available TRPM7 channels is severely depleted. Thus, as the authors concluded, TRPM7 is a critical and important player in OGD-mediated neuronal death. It will be of interest to know whether the I_{OGD} pathway mediates neuronal death in the presence of physiological and/or pathological (low) levels of Mg^{2+}. These are early days in the functional characterization of TRPM channels, especially in the context of complex cells such as neurons. Our best experimental attempts and interpretations are limited by what we know, and in the case of TRPM7 and TRPM2, there is much to learn. We need to know whether these two channels form heteromers, what splice variants are expressed in neuronal cells and under what conditions these are expressed, the electrophysiological signature of the heteromers, and the players involved in the modulation and activation of homomeric and heteromeric channels. I_{OGD} could be mediated by both TRPM7 and TRPM2. Only time and more studies will tell.

Nevertheless, given that this is a review on candidate susceptibility genes for a form of neurodegenerative motor neuron disease, the finding that TRPM7 is involved in neuronal death is relevant. Importantly, the recently published study describing the localization of TRPM7 in synaptic terminals of motor neurons and demonstrating TRPM7 involvement during neurotransmitter release in neuromuscular synapses [71] adds further justification for considering *TRPM7* as a good candidate susceptibility gene for Western Pacific ALS/PD.

TRPM7 in Guam ALS/PD

The T1482I TRPM7 variant in Guamanian ALS/PD

In an effort to identify a genetic variant that confers susceptibility to ALS/PD, we sequenced genomic DNA from ALS/PD samples and age-matched controls [93]. We found a heterozygous variant of *TRPM7* in a subset of Guamanian ALS/PD cases encoding a channel protein with a missense mutation, Thr^{1482} is replaced with Ile^{1482}. Alignment of TRPM7 sequences from various species showed that Thr^{1482} is evolutionarily conserved from zebrafish to humans, except in the mouse which has serine (Ser) in this position. Thr and Ser are not dissimilar since both can be phosphorylated. The evolutionary conservation of Thr/Ser in this position suggests that phosphorylation of this residue is important and underscores the potential significance of the Thr-to-Ile substitution because isoleucine cannot be phosphorylated. TRPM7 is a Ser/Thr α-kinase, it phosphorylates Ser and Thr residues in α-helices. We compared phosphothreonine levels between wild-type (WT) and T1482I TRPM7 and found that Thr^{1482} is indeed autophosphorylated. The Thr-to-Ile substitution did not affect TRPM7 kinase activity but increased the channel's sensitivity to intracellular Mg^{2+} block. Since one of the primary functions of TRPM7 is reportedly the cellular import of Mg^{2+} and Ca^{2+}, cells expressing T1482I will conceivably become more deficient in these ions. This deficiency will be more pronounced in an environment severely depleted of Mg^{2+} and Ca^{2+} to begin with, such as found in the hyperendemic ALS/PD foci in the Western Pacific. It is our hypothesis, therefore, that the *T1482I* variant confers a genetic predisposition to ALS/PD. In an environment with normal levels of Ca^{2+} and Mg^{2+}, severe deficiency does not occur, but prolonged exposure to a conducive environment (in this case one with very low Mg^{2+} and Ca^{2+} levels) unmasks the deleterious effects of the mutation. The resulting imbalance in Mg^{2+} and Ca^{2+} homeostasis could affect many cellular processes including those responding to increased oxidative stress and those causing activation of proinflammatory pathways. Unfortunately, testing this hypothesis is not so simple. *TRPM7* knockout in mice is embryonic lethal (A. Ryazanov, personal communication). Moreover, there is the additional complication that Thr^{1482} is replaced by Ser in the mouse. We are exploring other possibilities such as using a DT20 chicken lymphocyte cell line with the endogenous TRPM7 knocked-out and replaced with inducible T1482I TRPM7 (in collaboration with Drs. C. Schmitz and A. Perraud, National Jewish Medical and Research Center), although we fully recognize that this is an over-expression model. Given the newly described role of TRPM7 in neurotransmitter release in the neuromuscular junction, it will also be of interest to investigate whether synaptic

transmission is affected in the presence of T1482I, especially given that disruption and loss of neuromuscular synapse has been reported as one of earliest pathological events in ALS and occurs long before the appearance of symptoms [94,95].

Mg^2 deficiency increases oxidative stress

The demonstration that low Mg^2 conditions caused significant loss of rat dopaminergic nigral neurons lends support to the notion that neuropathological changes could ensue simply as a result of prolonged exposure to low Mg^2 [27]. What is the possible mechanism behind this? Low Mg^{2+} caused lipid peroxidation and activation of NF-κB in canine primary cerebral vascular smooth muscle cells [59]. Lipid peroxidation disrupts membrane integrity by damaging phospholipids and furthermore, increases oxidative stress through the production of reactive byproducts such as lipid hydroperoxides and reactive aldehydes [60]. The peroxidation of mitochondrial membrane lipids can alter the ionic balance in the mitochondria, causing mitochondrial swelling and increased production of ROS [96]. NF-κB activation induced by low Mg^{2+} conditions can initiate proinflammatory reactions such as induction of COX2 and iNOS in microglia, keeping them in a persistent activated state that is detrimental to surrounding neurons. Still another way that low extracellular Mg^{2+} levels can impact the cell negatively is through increased Ca^{2+}, Na^{+}, and metal influx through TRPM7, as a result of there being less Mg^{2+} to impose external block. Indeed we have seen increased Mn^{2+} influx through recombinant TRPM7 when extracellular Mg^{2+} is lowered (M. Hermosura and S. Thompson, unpublished data). Metals are redox catalysts, and will therefore contribute to the rise in cellular oxidative stress. That Mg^{2+} deficiency causes possible alterations in ion fluxes and increased oxidative stress was also suggested by gene expression studies in weaning rats exposed to Mg^{2+}-deficient diets [97].

Thus, the overall effect of low Mg^{2+} is to create conditions of high oxidative stress that is conducive to the activation of TRPM2, nonselective cation channels that have been linked to the activation of cell death pathways [46,47,90,98,99].

Oxidative stress in Western Pacific ALS/PD

Factors emanating from the unique mineral composition of the environment in the ALS/PD foci in the Western Pacific converge to produce a state of high oxidative stress. Oxidative stress is increasingly recognized as a key player in the initiation and amplification of the disease process in many disorders including neurodegenerative diseases [100] and underlies the growing interest in our next candidate gene, *TRPM2*.

TRPM2 is an ion channel that is activated by oxidative and nitrative stress

TRPM2 channels are cation nonselective channels that are highly expressed in the brain and in immune cells, especially those of monocytic lineage [101,102]. In a recent report, RTPCR was used to demonstrate widespread distribution of TRPM2 mRNA in the CNS [103]. In situ hybridization studies showed TRPM2 expression to be highest in the hippocampus, cerebral cortex, thalamus, and midbrain [104]. On a cellular level, TRPM2 was shown to be expressed in neuronal cells and microglial cells [46,104,105]. As mentioned earlier, TRPM2 is one of only three known *chanzymes*, channels intrinsically coupled to an enzyme domain. In TRPM2, the enzyme is in the C-terminal region and is designated NUDT9-H to reflect its homology to NUDT9 ADP-ribose hydrolase [106]. Although low level NUDT9-H enzymatic activity has been demonstrated, experimental data indicate that enzyme activity is not a requirement for channel gating [107]. Instead, studies indicate that NUDT9-H provides a binding site for adenine 5'-diphosphoribose (ADPR), the physiological activator of TRPM2 [108]. In addition to ADPR, millimolar concentrations of β-NAD have been shown to activate these channels [90,101]. Because of the high, non-physiological dose needed, the suggestion that β-NAD

could directly gate TRPM2 has been questioned. It was instead proposed that TRPM7 activation by β-NAD occurs as a result of β-NAD breakdown into ADPR [109].

ADPR activation of TRPM2 is modulated by intracellular Ca^{2+} [110,111]. Ca^{2+}, by itself, cannot gate TRPM2 [111]. However, Ca^{2+} is required for basal levels of ADPR to gate the channel. In granulocytes, basal ADPR was determined to be ~ 5 μM, and it remained around this level even after agonist stimulation. Ca^{2+} enhanced TRPM2 activation by ADPR. At a given ADPR concentration, higher $[Ca^{2+}]_i$ levels elicit larger TRPM2 currents, with a reported EC_{50} of 340 nM [110]. It was thus proposed that APPR and Ca^{2+} act in concert, as a second messenger system, to transduce the effects of agonist binding. The enhancing effect of Ca^{2+} on TRPM2 activation may be relevant in connection with oxidative stress-mediated neuronal death as discussed below.

TRPM2 in oxidative stress-mediated cell death

Accumulating evidence suggests that TRPM2 plays a central role in oxidative stress- mediated cell death [46,98,99,112]. H_2O_2-mediated death of cerebral cortical neurons was significantly reduced following siRNA inhibition of TRPM2 [46]. Similarly, H_2O_2-induced toxicity in striatal neurons was attenuated upon transfection with a dominant negative short form of TRPM2 (TRPM2-S) as well as TRPM2-specific siRNA inhibition [113,114]. In both cases, death was preceded by a rise of intracellular Ca^{2+} as a result of massive Ca^{2+} influx through TRPM2 channels. How oxidative and nitrative stressors activate this channel remains controversial as different studies support different candidate pathways. Nevertheless, existing possibilities can be broadly classified as direct and indirect. In the former, H_2O_2 gates the channel directly [91,104]. In the indirect pathway, TRPM2 activation is ADPR-gated, with oxidative stress simply increasing production of this metabolite. Experimental evidence supports at least two different mechanisms through which intracellular ADPR is produced by oxidative stress: one mediated by poly(ADP-ribose) polymerase (PARP) and the other through production of ADPR in the mitochondria by way of β–NAD breakdown [115,116]. Finally, it is also possible that oxidative stress-induced $[Ca^{2+}]_i$ rise could simply enhance ADPR-mediated activation of TRPM2, without any added production of ADPR [111]. As is often the case, existing in situ conditions, including cell type-specific metabolic factors dictate which pathway or pathways are recruited and it is highly likely that more than one is operating in a given situation. For example, PARP enzymatic activity and β–NAD breakdown could be happening simultaneously, increasing cytoplasmic ADPR levels. At the same time, oxidative stress-induced $[Ca^{2+}]_i$ rise enhances ADPR-mediated activation of TRPM2 channels. All these pathways could converge to create a situation where sustained Ca^{2+} influx occurs.

TRPM2 is permeable to Na^+ and other cations

It must be remembered, however, that TRPM2 is cation nonselective and that other ions (propelled by their own electrochemical driving force) permeate the channel alongside Ca^{2+}. Foremost among these is Na^+. The impact of massive Na^+ influx through TRPM2 has not been carefully assessed but one can envision that the ensuing membrane depolarization will exert a significant effect especially in excitable cells such as neurons and pancreatic β-cells. Sudden large changes in cytoplasmic Ca^{2+} and Na^+ levels after TRPM2 activation could be severely detrimental to proper mitochondrial function.One of these functions – buffering pathophysiological $[Ca^{2+}]_i$ rises, such as those evoked by oxidative stress, could be particularly susceptible because it is partly dependent on a Na^+/Ca^{2+} exchanger in the mitochondrial inner membrane [117]. Disruption in exchanger function could affect ionic homeostasis in the mitochondria, causing mitochondrial swelling and other adverse effects, among them reducing the half-life of mitochondrially encoded RNA [96,118]. Mitochondrial swelling is an early preclinical feature in neurodegenerative diseases, including ALS [119]. Strongly supporting this thesis is the recent demonstration that brain and spinal cord mitochondria in two familial

ALS (SOD1) mouse models exhibit reduced Ca^{2+} buffering capacity. Importantly, this decrease occurred very early in the course of the disease, prior to the onset of symptoms [120].

TRPM2 channels are cation nonselective and could therefore conduct transition metals, in addition to Na^{+} and Ca^{2+}. Indeed, Mn^{2+} permeation has been demonstrated using fura-2 quench experiments [90]. We recently completed studies where we used the zinc-specific dye, FluoZin-3, to demonstrate Zn^{2+} influx through TRPM2 (R. Go, C. Shetler, S. Thompson and M. Hermosura, unpublished data). The finding that TRPM2 could allow permeation of Mn^{2+} and Zn^{2+} is especially significant when viewed in the context of the possible role of TRPM2 in neurodegeneration because the abnormal accumulation of these metals has been associated with neuropathology [121]. A seminal paper on ALS/PD reported that the Mn^{2+} content in the spinal cord was 10-20X higher in ALS patients than in controls [13]. The abnormal cellular accumulation of Zn^{2+} has been linked to acute neuronal damage during ischemia and epilepsy, as well as with pathologic changes in neurodegenerative diseases [122]. A recent reassessment of metal accumulation in ALS/PD reported significantly elevated Zn^{2+} levels in the PD brain samples [123]. Altered metal homeostasis has also been described in sporadic as well as familiar ALS [124,125]. The proper regulation of metal homeostasis relies on a delicate balance between influx, sequestration by cellular buffer systems, and transport out of the cell. Transition metals are redox-active, capable of stimulating free radical formation thus increasing cellular oxidative stress. Therefore, transition metals entering through TRPM2 (and other influx pathways) create conditions conducive to more TRPM2 activation, further tilting the balance in favor of ion influx which is dominated by the physiologically abundant Ca^{2+} and Na^{+} ions. The ensuing increase in cytoplasmic Ca^{2+} and Na^{+} levels could cause mitochondria to generate more ROS, thus creating a vicious loop that could overwhelm the cell's protective machinery.

Mitochondrial Dysfunction in ALS

We have seen that as a consequence of its activation, TRPM2 can affect the proper functioning of the mitochondria. Mitochondrial dysfunction has been reported as a potential contributory factor in sporadic and familial ALS [118-120,126-129]. Unfortunately, this subject was not investigated early on in connection with Western Pacific ALS/PD, except for one mention of a possible complex I deficiency [130] and reports of degenerating mitochondria in ultrastructure studies on brain sections from autopsy specimens and animal models fed low Ca^{2+} and Mg^{2+} diets [22-27]. It appears that brain and spinal cord mitochondria are particularly susceptible to the disease process in ALS. In SOD1 models of ALS, diminished Ca^{2+} buffering capacity was only detected in brain and spinal cord, not in liver mitochondria [120]. Possible reasons for the unusual sensitivity of spinal cord mitochondria are their significantly lower threshold for Ca^{2+}-induced change in mitochondrial permeability transition (MPT), increased susceptibility to lipid peroxidation, reduced mitochondrial mRNA levels, and significantly higher levels of oxidized mitochondrial DNA [131].

Somatic mutations could contribute significantly to mitochondrial dysfunction. Mitochondrial DNA (mtDNA) is a 16.5-kb circular genome that encodes 13 proteins of the respiratory chain and 22 tRNAs. It lacks protective histones and is therefore more susceptible to oxidative damage than nuclear DNA. The high oxidative state in this organelle is expected to heavily favor more mutations. Increased frequencies of mtDNA mutations have been reported in sporadic ALS and other neurodegenerative diseases [118,127,132]. It was proposed that some of these mutations reduce complex IV activity of the electron transport chain (ETC). MtDNA from ALS subjects transferred to mtDNA-depleted human neuroblastoma cells caused abnormal ETC function, perturbed Ca^{2+} homeostasis, and alterations in mitochondrial structure [133]. We recently sequenced mtDNA from ALS/PD and found somatic mutations in the light strand promoter (LSP) region in mitochondrial DNA of brain tissue from both PD [134] and

ALS patients [R. Garruto et al., unpublished data]. The LSP exerts regulatory control over mitochondrial transcription and replication. Its crucial role in maintaining the stability of the mitochondrial genome implies that mutations in the LSP could be detrimental to proper mitochondrial function.

It has been suggested that mitochondria could act both as target and trigger in sporadic and familial ALS [135]. The apparent functional connection between TRPM2 and mitochondria lends support to this suggestion. Sudden increases in cytoplasmic Ca^{2+} and Na^{+} mediated by TRPM2 that could be handled routinely by normally functioning mitochondria could be detrimental to mitochondria where somatic mutations have caused dysfunctional ETC activities, or where lipid peroxidation has affected membrane integrity, rendering the organelle much less capable of handling ion imbalances. It is possible that higher levels of ROS are produced in these situations, causing persistent TRPM2 activation, and sustained massive Ca^{2+} and Na^{+} influx, that could overwhelm cellular buffering systems. The self-sustaining cycle of oxidative stress, $[Ca^{2+}]_i$ rises, elevated ROS, and increased TRPM2 activation, could be responsible for generating sustained Ca^{2+} influx. In the presence of dysfunctional mitochondria with diminished Ca^{2+} buffering capacities, abnormally high Ca^{2+} will accumulate. This series of events may underlie the abnormal accumulation of Ca^{2+} observed in the cytoplasm and mitochondria in brain and spinal cord tissues in ALS/PD and the other clinical variants of ALS [19,23,136]. Motor neurons are particularly vulnerable to Ca^{2+} overload because of a low cytosolic Ca^{2+} buffering capacity [119,137]. It is therefore reasonable to assume that oxidative stress and persistent activation of TRPM2 channels in motor neurons will be particularly damaging. The recent demonstration that L-BMAA-induced cytotoxic effects, $[Ca^{2+}]_i$ rises, and ROS production were substantially greater in motor than in other spinal neurons strongly support this hypothesis [44]. Importantly, the finding that L-BMAA caused substantial $[Ca^{2+}]_i$ rise and ROS production in motor neurons, conditions conducive to TRPM2 activation, suggests a possible role for TRPM2 in L- BMAA mediated neurotoxicity. TRPM2 function in motor neurons, especially in the presence of L-BMAA and other oxidative stressors, is a particularly important topic for future research.

Immune involvement in ALS: TRPM2 in Microglia

There is increasing evidence that the degeneration and death of motor neurons in sporadic and familial ALS is not limited to pathogenic processes within the neurons themselves, but involves an immune component contributed by neighboring cells, particularly microglia, the resident immunocompetent cells in the CNS [138-144]. Microglia provide early response to brain injury by releasing proinflammatory cytokines and toxic factors such as nitric oxide (NO), H_2O_2, and ROS. Activated microglia are phagocytic, possess enhanced migratory abilities and have been observed in the spinal cord ventral horn of ALS patients [145]. Microglial activity was not investigated in connection with Western Pacific ALS/PD, although defects in humoral and cellular immunity were reported [146,147]. TRPM2 is highly expressed in microglia and more importantly, it has been suggested that TRPM2 is involved in microglial activation during oxidative stress [104]. In the absence of information on microglial activation in ALS/PD, we will discuss what is known about this topic in familial and sporadic ALS.

Studies in sporadic ALS and in mouse models of familial ALS suggest that immune events occur at a very early stage in the disease process. Microglial proliferation and activation has been shown to precede and contribute to neuronal death [141]. Proinflammatory factors, presumably secreted by microglia, were detected at an early stage, when no motor neuron loss had yet occurred. Cerebrospinal fluid (CSF) in sporadic and familial ALS contains cytotoxic factors [142], one of which is 4-hydroxynonenal (4-HNE), a reactive aldehyde byproduct of lipid peroxidation [143]. It is becoming increasingly clear that interactions between motor neurons and surrounding glial cells are integral to ALS pathogenesis [138-140,148-150]. For

instance, a study using the G93A SOD1 model of familial ALS reported that disease only ensued when the mutation was expressed in both human glioblastoma and neuroblastoma cells, not when expression was restricted to either cell type [148]. Another study used a cell culture system to demonstrate motor neuron toxicity induced by lipopolysaccharide (LPS)-activated microglia [149]. Toxicity appeared to be caused by microglial-induced oxidative/nitrative stress activating a Ca^{2+} influx pathway, because it was prevented by a microglial inducible nitric oxide synthase (iNOS) inhibitor, the removal of extracellular Ca^{2+}, or by the addition of catalase or gluthathione. Finally, definitive involvement of microglia in the disease process in familial ALS was demonstrated using transgenic mice with deletable mutant SOD1 [139]. 'Turning down' mutant SOD1 expression in motor neurons substantially delayed disease onset and early progression. Applying the same manipulation to microglia did not affect disease onset but greatly slowed later disease progression and extended overall survival suggesting that microglia with mutant SOD1 accelerate the disease process.

Microglial activation is mediated through increases in cytoplasmic Ca^{2+} originating from intracellular stores and from the extracellular environment [104,151]. TRPM2 channels were shown to contribute significantly to the latter. Importantly, TRPM2 in activated microglia were shown to exhibit heightened sensitivity to H_2O_2 [104]. Activated microglia, however, produce H_2O_2 themselves (a process called respiratory burst). Therefore, the heightened sensitivity of TRPM2 to H_2O_2 in activated microglia will likely serve as a positive feedback signal, promoting more TRPM2 activation and sustained Ca^{2+} and Na^{+} influx - conditions that can keep microglia in a prolonged activated state. Such an occurrence will generate oxidative stress in neighboring neurons and glia. The combined effect of oxidative stress, subsequent TRPM2 activation, and drastic increase of cellular Ca^{2+} and Na^{+} levels will be particularly detrimental to motor neurons because of their inability to buffer huge cytoplasmic Ca^{2+} changes. Abnormal Ca^{2+} accumulation in motor nerve terminals in ALS lends support to this possible scenario [152] and to the hypothesis that increased intracellular Ca^{2+} triggered by immune mechanisms is involved in motor neuron cell death in ALS [153]. Dense populations of microglia are reportedly present in the hippocampus, olfactory telencephalon, basal ganglia, and substantia nigra [154]. Following the reasoning presented above, it can be surmised that these brain regions will be more susceptible to damage due to prolonged microglial activation. The selective loss of nigral cells after prolonged exposure to low Mg^{2+} lends support to this possibility [27]. Increased oxidative stress and NF-κB activation brought about by low Mg^{2+} could cause persistent microglial activation and nigral cell damage.

It is certainly likely that other Ca^{2+} influx pathways such AMPA/Kainate and glutamate receptors are also involved. However, the direct relationship evident in the 'microglia - oxidative stress - motor neuron TRPM2 – Ca^{2+} influx pathway' appears to be a plausible hypothesis, particularly in explaining the relationship between microglia and motor neurons and immune involvement in the pathogenesis of ALS [155,156].

Other intriguing clues to TRPM2 involvement in ALS/PD

β-amyloid peptides

These peptides have been linked to the etiology of AD and have also been found in ALS/PD. A recent study reported that β-amyloid activity is mediated through TRPM2 channels [111].

Diabetic peripheral neuropathy in ALS/PD and the role of PARP activation

In 1997, a possible relationship between diabetes mellitus and ALS/PD in Guam was noted [130]. A study published in 1999 reported that peripheral neuropathy may be a manifestation of Guamanian ALS/PD [157]. The activation of poly(ADP-ribose) polymerase (PARP) has recently been identified as an important mechanism underlying peripheral diabetic neuropathy

[158]. PARP activation has also been identified as one of the major pathways whereby oxidative stress activates TRPM2 channels [115] suggesting that Ca^{2+} influx via TRPM2 may be involved in peripheral diabetic neuropathy. Importantly, these results strongly support that suggestion that Guam ALS/PD is a multisystem disorder with a subclinical involvement of the peripheral nervous system and that unregulated TRPM2 activation, perhaps due to high oxidative stress, may be a key etiological factor.

TRPM2 mutants in ALS/PD

Sequence analysis of TRPM2 revealed the presence of three variants associated with ALS/PD. The results are being written for publication and will not be covered in this review.

Summary

A great deal of effort and major resources have been dedicated to the study of Western Pacific ALS/PD. The substantial amount of neuropathological, clinical, and epidemiological data generated over the years represents a treasure trove of useful information and clues that could be analyzed and interpreted in conjunction with more recent studies on sporadic and familial ALS. It is generally accepted that the different clinical variants of ALS likely share similar molecular pathogenic mechanisms and of these, the preponderance of evidence suggests the involvement of oxidative stress, mitochondrial dysfunction, aberrant Ca^{2+} homeostasis, and immune mechanisms, primarily mediated by activated microglia.

By virtue of their activation and permeation properties and the fact that they are expressed in motor neurons and microglial cells, we present *TRPM7* and *TRPM2* as candidate susceptibility genes in ALS/PD, and perhaps for sporadic ALS, as well. TRPM7 and TRPM2 channel activities are interconnected with the principal factors suspected of involvement in these diseases – aberrant Ca^{2+} and Mg^{2+} homeostasis, mitochondrial dysfunction, oxidative stress, and inflammatory responses. It can be hypothesized, therefore, that genetic variants of these channels could affect any or all of the suspected mechanisms and thus contribute to the disease process. While there are certainly conjectures and suppositions based on circumstantial evidence, we nevertheless hope that we have identified two promising targets for future research.

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