Comparative Analysis of Competing Hypotheses Regarding the Molecular Pathogenesis of Huntington's Disease

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Abstract

The hallmark molecular mechanism of Huntington's Disease is neuronal intranuclear inclusion bodies consisting of polyubiquitinated mutant huntingtin (mhtt) aggregates expressing an expanded polyglutamine tract. Aggregate-prone fragments of mhtt bind to several intracellular proteins in addition to the glutamate neurotransmitter receptor N-methyl-D-aspartate (NMDA). This review will detail the molecular and cellular pathways by which mhtt aggregates form clumps in inclusion bodies (IBs) by the impairment of the ubiquitin-proteasome system and the autophagy-lysosome system. It will also review the manner by which intracellular mhtt causes neuronal excitotoxicity via NMDA-receptor mediated excitotoxicity. Numerous studies on models of HD have presented various hypotheses on the pathogenesis of neurodegeneration, such as the debate on whether IB formation is a response mechanism of HD or its cause. This paper will discuss the ongoing debate and suggest future research that may reconcile the competing theories. Finally, this article will highlight current and future therapeutic targets for HD in the context of these pathogenesis theories.

(1) Introduction

Huntington's Disease (HD) is a fatal autosomal dominant neurodegenerative disease with a major hallmark of neuronal death in the striatum. GABAergic medium-sized spiny neurons (MSNs) in the striatum area of the brain's basal ganglia are the most susceptible to neurodegeneration (Albin 1995), however neural death also takes place in the cerebral cortex and along the corticostriatal pathway of HD brains. Patients diagnosed with HD display a variety of motor, cognitive, and psychiatric symptoms that impair everyday wellbeing, including but not limited to dementia and chorea (uncontrollable movements). Its primary pathological feature is cell death due to protein aggregation and neuronal excitotoxicity. The leading theory is that protein aggregates interfere with N-methyl-D-aspartate (NMDA) receptors via multiple pathways and lead neuronal excitotoxic processes.

The disease is caused by a CAG repeat mutation in exon 1 of the gene *huntingtin* (htt), resulting in an expanded polyglutamine (polyQ) tract near the N-terminus. The mutated form of this protein is known as mhtt. The expanded polyQ tracts cause the mutated protein to be more prone to misfold and form intracellular aggregates which clump in neuronal intranuclear inclusion bodies (NIIs). However, whether the presence of IBs directly correlates to HD pathology is widely disputed. This debate is widespread in the study of HD pathogenesis and many other competing hypotheses revolve around this. For instance, there is a major

controversy around whether mhtt is a gain-of-function mutation or a loss-of-function. Additionally, there are multiple supported theories pertaining to the exact molecular mechanisms behind IB formation, such as the transglutaminase hypothesis and the polar zipper hypothesis.

Since the discovery of the *huntingtin* gene in 1993, researchers have developed efficient genetic models of HD to study its neuropathology and symptomatology. Most HD models fall into two categories: genetic and nongenetic. In nongenetic models, researchers are able to induce cell death by excitatory mechanisms and by disrupting mitochondrial function (and thus ATP production). Transgenic and knock-in models are also used frequently in HD research (the R6/2 transgenic mouse model first reported in 1996 is the most widely used to date for HD) (Mangiarini et al.1996). HD models are differentiated in terms of variations of length in the htt polyQ, and the length of the polyQ tract is directly proportional to the severity of neurodegeneration. The R6/2 model has around 150 CAG repeats (polyQ=150) while the R6/1 has polyQ=115.

Later in the review, these various competing hypotheses will be discussed in order to point a way forward for future research. Solidifying knowledge regarding molecular pathways and the various protein structures that describe HD neurodegeneration would provide the tools to develop specifically targeted therapeutics for the disease.

(2) Genetic basis of HD

HD is caused by a mutation in the IT-15 (or htt) gene in exon 1 located at the chromosomal location 4p16.3 coding for an expanded polyglutamine tract (polyQ). Glutamine is coded for by the trinucleotide sequence “CAG”, and therefore this mutation can be classified as a CAG repeat mutation, which is common in neurodegenerative and late onset diseases like Huntington’s. There is much debate relating to the exact functions of the non-mutated htt, however most scientists agree that it plays an important role in neuronal homeostasis and prenatal development. Some studies suggest it might be used in repairing damaged DNA.

The length of the polyQ is the main factor affecting age of onset and severity of neurodegeneration. In normal htt, there are between 10 and 36 CAG repeats, however variation exists. If there are less than 26 repeats, the person will not express the HD phenotype. If there are between 27 and 35 repeats, the person will not show symptoms, however this number can increase in subsequent generations with paternal inheritance. CAG repeats between 36-39 usually have variations where some people do get HD and some don’t. Finally, if the length of the polyQ is greater than 40 repetitions, the person will display the HD phenotype (Myers 2004). After this threshold, polyQ tract length positively correlates to onset of the disease and the more severe the neurodegeneration.

(3) Mhtt aggregates and inclusion body formation

HD is primarily caused by a CAG repeat mutation in the htt gene, resulting in an expanded glutamine amino acid sequence (polyQ) near the N-terminus of the resulting polypeptide. This modulation of the htt polypeptide causes it to misfold and become insoluble and prone to aggregate, thus creating intranuclear inclusion bodies (Hoogeveen et al. 1993) and cytoplasmic aggregates. Many analyses of htt protein aggregates *in vitro* and *in vivo* have linked a higher concentration of intracellular inclusions with a faster onset, generally due to the observation that inclusions interfere with intracellular and peripheral proteins (such as intranuclear proteins and glutamate receptors) involved in cellular homeostasis and transcriptional regulation. It follows that a higher quantity of aggregates will result in a faster neuronal death.

(3.1) Molecular structure of aggregates

The huntingtin protein is very important for cellular homeostasis and prenatal development, with functions including vesicular transport and chemical signaling. It interacts with a variety of intracellular proteins, including one that is related to htt closely. HAP-40 (huntingtin associated protein of 40kDa) has endosomal function, supporting the hypothesis that htt is used in cellular transport. Using cryo-electron microscopy (EM), the structure of huntingtin was determined to contain three domains wrapped compactly around HAP-40 (Guo et al. 2018) with an N-terminus, a bridge domain consisting mainly of supercoiled α-helical structures, and a C-terminus. However, the N-terminal domain was unfortunately not fully resolved in cryo-EM structure. Even so, proteolytic loops in this domain containing the polyQ tract are said to be the root of htt toxicity and the site of potential post-translational modifications.

Using electron microscopy and glutathione S-transferase-huntingtin (GST-HD) staining, Scherzinger et al. (1997) was able to conclude that amyloid-like protein fibrils (similar to that in other neurodegenerative diseases) formed by proteolytic cleavage of GST-HD51 with polyQ=51 are present *in vitro*. Using this data, the same study used Western blotting and an anti-HD1 antibody in a line of R6/2 brains and found that a surprisingly high molecular weight was found in the nuclear fraction *in vivo*, similar to that obtained by the proteolytic cleavage of GST-HD. However, this method did not reveal immunoreactive bands in the cytosol of R6/2 brains, contradicting the common consensus that htt aggregates are present in both the cytoplasm and the nuclear regions.

(3.2) Transglutaminase aggregation hypothesis

One hypothesis that has been developed to explain mhtt aggregate formation is the transglutaminase hypothesis. First proposed in 1993, Green et al. (1993) suggested that as a result of excessive polyQ length, htt proteins could become aggregated by transglutaminases. Transglutaminases (TGs) are multifunctional enzymes that catalyze the bonding between γ-carboxamide glutamine residues in peptide bonds as substrates along with lysine residues,

resulting in post-translational modification. The mutant htt elongated polyQ tracts can bond with neighboring lysine polypeptide chains in order to aggregate. Several TG isoforms have been classified, however the function hypotheses have only been developed for TG2 and TG6 (Lorand and Graham 2003, Iismaa et al. 2008). In the case of HD pathogenesis, several studies have shown that TG2 and TG6 are involved.

Transglutaminase 2, or TG2, has been postulated to play a role in autophagy, cell adhesion, signal transduction and cell stress response (Fesus and Piacentini 2002, McConoughey et al. 2010, D'Eletto et al. 2009, D'Eletto et al. 2012). TG2 has been hypothesized to be involved in neurodegenerative diseases such as Alzheimer's disease (AD) and HD, since TG2 activity increases in AD and HD brains (Jeitner et al. 2009). In support of this hypothesis, substrates for TG2 include Tau protein, amyloid-β peptide (Aβ), and mhtt. Tau protein and Aβ are hallmarks for AD and other neurodegenerative diseases such as frontotemporal dementia (Pick's disease) and corticobasal degeneration. Additionally, evidence was presented that TG2 ablation leads to a decreased neuronal intranuclear inclusions (NIIs) concentration *in vivo* (R6/1) as well as *in vitro* with red-staining immunohistochemistry on aggregates. Overall, it is clear that transglutaminase-2 may play a significant role in HD pathology when it comes to formation of high-molecular weight (HMW) N-terminal fragments and their resulting NIIs.

Transglutaminase 6 (TG6) is another major player in the neurodegenerative processes in HD. TG6 is solely confined to neurons, but the exact function of the enzyme is not fully known, however it may be involved in neurogenesis and motor-neuron homeostasis (Thomas et al. 2013). In the BACHD mouse model (human mhtt exon 1 with loxP added; polyQ=97), TG6 distribution was widespread and prominent, however protein expression was highest in the cerebral cortex (Schulze-Krebs et al. 2021). TG6 concentration was higher than any other transglutaminase isoform. In the same study, TG6 was also very prominent and widespread in brains of tgHD rat models (polyQ=51) compared to *wt* (wild-type) htt.

(3.3) Protease aggregation hypothesis

Mhtt can be cleaved by a number of enzymatic proteins, namely caspases (cysteine-aspartic proteases), calpains (calcium-activated proteases), and MMPs (matrix metalloproteinases).

Initially, htt was first identified as a caspase substrate cleaved during apoptosis and linked to a neurodegenerative disorder (Goldberg et al. 1996). This study also presented evidence that cleavage efficiency is dependent on polyQ length. It has also been shown that proteolytic htt fragments cleaved by caspases were detected in HD brains before striatal neurodegeneration, supporting the hypothesis that caspase-dependent cleavage of htt may correspond to HD pathogenesis through formation of aggregation-prone fragments (Wellington et al. 2002).

Calpains have also been shown to catalyze the proteolysis of htt (Gafni and Ellerby 2002). These are proteases activated by increased intracellular Ca^{2+} levels, such as from excessive NMDA receptor activation and glutamate intake). Intracellular aggregates can then form due to cleavage by activated calpains. Additionally, recent RNAi and chemical compound *in vitro* screening have revealed that calpains can control levels of htt aggregates via the autophagy-lysosome pathway (Williams et al. 2008). Inhibition of calpains may activate aggregate degradation by lysosomes in addition to stopping aggregate production. This concept has resulted in potential therapeutics targeting the autophagy-lysosome calpain pathway to decrease htt aggregate accumulation.

Lastly, MMPs are another possible moderator of htt aggregation by proteolytic cleavage. Decreased MMP activity in HD mouse models has shown a subsequent decrease in aggregate concentration and by effect a decrease in neural degeneration (Miller et al. 2010). Specifically, they found that MMP-10 and MMP-14 (two members of the MMP class) are expressed in striatal HD models using Western blotting analysis. Additionally, they reported that MMP activation frequency was significantly higher in HD models where polyQ=111 than those where polyQ=7. For this study, tests were performed *in vitro* on cultured model systems as well as *in vivo* on members of the *Drosophila* genus, and R6/2 and YAC128 mouse models.

(3.4) Other aggregation hypotheses

Another proposal to the mechanism of htt aggregation is also known as the polar zipper hypothesis. A polar zipper is a β-pleated sheet of antiparallel strands held together with strong hydrogen bonds at the side-chain. Perutz et al. (1994) first proposed the hypothesis that htt aggregates form by these polar zippers, and since then, various *in vitro* and *in vivo* studies have presented evidence justifying it. For example, it was found that GST-HD51 formed β-sheet amyloid-like fibrils as a result of its proteolytic cleavage (Scherzinger et al. 1997).

Lastly, another hypothesis on inclusion formation has been studied, one that describes IB accumulation in 4 distinct phases characterized by formation of oligomers (oligomerization) (Ossato et al. 2010). The first phase (accumulation phase) initially occurs when mhtt begins to be expressed at a low intracellular protein concentration where there are only monomers in the cell. In the second phase (oligomerization phase), the monomer intracellular concentration reaches a certain value and it begins to form into oligomers. This occurs when polyQ ≥ 46. In the third phase (nucleation phase), monomers and oligomers start forming nucleation sites in the cytoplasm. In the final phase, the majority of the mhtt is recruited by the neuronal inclusions and the remaining monomers are left at a lower concentration.

(3.5) Neuronal intranuclear inclusions (NIIs)

NIIs are clumps of polyubiquitinated htt proteins located in the nucleus of neurons and are an extremely controversial and debated topic among HD researchers due to the controversy around their pathogenic or beneficial role. A pronounced correlation between the frequency of NIIs and the length of N-terminal polyQ can be observed in striatal MSNs expressing the HD phenotype. NIIs can be located and measured in neurons using anti-ubiquitin N-terminal huntingtin antibodies. Using ultrastructural analysis, Davies et al. (1997) was able to gleam a novel mechanism of NIIs in which the appearance of these inclusions produces a pronounced indentation in the nuclear membrane as well as an increased concentration of nuclear pores.

The structure of NIIs was determined near the end of the 20th century, using methods such as immunocytochemical ubiquitin staining to observe size and composition. It was found that these lesions appeared as round inclusions approximately the size of the nucleolus (1-2 μm), however sometimes round and elongated forms were identified (Becher et al. 1998). This study used 20 human patients who had HD for several years before the subjects' brains were autopsied, and not mouse models, therefore it is likely that the validity and accuracy of their results is high.

Some of the significant features of cytoplasmic and nuclear inclusion morphology have been revealed through correlative light and electron microscopy. As measured by Riguet et al. (2021), in HD neurons where polyQ=72, ~85% of inclusions were located in the cytoplasm, and ~15% in the nucleus. They found a distinct difference between the two: nuclear inclusions contained fibrillar structures surrounding its main complex, however it lacked the core/shell organization and the membranous structures embedded in the compound that cytoplasmic inclusions did express. These results suggest that the location of polyubiquitinated neuronal inclusions is a factor in determining its molecular structure, which may in turn form causation with the molecular pathology of HD.

Additionally, a pronounced correlation between the frequency of NIIs and the length of N-terminal polyQ can be observed in striatal MSNs expressing the HD phenotype. It is already well known that a longer polyQ tract results in a more compact polyQ domain that accelerates aggregatory processes. No inclusions formed in cells overexpressing $HTT_{ex1}16Q$, consistent with previous data. A sparse population of inclusions were observed in cells overexpressing $HTT_{ex1}39Q$, however these were only detected in the cytoplasm. Finally, in neurons expressing mhtt with a polyQ=72, a significantly more dense reading of inclusions were made, with an exaggerated core in each inclusion. These cultured neurons with 72Q mhtt also underwent apoptosis after 96 hours post-transfection.

(3.6) Ubiquitin-proteasome system (UPS) impairment

One of the major hallmarks of HD progression and pathology is the presence of NIIs, which themselves are tagged by a presence of ubiquitin (Ub). It is well known that impairments in the ubiquitin-proteasome system have caused many neurodegenerative disorders, and thus it may additionally be a cause of HD. This protein degradation system operates both in the nucleus and cytoplasm of all cells, targeting, recycling, and degrading short-lived and misfolded soluble proteins (Hershko and Ciechanover 1998) tagged with the ubiquitin post-translational modification. While the autophagy-lysosomal system (including the double-membraned autophagosome) is also important in HD progression, it has been suggested that the UPS system impairment plays a more prominent role in removing toxic and aggregate-prone N-terminal htt fragments (Li et al. 2010).

UPS is extremely important for functional neurons and is involved in processes like neuroplasticity, memory, and neurotransmitter regulation in the synaptic cleft (Krug et al. 1987, Fonseca et al. 2006, Karpova et al. 2006). It consists of two major steps in the degradation pathway. First, proteins are tagged for degradation by Ub. Second, a proteasome lyses the substrate into fragments with the help of many cofactors and coenzymes. In the first step, Ub must be covalently conjugated at the C-terminus, forming chains ($n \geq 4$). Three enzymes are required for this step to be functionally complete, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase), which act together to form a tight conjugation between Ub and the substrate (Hershko and Ciechanover 1998, Pickart 2001). In the proteasome complex, two main subunits administer the ubiquitinated protein degradation, the 19S and the 20S subunits. The 19S subunit recognizes, unfolds, and translocates the substrate before being placed in the 20S subunit for decomposition (Voges et al. 1999, Hartmann-Petersen et al. 2003). Deubiquitinating enzymes are used before degradation to remove the ubiquitin tags and their corresponding chains from the substrate once it has been recognized by 19S. (Kawakami et al. 1999).

Several instances of genetic and cellular evidence support the hypothesis of UPS impairment in HD. For one, many other neurodegenerative diseases are triggered by mutations in different stages, such as ubiquitylation, deubiquitylation, and substrate delivery (Van Leeuwen et al. 1998). Impairment in any of the steps of the UPS pathway may deter the ability of the system to degrade the necessary proteins required for cellular homeostasis. For another, when UPS was inhibited, an increase in htt aggregate formation followed, forming inclusions similar to those in Parkinson’s Disease (PD), such as Lewy body-like inclusions (McNaught et al. 2004).

One hypothesis describes a mechanism of proteasome sequestration resulting in impaired UPS function. NIIs are labeled with various UPS antibodies, suggesting that there is either a direct or indirect interference with the proteasome, either from htt itself or htt-associated proteins (HAPs). In one study, it was shown that the 26S proteasome is continuously assembled to degrade a

substrate just to be disassembled again (Konstantinova et al. 2008). This may prove to be data that supports this hypothesis since IBs could directly limit the subunit from assembling into its degradation-active state. However, newer evidence is arising that counters this hypothesis with the notion that UPS proteasomes are already dynamically active and remain so for substrates without interference.

Another study presented evidence that completely disrupts the UPS hypothesis. The three proteolytic activities of the proteasome work by cutting different types of peptide bonds, respectively, after the basic, hydrophobic, and acid residues (DeMartino and Slaughter 1999). However, glutamine does not fall into any of these categories, and thus, it may not be a substrate of proteolytic cleavage by UPS. Instead, its function in proteostatic imbalance may be to effectively clog up and block the proteolytic subunit of the proteasome, rendering other substrates undegradable.

(3.7) Autophagy-lysosome pathway impairment

The autophagy system is another major factor in maintaining cellular homeostasis via protein degradation pathways. Its main feature is the autophagosome, a double-membraned vesicular structure that transports cytosolic fluids and materials to the lysosome for degradation and recycling. In the context of neurodegenerative disorders, it is essential in degrading aggregate-prone proteins (Williams et al. 2006).

One of the main regulators of autophagy is the “target of rapamycin,” or TOR. This protein detects energy status and proteins for degradation by interacting with class I phosphoinositol 3-kinase (PI3K), serine/threonine kinase Akt, and 5′-AMP-activated protein kinase (AMPK) (Kroemer et al. 2010). Prospective therapeutic implications of the mTOR (mammalian target of rapamycin) signaling pathway *in vivo* may prove effective in the clearance of mhtt aggregates (Sarkar et al. 2009). Specifically, targets such as Ca^{2+}–calpain–Gsα and cAMP–Epac–PLC-ε–IP_3 signaling are promising. It has been proven that calpain activation inhibition promotes autophagy *in vitro* (Xia et al. 2010), however further study regarding the mTOR therapeutic target must be done before *in vivo* mammalian models show results.

(4) NMDA receptor-mediated neuronal excitotoxicity

Arguably the most well-known pathogenic protein that has been determined to cause neurodegeneration in HD is the N-methyl-D-aspartate receptor (NMDAR), which binds to the glutamate neurotransmitter. This is the primary excitatory receptor in humans and is activated by glutamate. Htt may elicit pathogenesis by excitotoxicity following aberrant activation of NMDA. The excitotoxicity process occurs due to excessive glutamate intake and NMDA firing, causing a cascade of intraneuronal failures, including mitochondrial dysfunction and apoptotic caspase

activation. In the case of HD, various mediums may dictate how mhtt interferes with *wt* NMDA activity and how it results in excitotoxic cell death.

(4.1) NMDA receptor structure and function

NMDARs are ionotropic tetrameric structures consisting of two NR1 subunits and two NR2 subunits forming functional heteromultimers in the endoplasmic reticulum and are expressed virtually everywhere in the CNS (Monyer et al. 1994). However, immunohistochemistry staining found a high concentration of NMDARs in striatal neurons of R6/2. This may be because striatal neurons receive large amounts of glutamatergic inputs from thalamostriatal and corticostriatal synapses, requiring copious amounts of NMDARs to accept them. NMDA subunit differentiation is based on what it binds to, glycine and glutamate, where NR1 binds to glycine and NR2 binds to glutamate. The NR subunits have several splice variants, such as NR2A, NR2C, and NR1 C-terminus, causing functional differentiation in NMDA (Cull-Candy et al. 2001). Another known property of NMDARs is that they are especially sensitive to prominent cations like Zn^{2+}, H^{+}, and Mg^{2+}.

NMDARs have high permeability to calcium ions (Ca^{2+}) and have low activation/deactivation kinetics, which may be one of the significant reasons why Ca^{2+} is found so excessively in post-mortem HD brains (Kew and Kemp 2005, Nowak et al. 1994). However, Ca^{2+} influx via NMDA is crucial for synaptic development and plasticity (important for learning and memory consolidation) in healthy striata.

(4.2) Mhtt interaction with NMDA via PSD-95 and phosphorylation

It is clear that mhtt is involved in some form of interaction with NMDA, either by directly binding to the C-terminus or activating and interacting with various proteins along a vesicular or exocytotic pathway. While there are numerous neurodegenerative processes, mhtt's role in excitotoxicity is not disputed among the scientific community due to the overwhelming amount of cellular and genetic evidence. For example, Milnerwood and Raymond (2006) suggested that mhtt may play a role in interfering with the timing of vesicle release, a key process in neurotransmitter (glutamate, for instance) release into the synaptic cleft. Alterations in the biophysical properties of vesicles and its recovery cycle may impede NMDAR's action potentiation and as a result, the neuron's signaling.

One idea that has been proposed that points to potential targets of mhtt is the postsynaptic density protein 95 (PSD-95). This particular membrane-associated guanylate kinase (MAGUK), along with another member of the MAGUK family PSD-93, has been known to bind the C-terminus of NMDA NR2 subunit tails and stabilize them in neuronal surface clusters (Kornau et al. 1995). Clustering of receptors like NMDA is involved in long term potentiation and depression, integral to functional spatial learning (as reported from transgenic mouse models). This study was aimed to determine the intermediary protein that connects *huntingtin* to PSD-95.

They found that the PDZ domain of PSD-95 binds to a 7-amino acid carboxyl domain with the terminal tSXV motif (S-serine, X-any amino acid, V-valine). It was discovered that htt interacts with PSD-95 in transfected 293T cells, and *in vitro* studies of human HD cortical tissue revealed that non mutated htt interacts with the SH3 domain of PSD-95 (as a mediary protein) and additionally with NR1, NR2A, and NR2B subunits (Sun et al. 2001). Although indirectly, this line of evidence indicates that polyQ-expanded htt would have the opposite effect on neuronal toxicity, inhibiting PSD-95 and therefore overactivating NMDA.

Other than interaction with NMDA via postsynaptic density proteins along its pathway (however somewhat related), mhtt modulates NMDA function via post-translation modifications such as phosphorylation, the chemical process where a phosphate group (PO_3) is added to a biomolecule. In the case of HD brains, the specific Src family of protein kinases and the protein tyrosine phosphatase may be involved in synaptic activity increases and regulation of NMDA populations on the neural membrane (Li et al. 2002). Tyrosine phosphorylation stabilizes NMDA receptors on the neuronal surface by inhibiting endocytosis via binding with NR2B and co-expression with PSD-95 (Roche et al. 2001). Moreover, levels of phosphated and activated tyrosine kinase Src were found in HN33 neurons where htt^{48Q} was expressed increased relative to htt^{16Q} (the non-symptomatic genotype) (Song et al. 2003), i.e. polyQ-expanded huntingtin may induce a positive change in tyrosine phosphorylation of NMDA via PSD-95 and its resulting dysfunction.

(4.3) NMDA receptor dysfunction

Excitotoxicity occurs when glutamate over-accumulates and binds to ionotropic NMDA receptors. This results in excessive synaptic excitation, an imbalance of intracellular calcium ions, and a chain reaction of apoptotic events leading to cell death.

Many biomolecules play vital roles in this act, however probably the most crucial to excitotoxic initiation is the glutamate neurotransmitter. Decreased uptake by the neuron in question may correlate to increased concentration in the synaptic cleft. In the case of HD, glutamate occurs frequently in thalamostriatal synapses, thus a vast amount of research has been dedicated to studying its implications on synaptic plasticity. Indeed, it was found in YAC128 and R6/2 mice that an increase in glutamate release into thalamostriatal synapses causes overactivation of extrasynaptic receptors (Kolodziejczyk and Raymond 2016).

(4.4) Calcium homeostasis disruption causes mitochondrial dysfunction

Invariably, altered NMDAR function will result in a change in intracellular calcium levels, found *in vivo* (YAC72 and R6/2) as well as in cultured striatal MSNs from YAC transgenic models expressing polyQ=46 and polyQ=72) (Zeron et al. 2004). They also found that cultured neurons from these models were found at birth, suggesting calcium dysregulation as a result of mhtt expression from an early age, and not from age of onset. The early start of calcium

dysregulation may not immediately result in neural death, however it may be a stepping stone for other neurodegenerative processes after the age of onset.

The mitochondria (and specifically its matrix) is vital to the survival of any eukaryotic cell due to the necessary process of cellular respiration that generates adenosine triphosphate (ATP), the energy unit of the cell. The electric potentiation achieved across the intramitochondrial membrane drives oxidative phosphorylation, pumping in Ca^{2+} ions from the cytosol and eventually returning back to baseline calcium levels after a moderate depolarization. However, excessive glutamate stimuli to NMDARs may in turn allow large quantities of Ca^{2+} into the cytoplasm and mitochondria, resulting in activation of the mitochondrial permeability transition (mPT) and decrease in ATP generation. mPT is one of the first steps to cellular apoptosis, after its rapid depolarization and expulsion of Ca^{2+} into cells, which may in turn activate the caspase apoptotic pathway (Orrenius 2004).

Other than mPT activation, a variety of abnormalities may arise in mitochondrial function. HD patients and post-mortem brains have had decreases in glucose metabolism and oxygen consumption, as well as reduced cAMP levels in the cerebrospinal fluid (CSF), proposing a possible cellular respiration dysfunction (Leenders et al. 1986, Cramer et al. 1984, Gines et al. 2003). Furthermore, HD mitochondria have been found to display another dysfunctional phenotype interfering with its Ca^{2+} clearance. Mhtt aggregates inhibit mitochondrial expression of PPARG coactivator-1 alpha (PGC-1α), an important protein regulating mitochondrial function (namely respiration and biogenesis) (Cui et al. 2006). Specifically, it associates with the promoter sequence and interferes with the CREB transcriptional pathway required for PGC-1α gene expression. They also found by assessing wild-type and mutant STHdhQ111 (polyQ=111) cells that upregulation of PGC-1α may be able to rescue the effects of mitochondrial dysfunction. To conclude, mitochondrial dysfunction could be impaired by mhtt via two main molecular mechanisms, 1) interaction with NMDARs causing excessive Ca^{2+} influx, mitochondrial membrane depolarization, and mPT activation, and 2) inhibition of PGC-1α causing an impairment in mitochondrial respiration and biogenesis (impaired clearance of cytosolic Ca^{2+}).

(4.5) mPT activates caspase apoptotic pathway

We already know that caspases are involved in proteolytic cleavage of proteins, such as of mhtt. However caspases are also integral to the cellular degenerative pathway when presented with apoptotic stimuli. In the case of HD, one of the main stimuli underlying caspase activation is mPT activation following the rapid Ca^{2+} influx from NMDARs and the depolarization of the mitochondrial membrane.

mPT activation is generally followed by the release of several mitochondrial proteins, one of which is cytochrome *c*. When released into the cytosol, it binds to apoptotic protease activating

factor 1 (APAF-1), forming an apoptosome capable of binding to caspases and initiating the apoptotic cascade. In the case of HD, the cytochrome *c* apoptosome activates caspase-3 and caspase-8 (Sawa et al. 1999). These modulated caspases are eventually responsible for the degeneration of the cell. Binding to specific sequences cleaves poly-ADP ribose polymerase (PARP) among other nuclear enzymes crucial for DNA fragmentation and replication.

(4.6) Effect of glial cells in NMDA receptor-mediated excitotoxicity

Glia (or gliocytes) are non-neuronal cells in the CNS and PNS responsible for supporting the function of neurons. These can be categorized broadly into microglia, astrocytes, and oligodendrocytes. Microglia are known for their highly condensed nuclear membrane, while astrocytes and oligodendrocytes are known for their intracellular fibrils and their role in associating with myelinated nerve fibers (or axons). All glial cells have common features that differentiate them from neurons, such as a limited and sparse cytoplasmic space and a smaller shape.

It is known that htt is widely expressed throughout the body, however selective neurodegeneration occurs mainly in the neostriatum and parts of the corticostriatal pathway. Htt is also expressed in glia, which may contribute to another hypothesis that HD pathology is not directly caused by neuronal degeneration, but rather by glial dysfunction. It was reported that nuclear htt appearance occurs much later in glia than in neurons, suggesting that glia might be better at clearing soluble misfolded htt than neurons (Shin et al. 2005).

Mhtt aggregates may interfere with astrocytes by inhibiting the transcriptional pathway to synthesize the glutamate amino acid transporter GLT-1. This results in reduced uptake by astrocytes and other glial cells, which in turn causes the excitotoxic pathway discussed previously. Mhtt can cause glia to negatively interfere with neurons and increase their vulnerability, not by physically modulating its shape, but by modulating the extracellular glutamate density in the synaptic cleft.

(4.7) Mhtt modulates BDNF and synaptic vesicles to cause neuronal death

While the main protein hallmark of HD pathology is the NMDA receptor, other proteins that interact with mhtt may contribute to neurodegenerative processes. BDNF is an important precursor protein involved in moderating intracellular traffic and its interaction with polyQ-expanded htt is also involved in HD's pathologic features. It was shown that mhtt (specifically FL-75Q-htt-ex1, polyQ=75) impairs the post-translational intracellular traffic functions by BDNF more than its polymorphism variant Val66Met (del Toro et al. 2006). By using Golgi fluorescent targeting, they were able to conclude that mhtt impairs the post-Golgi vesicular transport of Val66Val more as compared to *wt* htt, and moreover less Golgi fluorescence was found in Val66Met, suggesting a less concentrated interaction by mhtt. This may suggest an

impaired attribute of HD neurons as a result of mhtt-mediated BDNF modulation that they slowly lose the ability to transport vital cellular materials, possibly resulting in apoptosis.

Other than modulating BDNF, mhtt may cause neuronal dysfunction by inhibiting synaptic vesicles (Li et al. 2003). Mhtt aggregates are known to be mainly localized in the nucleus of striatal MSNs. However they also accumulate into IBs in axons and, more importantly, the axon terminal. This colocalization at the terminals of neuronal axons has correlated with the data supporting interference by mhtt with synaptic vesicles, however the direct link is still unknown. From Li et al. (2003), electron immunogold labeling and traditional Western blotting revealed 4 main pieces of evidence supporting this hypothesis. First, a decreased concentration of synaptic vesicles in mhtt-containing axon terminals was revealed in R6/1 and repeat knock-in models. Second, huntingtin-associated protein 1 (HAP1) was found to be colocalized with soluble htt in extracted synaptosomes, however at a lower concentration than in *wt* neurons. Third, aggregated htt (insoluble) does not bind with HAP1 as shown through immunostaining of R6/2 cortical tissue and thus may inhibit its interaction with synaptic vesicles. Finally, accumulation of mhtt in corticostriatal tissue slices of HD mouse brains caused a decrease in glutamate release. This evidence suggests a strong correlation between aggregate concentration in striatal axon terminals and the leading cause of glutamate release depletion, which leads to the impairment of NMDA uptake of glutamate and excitotoxic cell death.

(5) Ongoing debate

This paper has frequently mentioned one hypothesis regarding the role of inclusions in HD pathology. It is crucial that scientists in this field find a way to bridge these theories in order to develop more efficient therapeutics. This section will focus on arguably the most prominent and widely debated topic in the HD scientific community, centered around the role of inclusion bodies in HD neuronal death.

One proposal hypothesizes that IBs are not a pathogenic factor in the progression of HD and instead may mediate aggregation of toxic mhtt. In Arrasate et al. (2004), automated microscopy revealed that levels of diffuse Httex1-Q47-GFP (polyQ=47, fused with green fluorescent protein) decreased rapidly following the formation of an IB, and the rate of Httex1-Q47-GFP decrease was inversely proportional to the rate of IB formation. This suggests that IBs may form as a “coping response” to an increase in toxic intracellular mhtt, by sequestering toxic diffuse-forms of mhtt or by other inhibitory means. It was additionally found in the same study that the presence of IBs predicts neuron survival and leads to a diminished concentration of mhtt in other parts of the neuron. Instead of IBs as a mediating factor in the progression of the disease, it was suggested that levels of diffuse htt and the length of the polyQ best predict the death of neurons.

The *in vitro* neuron culture for HD from Arrasate et al. (2004) was later used in conjunction with observations from animal models with aggregate-type pathogenic properties (Johnston et al. 2000) to formulate another line of evidence to support this hypothesis. In Bennett et al. (2005), data from close examination of these cell lines suggested that the neurodegenerative deficits reported in HD neurons occurred prior to significant development and growth of IB concentration. While IBs do represent aggregate concentration, it is possible for UPS impairment to be detected without the formation of IBs. However this argument may be flawed due to the error formed by the possibility of not detecting all instances of an inclusion.

On the other hand, Western blotting analysis using antibodies that specifically targeted polyQ sequences showed NIIs marked with polyubiquitinated domains and mhtt increase prior to symptomatic development in R6 lines with expanded polyQ (Davies et al. 1997). The appearance of NIIs rapidly increases the density of nuclear pores and a pronounced indentation in the nuclear membrane. These changes in morphology in neuronal nuclei have also been reported in human patients, suggesting a proportional correlation between IB formation and HD pathogenesis.

(6) Current and future therapeutic options

(6.1) Current therapeutic options

As of right now, there are no FDA-approved drugs or therapeutic options that can stop or slow the progression of the disease by direct targeting of the htt gene or aggregates, however some medications can ameliorate the symptoms. However, the extent to which toxic mhtt must be eliminated in order to lessen cognitive/motor deficits is still unknown. Further research is required to more accurately discover drugs that will impact HD symptoms in a significant manner. Most of the current drugs, such as tetrabenazine and haloperidol, are used to treat the numerous chorea-like symptoms. Table 1 summarizes some of the current therapeutic options for patients with HD.

Current care for HD patients includes mostly treatment of symptoms by use of drugs mentioned below in order to lessen its severity. The major effort of nurses, physicians, and therapists to help those with HD is to maintain and improve quality of life (QOL). Physiotherapy can be utilized to maintain balance, coordination, and mobility. As the disease advances in severity, more extensive palliative care is often an option, as living at home may become impossible. However, through the efforts of an effective and willing healthcare team, QOL of HD patients can be maintained.

Medication	*Symptom treated*	*Molecular mechanism*
Tetrabenazine/ Deutetrabenazine (Kumar et al. 2020)	Chorea	Inhibits vesicular monoamine transporter type 2 (VMAT2)
Haloperidol (Unti et al. 2017)	Hallucinations and delusions	Inhibits dopamine D2 receptor
Citalopram (no short term treatment) (Beglinger et al. 2014)	Depression	Inhibit reuptake of serotonin
Lamotrigine (Kumar et al. 2020)	Mood symptoms	Inhibits Na channels and glutamate release
Clonazepam (Coppen and Roos 2017)	Chorea and anxiety	Binds to and enhances GABA function

Table 1. Current therapeutic options for HD symptoms

(6.2) Potential therapeutic options

Many ongoing clinical trials are using therapeutics that target the disease at the RNA level. Current research focuses on lowering mhtt levels with post-transcriptional interference such as RNAi therapies and RNA targeting molecule therapies. Additionally, researchers are studying potential therapeutics for HD that target mhtt aggregation, attacking the ubiquitin-proteasome system and the autophagy-lysosome system. Examples of these potential methods are proteolysis-targeting chimera, autophagy-targeting chimera, and autophagosome-tethering compound.

The medical and scientific community is determined to pave a way forward in treating HD patients. Several ongoing clinical trials, as reported by the NCBI, are testing various drugs that aim to ameliorate symptoms in early-onset HD.

Sage Therapeutics is currently in phase II of their double-blind, placebo-controlled PRECEDENT study that is testing the efficacy and safety of SAGE-718 (Dalzanemdor) as a potential therapeutic for HD and AD. SAGE-718 is a biological derivative of the protein 24(S)-hydroxycholesterol, which has a complex role in AD and other neurodegenerative disorders, having been shown to have beneficial and detrimental effects on neural function and amyloid or tau protein pathology respectively.

In addition, many researchers in the field of HD are interested in the application of stem cells to regenerate striatal tissue that has been lost from neuronal death. Cellavita HD is in phase III of a

clinical trial aiming to analyze the efficacy of this therapeutic on HD (Van de Roovaart et al. 2023). This therapeutic function is achieved by utilizing dental-pulp-derived mesenchymal stem cells (DMSCs). Due to its ability to bypass the blood-brain barrier, it can promote the proliferation of neural stem cells so that they will not be susceptible to mhtt-induced excitotoxicity. These stem cells are able to differentiate anti-inflammatory, neurogenic, antiapoptotic (a crucial one), and angiogenic/osteogenic mediators. It was found that the insertion of DMSCs increased BDNF levels in the corticostriatal region of the brain. Additionally, DMSCs can differentiate, not only into neurons, but also into glial cells, which are also impaired in HD brains. However, lack of geographical reach due to the clinical trial only being performed in Brazil limits the pool of applicants (currently at 35, on the lower end of phase III trials).

(7) Conclusion

While clinical research is developing therapeutics for HD by targeting various points along the biochemical pathway, it could also be beneficial to understand the complex competing hypotheses. By instead forming a stronger basis of knowledge about the relationship between polyQ length, huntingtin aggregation, inclusion formation, NMDA overstimulation, and glutamate-induced excitotoxicity, future pharmaceutical scientists may be able to form a stronger set of possible inhibitors of aggregatory impairment of key intracellular systems. Additionally, due to the extent of healthy htt's interaction with other crucial proteins, it is important that future therapeutics are specifically targeted at mhtt and not at healthy htt to prevent harmful side-effects.

One example of a promising and widely popular tool to determine the root of HD pathogenesis is the genome editing tool CRISPR-Cas9. In Shin et al. (2016), they permanently deactivated the mutant HTT allele using CRISPR-Cas9 using the locating principle of Protospacer Adjacent Motif (PAM)-altering SNPs personalized for each patient. This strategy may be effective in diminishing the effects of HTT's gain-of-function by inhibiting the production of mhtt. This method is currently under clinical trials, though it has proven successful on mouse models.

However, due to the widespread neuronal loss characterized by HD, it may be difficult to target every striatal neuron expressing mhtt. The downside to this technology is that due to the use of the *huntingtin* protein as a positively impacting protein (also used by the rest of the body), using CRISPR/Cas9 to directly deactivate the mhtt allele is dangerous. If the other healthy allele were to be impaired, other important developmental functions would also be impaired. Additionally, due the importance of healthy htt to prenatal development, this technique may not be viable as a prenatal treatment of HD.

Ultimately, HD presents a complex pathology between protein aggregation and neuronal excitotoxicity, primarily driven by mhtt and its interaction with NMDA. This review has examined

multiple competing hypotheses regarding the role of IBs and mhtt aggregation. Additionally, the impairment of critical cellular systems including the UPS and the autophagy-lysosome pathway further worsens neurodegeneration in HD, contributing to the toxic intracellular buildup of misfolded proteins. The ongoing debate around whether inclusion bodies serve a protective or deleterious role remains unresolved, indicating that further research is required to clarify their function and their implications. Understanding the exact mechanisms by which mhtt induces neurodegeneration, including NMDA receptor-mediated excitotoxicity, mitochondrial dysfunction, and caspase activation is crucial for developing targeted therapies. Current therapeutic options focus primarily on symptom management, but advances in genetic and molecular research, such as mRNA-targeting therapies and stem cell treatments, offer promising roads to future interventions. Ultimately, reconciling the diverse molecular pathways involved in HD pathogenesis will be essential for designing effective treatments that can slow, halt, or even reverse the progression of this disease.

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