Review

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Review

Neurotrophic Viruses in the Pathogenesis of ALS

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Abstract: Treatment of neurological disease is hampered by the lack of validated specific and sensitive biomarkers, resulting in delayed diagnosis and nonspecific disease modifying therapies. For example, in ALS the lack of a sensitive and specific biomarker impedes the ability to administer a treatment prior to or at the onset of motor neuron dysfunction. Although viral or other infectious etiologies have been proposed as a contributing factor to neurodegeneration, it can be argued that these are casual and not causal associations. In the case of ALS, evidence for direct causality would require *in vivo* validation of specific nucleotide sequences of microbial origin which are known to be neuroinvasive with tropism for motor neurons, such as polio and non-polio. Several viral enteropathogens recapitulate the pathological events in sporadic ALS, including the ability to cleave TDP-43 resulting in accumulation of misfolded proteins in the cytoplasm. This review provides supporting evidence that motor neuron disease is causally related to specific enteroviruses and argues that prospective validation is imperative for effective prevention and treatment.

Keywords: ALS; TDP-43; misfolded proteins; enterovirus; polio; motor neurons; retrotransposons; prions; Alzheimer's

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the loss of upper and lower motor neurons in the motor cortex, brain stem and the anterior horn of the spinal cord. The etiology ALS is currently unknown, however the incidence in the number of cases across the world is projected to increase by almost 70% in the next two decades [1], suggesting a confluence of genetic and sporadic factors. Among several sporadic factors, a viral etiology has previously been proposed based upon the similarities between the molecular phenotype of ALS and enteroviruses [2].

Several related enteropathogens including polio, Coxsackie and other enteroviruses such as EV-71 have been associated with motor neuron dysfunction, but these are normally acute bases which do not progress in a step wise progression as does ALS. Further, the findings of viral genetic material in the CSF of ALS patients as previously reported can be argued as being incidental and not causal [3]. Causality based biomarkers in ALS require the fulfillment of the revised Koch criterion where a specific nucleic acid sequence corresponding to a putative pathogen is present in most cases of affected patients, but not present in unaffected patients [4]. An additional criterion to fulfill the revised Koch postulate is whether the pathological mechanisms of a particular infection are consistent with protein misfolding of RNA binding proteins which are characteristic hallmarks of ALS. In the following paragraphs we will argue that enteroviruses exert alternative splicing of host RNA resulting in post translational modifications of proteins which recapitulate the hallmark features of neurodegeneration including the accumulation of intrinsically disordered proteins which self-aggregate and cross seed unaffected cells in a prion like manner.

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TDP-43 hyperphosphorylation in ALS

Among genes linked to ALS, mutations in the gene encoding for Transactive response DNA binding Protein of 43 kDa (*TDP-43*) have been linked to familial and sporadic cases [5]. The involvement of TDP-43 in neurodegeneration was first reported when hyperphosphorylated TDP-43 was observed in the majority of patients with ALS and frontal lobar degeneration [6]. As an RNA binding protein, TDP-43 binds to more than 6,000 mRNA targets, representing nearly 30% of the entire transcriptome [7–9].

TDP-43 hyperphosphorylation leads to protein aggregation and misfolded RNA binding proteins as the hallmark of ALS and other neurodegenerative diseases, such as Niemann-Pick type C (NPC) [10,11].

As early as 1995, it was noted that TDP-43 acts as a transcriptional repressor of HIV-1 infection by inhibiting viral assembly [12]. More recently, TDP-43 has been shown to affect viral fusion and infection capacities in an epigenetic manner by altering HDAC6 levels and tubulin-deacetylase levels involved in a microtubule function [13]. Subsequent studies confirmed that hyperphosphorylated TDP-43 impairs small interfering RNA silencing, which is the major post-transcriptional mechanism of retrotransposable elements or genomic parasites as a novel mechanism of neurodegeneration in ALS [14]. TDP-43 has been shown to contain a prion-like domain that can form a self-replicating amyloid conformation [15] TDP-43 possesses intrinsically disordered domains whereby the rate α-helix-to-β-sheet transition is dependent upon post translational modifications of phosphorylation-dephosphorylation pathways [16]. Furthermore, prion-like domains may directly originate during viral genomic RNA processing involving nucleocapsid (NC) proteins responsible for virus capsid assembly and structure [17]. To date, approximately 70 human RNA-binding proteins have been reported to possess a prion-like domain and render intracellular proteins more prone to misfolding and aggregation [18].

For example, prion-like RNA binding domains have also been recognized in fused sarcoma (FUS), the most aggressive, early-onset forms of ALS [19]. Mutations in *FUS* are also associated with misfolded RNA bindings proteins in an identical matter as TDP-43 [20]. In a similar manner, FUS mutations are more sensitive to retroviruses and exacerbate cytoplasmic mislocalization and protein misfolding [21]

The misfolding of proteins in ALS gives rise to hydrophobic PrP^{Sc} aggregates which are insoluble filaments comprised of amyloid and tau like plaque. Prior studies have demonstrated that β-amyloid aggregates exhibit many properties indistinguishable from those of prions, including the ability of amyloid fibrils to self-propagate [22].

These observations suggest that common mutations in ALS disinhibit host mediated viral repression by targeting prion like domains, resulting in protein aggregation and motor neuron degeneration in a prion like manner.

Part I: Enteroviruses in the etiology of motor neuron disease

Enteroviruses are now regarded as the most important neurotropic enterovirus in the Asia-Pacific region in the post-polio eradication era [22,23].

Collectively, Enteroviruses are single-stranded positive-sense neurotrophic RNA viruses including polio, coxsackievirus A and B types, and numerous other enteroviruses, including EV-68 and EV-71 [24]. EVs are non-enveloped viruses comprised of a viral capsid that contains a single open reading frame (ORF) which encodes four structural proteins, VP1–4 and seven non-structural proteins (2A–C and 3A–D) including RNA-dependent RNA polymerase ($3D^{Pol}$) [25].

Enteroviruses are primarily transmitted through the fecal–oral route where infections can be limited to febrile illnesses but in more severe cases can result in meningitis, brainstem encephalitis, and acute flaccid myelitis through retrograde transmission from the gut to the brain [26]. In the case of Polio, the enterovirus specifically targets motor neurons, resulting in cases that are clinically indistinguishable from ALS. Coxsackie and enterovirus 71 also target motor neurons and have been associated with outbreaks of acute flaccid paralysis in several regions of the globe, including in the US [27].

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The establishment of a causal relationship between enteroviruses and motor neuron disease has resulted from epidemiological studies where the isolation of viral particles was discovered in spinal cord samples of affected patients with motor neuron disease [28]. In non-polio enteroviruses, amplification of a 414 base RNA target sequence in the conserved enterovirus 5' untranslated region was found in eight of 11 cases of sporadic motor neuron disease, suggesting that the class of enteroviruses display neurotrophic features similar to polio [29]. Subsequently, in later studies, qRT-PCR analysis of cerebrospinal fluid of EV-71, a newly emergent enterovirus detection was detected in 14.5% of 242 ALS patients and 7.6% of 354 controls [3].

It is believed that EV-71 emerged in the mid-20th century, as indicated by an analysis of viral capsid sequences similar to those found in other enterovirus genotypes belonging to common ancestral genogroups. The emergence of EV-71 was likely due to a recombination event between a portion of the genome of CV-A16 and the 5′-NTR of poliovirus. [30,31].

These shared nucleotide sequences between phenotypically different viral pathogens are likely based upon evolutionarily events between host and pathogen. Darwinian like pressures most likely have driven enteroviruses to “best fit strategies” in the context of population bottlenecks imposed by competitive forces in host- infection dynamics and mitochondrial energy barriers. As enteroviruses have high mutation rates, “best fit” sub-strains of enteroviruses categorically lead to the formation of quorums, where structural genes within the viral capsid maintain a degree of molecular fidelity across different viral species, which are noted as quasi-species [32].

In neurotrophic viruses including all enteroviruses, redundant RNA sequences within the viral capsid act as an “ancestral” hub for successful retroviral replication by encoding Activity-regulated cytoskeleton-associated protein (Arc-Gag complexes). The Arc-Gag complex facilitates the assemble of viral RNA genomes into a spherical virion [33] Interestingly, evolutionary analysis indicates that Arc protein complexes are derived from retrotransposons, which are also ancestral to retroviruses with highly conserved homology to human protein isoforms, repurposed during evolution to mediate intra and intercellular communication at synaptic junctions via specific RNA sequences [34].

Part II: Raiders of the lost Arc: How viral Arc-Gag complexes produce neurodegeneration:

Neurotrophic viruses maintain a rudimentary level of “consciousness” with the capacity to “communicate” between separate species, “perceive” time and space at a molecular level and engage in “quorums” through neurotransmitter signaling through selective gene editing. These “best fit” strategies in the viral quorum are driven by phosphorylation- dephosphorylation pathways encoded within Arc to ensure viral particles to infect cells at the neuromuscular junction [35] .

Within the viral capsid the Arc-Gag complex encodes several ATPase-dependent viral helicases which directly participates in enteroviral RNA replication [36]. It is interesting to note that activation of Arc results in epigenetic based histone modifications such as H3K27Ac which have recently been shown to be upregulated in late-onset Alzheimer’s disease, suggesting its possible role in viral mediated neurodegeneration [37]. In support of this hypothesis, the Arc complex binds to the amyloid precursor protein and presenilin1 whereby genetic deletion of Arc reduces Aβ load in a transgenic mouse model of AD [38]. In humans, the Arc-Gag complex binds to calmodulin-dependent protein kinase II α and β (CaMKIIα, and CaMKIIβ, respectively) resulting in cellular depolarization and the activation of glutamate and calcium channels (Zhang et al., 2019), well described pathways involved in the pathogenesis of neurodegeneration (Figure 1).

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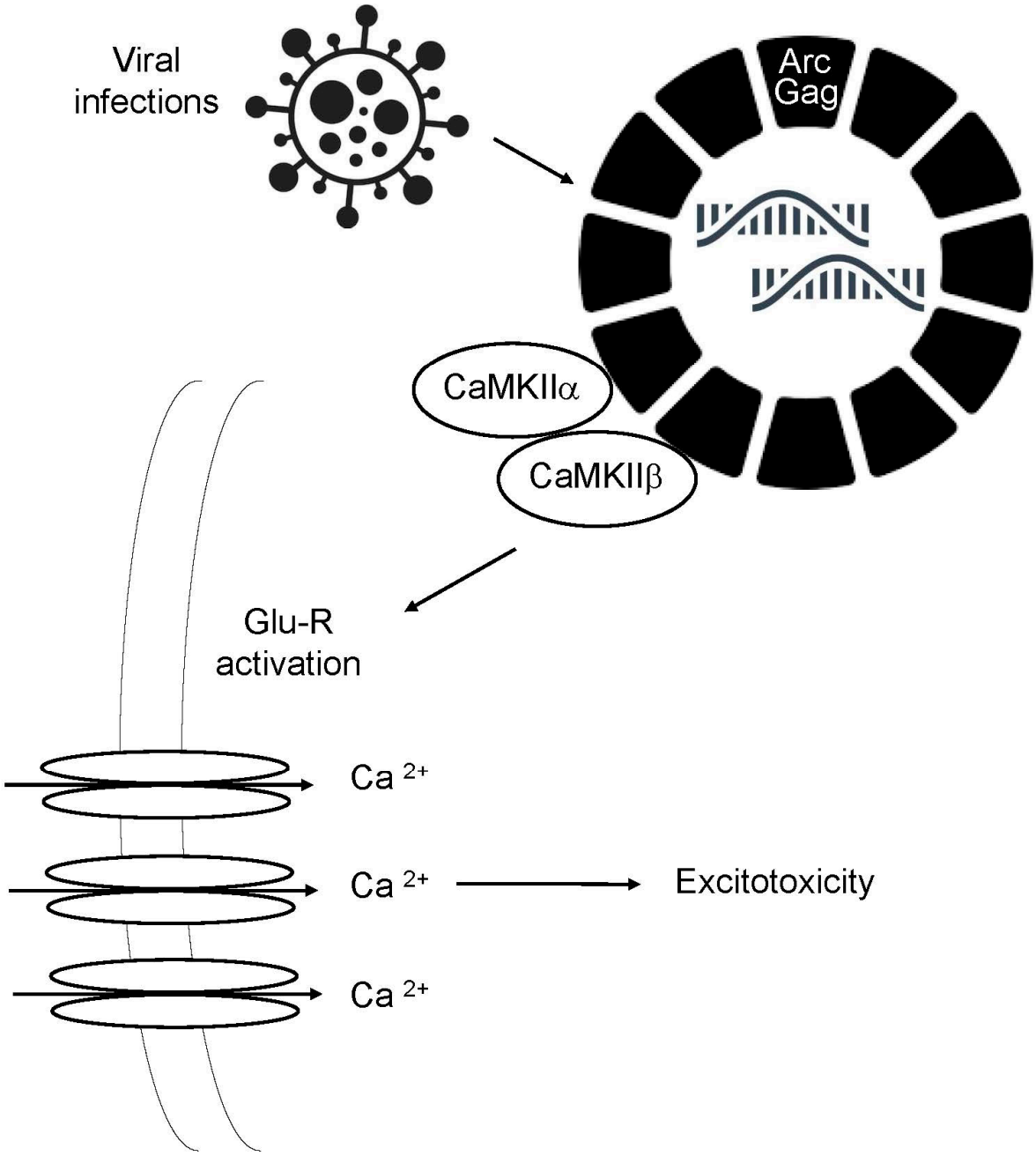

Figure 1. Virus-mediated excitotoxicity. The Arc-Gag complex normally interacts with the plasma membrane, weakening excitatory synapsis. CamKIIa and b phosphorylates the Arc-Gag complex of the virions, decreasing its canonical function, leading to the clustering and activation of glutamate receptors and calcium efflux, which mediates excitotoxicity.

Viral capsid containing Arc-Gag complexes also induces protein aggregation due to excessive activation of CaMK-II [40] particularly the 3C protease to hijack the Endosomal Sorting Complex Required for Transport-III. Viral proteases are capable of penetrate nuclear pores surrounding the nucleus of host cells as a common feature of many structurally different viruses, including HIV[41] Infection due to Coxsackievirus, for instance, has been shown to result in abnormal accumulation of insoluble, misfolded TDP-43 protein aggregates due to a 3C protease, which cleave TDP-43 rendering it incapable of acting as a critical anti-viral repressor protein [42,43]. In EV-71 infection, similar mechanisms relay to 3C proteases facilitates viral RNA replication [44]. Interestingly unbiased analysis of the Sars-Cov2 viral genome also has revealed capsid dependent release of protease 3C

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protease, which has a direct effect on TDP-43 ectopic expression [45]. Thus, divergent viruses exert similar mechanisms of cytotoxicity involving the activation of 3C proteases suggesting an evolutionarily preserved feature of the Arc-Gag complex.

Gag polyproteins are the only viral protein required for the formation of viral budding as the nucleocapsid domain of Gag is responsible for recognition of specific dimerization and packaging signals in viral RNA [46]. Conversely, incomplete processing at any of the Gag cleavage sites is highly detrimental to virion by disrupting individual steps in the Gag processing cascade [47]. The Arc-Gag complex target host nucleoporins, (including nup62 which forms the central core of the nucleocapsid domain) to promote transport from the cytoplasm to the nucleus to ensure viral replication [48]. It is interesting to note that truncated forms of nup62 have been detected in pre-symptomatic stages of ALS [49].

Part III: The devil is in the details: The Arc-Gag complexes specifically modulates viral helicases.

The Arc-gag complex also encodes Na/K ATPase, a sodium/potassium mitochondrial enzyme where binding of the ATP1A1 domain results in the induction of viral endocytosis by alternative RNA splicing mechanisms [50]. Na/K-ATPase acts as a key scaffolding protein which interacts with protein kinase C and phosphoinositide 3-kinase, both of which have been implicated in ALS by regulating the phosphorylation status of adducin- a protein essential for the stability at the neuromuscular junction. Na/K ATPase changes the phosphorylation of adducin reducing the synaptic integrity at the neuromuscular junction in ALS [51]. Enrichment of α2-Na/K ATPase (α2-NKA) has been reported in astrocytes expressing mutant superoxide dismutase 1 [52]. Additionally, astrocytic α2-NKA is elevated in postmortem human brain tissue from AD and progressive nuclear palsy, suggesting a common pathogenic link between overexpression of Na/K ATPase and neurodegeneration [53]. Furthermore, Na^+/K^+-ATPase also appears to modulate disease-specific conversion of prion proteins [54]. Whereas knockdown of α2-Na/K ATPase in $SOD1^{G93A}$ astrocytes markedly inhibited their ability to induce degeneration and protected motor neurons from degeneration in vivo [55].

Viral infectious also exert cytotoxicity through post translational mechanisms involving their ability to alter the phosphorylation status of RNA proteins, such as TDP-43, as mentioned earlier. These hyperphosphorylation events may occur as a result of infection-mediated changes in inorganic polyphosphates (PolyP). Polyp provides phosphate groups for ATP synthesis in all life forms, from viruses, bacteria and humans. As linear polymers, they are essential for the survival of enteropathogens, including E. Coli [56] and are used by bacterium to manufacture amyloid like biofilms [57] (Figure 2). In viral disease, PolyP provides phosphate groups for RNA polymerase enzymes to assist in the fidelity of the viral genome by providing phosphate groups to their nucleotide sequences. For example, in the case of poliovirus and enterovirus 71, genome maintenance and propagation are dependent on RNA-dependent RNA polymerases derived from polyphosphate [58].

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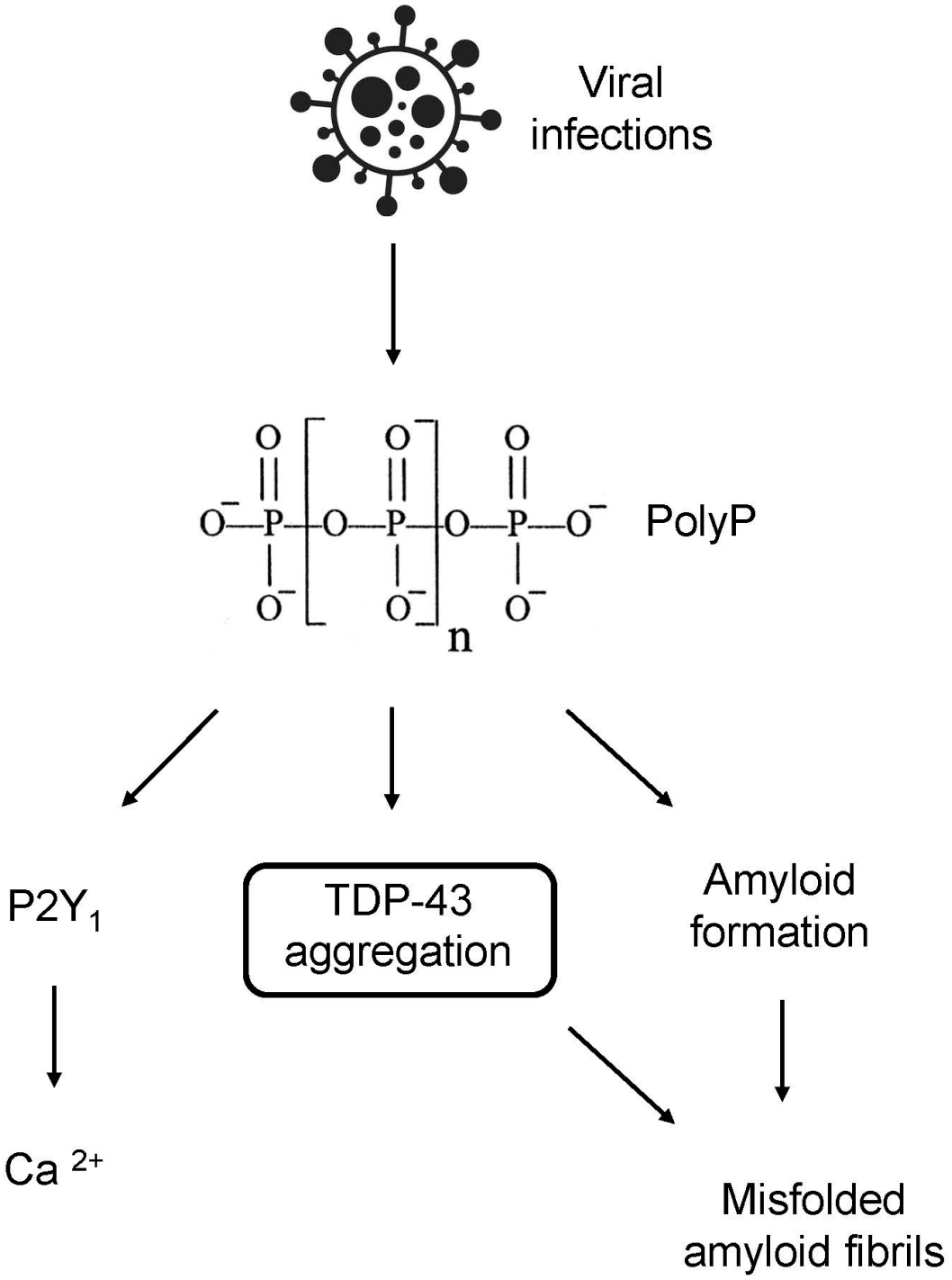

Figure 2. PolyP in neurodegeneration. Viral infections lead to increased levels of PolyP, triggering the phosphorylation and aggregation of TDP-43, the formation of misfolded amyloid fibrils, and activation of P2Y1 receptors and, subsequently, calcium influx. TDP-43 buildup also contributes to the formation of toxic amyloid fibrils.

In the human brain, PolyP act as a gliotransmitter in astrocytes, whereby it stimulates the activation of intracellular calcium by binding to purinergic receptors $P2Y_1$ in the brainstem, resulting in calcium spikes and excessive depolarization of neurons [59]. Elevations of PolyP have also been shown to impair pathogen clearance, antagonize phagocyte recruitment, and suppresses hundreds of type I interferons-necessary for host immune viral repression [60]. Elevations of PolyP were first observed in 1955 in the CSF of over one hundred and four patients with polio [61]. More recently, increased polyphosphate concentrations were found in ALS astrocytes and in the CSF, resulting in

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toxicity of motor neurons [62]. *In vitro*, PolyP binds to tau and accelerates its aggregation, in the presence of tubulin or microtubules [63]. Perhaps, this is the mechanism by which infusion of ALS-CSF in hTDP43^{WT}mice triggered motor and cognitive dysfunction, resulting in cytoplasmic TDP43 proteinopathy, neurofilament abnormalities and neuroinflammation [64]. Furthermore, TDP43 phosphorylation is mediated by defects in T cells immunity including specific subset receptors such as CD28 [65]. Defects in CD28 have been reported in ALS patients, suggesting a reduced capacity of naïve T cells to recognize common viral pathogens, resulting in immune senescent state [65]. Granulocyte-colony stimulating factor-CSF, an FDA-approved drug for cancer patients who are immune deficient, acts as a costimulatory factor for T cells CD receptors which when activated reduces TDP43 hyperphosphorylation[66].

Part IV: Unresolved questions

One of the most perplexing questions regarding the potential pathogenesis of ALS as a chronic infectious disease is why only certain individuals develop motor neuron disease and not others as these pathogens are ubiquitous in the population? According to the Koch hypothesis, only individuals who have evidence of a microbial infection should manifest a particular disease whereas those who do not have symptoms will not as otherwise the infection is not causal. Enteroviruses are common but only a small number of infected individuals develop signs and symptoms. Thus, viral infections are necessary, but not sufficient to trigger ALS. The likely explanation regarding only a subset of patients with enterovirus relates to the inherent capacity to develop ALS secondary to immunosenescent states imposed upon by viral mediated editing of adenosine to inosine, resulting in Adenosine deaminase deficiency and aberrant activation of antiviral innate immune For example, in ALS neuronal astrocytic progenitor cells with C9orf72 or sporadic mutation have demonstrated defects in the conversion of adenosine to inosine secondary to adenosine deaminase deficiency [67].

In conclusion, viral induced neurodegeneration involves a pathological cascade beginning with the Arc-Gag protein complexes released by retrotransposons and retroviruses, which encodes specific viral helicases shared by HIV, and enteroviruses such as polio and others, such as EV-71. Release of these helicases results in abnormal RNA editing of RNA based proteins such as TDP-43 and adenosine deaminases which disrupt host defense mechanisms, resulting in viral derepression to enhance viral replication. The fact that viral enteropathogens recapitulate motor neuron disease through similar mechanisms that involves PolyP resulting in the formation of insoluble protein aggregates in an amyloid like manner, supports our hypothesis that ALS is virally-mediated.

Part V: Concluding remarks: A call to Arms:

The prospective validation of the viral hypothesis of ALS can be enabled by viral specific nucleic acids may be quantified using qPCR methods or by newer digital PCR methods. These emerging technologies can detect spliced versus un-spliced RNA in affected individuals at risk or displaying evidence of neurodegeneration. However, most current viral sequencing technologies provide no coverage of noncoding regions, for example in the untranslated region of polioviruses [68]. These new sequencing methods will ultimately fulfill the Koch hypothesis by proving causality, and in the event, offering the promise of targeted therapies for neurodegeneration. Prospective studies of the CSF of ALS patients involving elevations of inorganic polyphosphates should be considered to replicate previous findings by Arredondo, et al [62]. Various FDA-approved phosphatases, in this regard, act to reduce PolyP by promoting CD28-response elements by enhancing calcineurin, which in turn can target TDP43 as a disease modifying therapy. As ALS is an invariably fatal disease without a cure, the primary goal of this paper is to implore clinicians and researchers to look beyond our current theories of ALS pathogenesis and consider a broader explanation regarding the cause of ALS being infectious mediated.

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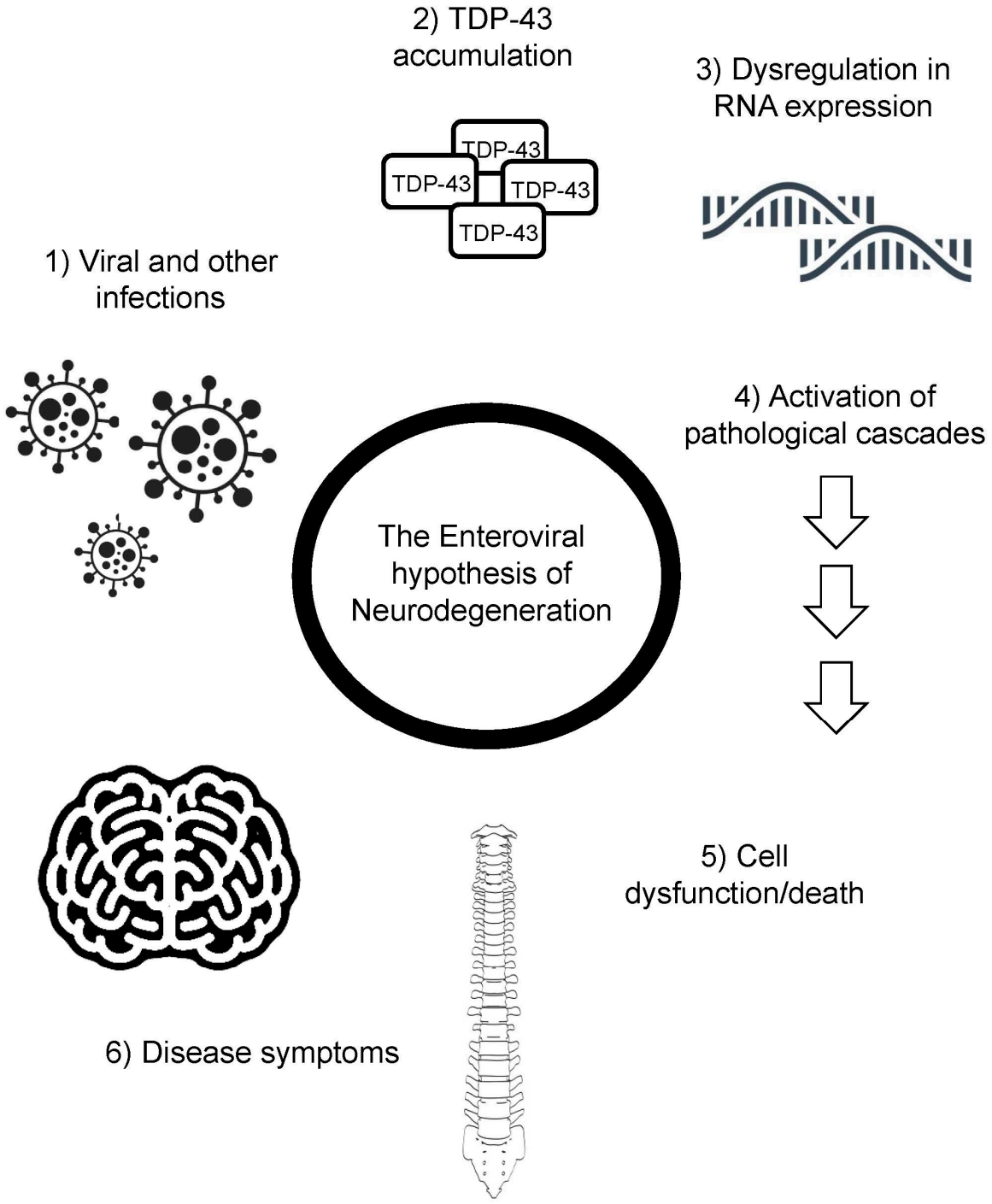

Figure 3. The enteroviral hypothesis of neurodegeneration. Viral infections lead to TDP-43 phosphorylation and aggregation, altering RNA expression, activating pathological cascades, leading to cell dysfunction/death, and the characteristic symptoms of ALS.

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