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Therapeutic Effect of Berberine on Huntington's Disease

Transgenic Mouse Model

By
Wenxiao Jiang

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Microbiology and Molecular Genetics

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Therapeutic Effect of Berberine on Huntington's Disease Transgenic Mouse Model

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Abstract

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By Wenxiao Jiang

Huntington's Disease (HD) is an incurable autosomal dominant, debilitating neurodegenerative disease caused by a CAG trinucleotide repeat expansion in exon 1 of the huntingtin (Htt) gene, which encodes a polyglutmine (polyQ) repeat tract in the N-terminal Htt. The disease is characterized by neurological symptoms such as chorea, dystonia, bradykinesia, dementia, and normally affects the mid-elderly. In severe cases, HD can onset at a much younger age because of large polyQ repeat expansions that are often in excess of 55 glutamines. Pathologically, HD is hallmarked by the formation of mutant Htt (mHtt) aggregate accumulation in the brain and neurodegeneration that preferentially affects the striatum. HD affects roughly 30,000 patients in the US, mostly of Western-European descent. Although there are efforts to treat HD by stemming and ameliorating its symptoms, so far, no treatments have been successful at preventing or delaying the development of this devastating disease. Berberine (BBR) is an abundantly-available, botanically-derived organic small molecule that is traditionally used orally to treat bacterial diarrhea and is well-tolerated in humans both in chronic and high-dosage use. Recently, BBR has shown protective effects on neurodegenerative conditions such as Alzheimer's Disease and Parkinson's Disease, which sparked our

interest in the possible effects of BBR against HD. In this study, we show that BBR treatment reduces mHtt aggregation both in a transfected cell model and in the brains of a transgenic HD mouse model via up-regulation of autophagic clearance of mutant proteins. The HD mice exhibited drastically improved motor function and a slightly increased lifespan. These encouraging results on HD mouse models also provide mechanistic insight into the broad effects of BBR on neurodegenerative diseases caused by misfolded aggregated proteins.

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Chapter I:

Introduction

1.1 Huntington's Disease

Huntington's disease (HD) is a severe, debilitating neurodegenerative disease that is characterized by chorea, dystonia, motor coordination loss and mental deterioration (Tab. 1-1). Onset of disease is usually mid-late life, but can be as early as in juvenile life [1, 2, 3]. Although measuring the true prevalence of HD is extremely difficult, it is estimated that in nations of predominantly Western ancestry, the incidence ratio is roughly 5-7 per 100,000 (neglecting some select small populations in Western Europe and Venezuela which exhibit highly exalted levels) [4]. In the US, it is thought that there are a least 30,000 people who suffer from HD, a number that is both rising and most likely underestimated [4]. The HD gene on 4p16.3 encodes the huntingtin (Htt) protein, of 350kDa, with a variable-length polyglutamine (polyQ) tract encoded in exon 1. Expansion of the polyQ tract (>36 CAGs) results in HD, and further-expanded tracts of over 55 CAG repeats lead to aggressive progression juvenile-onset HD [1] (Tab. 1-2). The reason for this threshold is most likely that expanded polyQ tracts of over 36 repeats cause Htt to misfold, leading to abnormal protein interactions and aggregation of Htt [5-7]. Although the role of Htt aggregates remains controversial, the accumulation of these toxic forms have been used as a marker to assess the therapeutic effects of drugs that may counteract HD [8]. The precise function of Htt remains to be fully investigated, though studies have implicated its roles in vesicular trafficking [9-11]. Silencing the htt gene results in embryonic lethality in mice, though heterozygous knock-out phenotypes vary depending on model and study [12-14]. Some have evidence that

heterozygous Htt knockouts present with moderate neurological abnormalities, [13, 14] while others offer dissenting results showing that they are phenotypically indistinguishable from wild type (WT) [12].

Clinically, one of the agents used today to treat HD is tetrabenzine [15, 16]. This compound is a vesicular monoamine transporter (VMAT) 2 inhibitor. By inhibiting vesicular transport of monoamines such as dopamine and γ-Aminobutyric acid (GABA), tetrabenzine can ameliorate chorea-like movements, and is thus a valuable symptom-based treatment for several neurological diseases including HD [15,16]. Unfortunately, as a dopamine antagonist, depression is one of the main side-effects of tetrabanzine [15, 16].

Another symptom-based treatment for HD is haloperidol [17-19]. This is a butyrophenone with strong antipsychotic properties, which also makes its application quite wide in range [17-19]. As a receptor antagonist of D2, Alpha 1, 5-HT2, haloperidol exhibits a long list of common side-effects including, but not limited to dystonia (one of the symptoms of HD), muscle rigidity, and Parkinsonism [17-19].

Although there are no approved disease-modifying drugs for HD (in large part due to the lack of understanding of the underlying mechanisms), there are 2 compounds in clinical trials with the potential to target some HD-associated imbalances.

Selisistat is one of the potential disease-modifying treatments under clinical testing [20-26]. This compound works by inhibiting a natural protein deacetylator,

SIRT 1, which causes an increase DNA acetylation and transcription to target the gene expression imbalance in HD [20-26]. There are widely recognized positive effects of Selisistat in HD cellular and animal models, and clinical testing is still ongoing. While Selisistat is generally believed to be safe, side effects involving hepatic hyper-function have been noted [20, 24]. Despite its relative success, the connection between gene down-regulation and HD pathology/ symptomology is unknown so it is still uncertain as to whether Selisistat actually addresses a root cause or simply targets a bio-molecular symptom or by-product of an actual underlying cause.

PBT2 is another drug that is classified as disease-modifying and works primarily through metal sequestration to defend against damage caused by reactive oxygen species (ROS) and counteracting metal-induced effects on mutant Htt (mHtt) aggregation [27-30]. The main targets of PBT2 are copper and zinc. Unfortunately, while seemingly successful in animal models, the effectiveness of PBT2 to significantly modify HD in a positive way in humans is still highly shrouded in uncertainty even after some clinical trials [29-30].

Despite all of the efforts today, a drug that is effective at treating or stopping HD progression still eludes modern science. Discovery of a safe and effective disease-modifying drug would be a major game-changer in the struggle against HD.

1.2 Huntington's Disease Research

In order to study HD, several different mouse models have been constructed to imitate the disease [31]. The design philosophy consists of making the mouse either

a knock-in (KI) model or a transgenic one. Then, a choice is made between having the model express full-length mHtt or N-terminus fragments. While the full-length HD mouse models may more accurately imitate what happens in patients, the models progress through disease more slowly, making it difficult to evaluate therapeutic effects of drugs on the mild and slowly-developing neurological phenotypes. Thus, the N-terminal Htt expressing mouse models are often chosen for their directness and speed of progression. Some notable and popular aggressive models are the R6/2 and N171 82Q transgenic N-terminal Htt expressing models [32-34]. While they express different lengths of pathogenic polyQ, both models exhibit tremors, loss of motor coordination, weight-loss, clasping (a behavior in which when held upside-down by the tail, the mouse's limbs, especially hind limbs, grasp each other and are held close to the body) and finally, early expiration (roughly 4 months) [32-34].

PolyQ expansion-mediated protein misfolding and pathology can be assessed in vitro and in vivo. *In vitro*, mHtt aggregation correlates with protein misfolding and can be determined by Western blot or microscopy. In the HD brain, de-myelination and neuronal degeneration can also be seen [35]. In the animal model, behavioral tests are used to evaluate disease progression. These mainly test for balance, depression, motor function, memory, and strength. For balance, the balance beam test can be used. This test records the duration necessary for the mouse to cross a narrow beam; greater balance results in a faster crossing [36]. For depression, the Porsolt swim test can be used in which the mouse is put into a pool of water and the

time that it spends swimming is measured against the time it spends floating, rationalizing that a less depressed mouse would have more desire to overcome the situation and thus would spend more effort attempting to escape [37]. Another possible test for depression is the open field test in which the mouse is released into a new environment and the amount of distance it covers exploring the area is measured, rationalizing that a depressed mouse would prefer to hide away while a normal one would be keen to scout the area [38-40]. Memory can be tested by various maze tests in which the mouse must first learn the area of a treat (or platform in the case of the water maze), then continuously relocate it in the same area to show how well the mouse remembers past experience [41]. Strength can be tested by the grip-strength test in which a mouse is allowed to grasp a metal mesh attached to a force-meter before being pulled off, recording how much force the mouse exerted onto the force-meter before being forcibly removed. Motor coordination could be assessed by the foot-fault test, which measures the number of times an animal makes a mistake in its gait when walking on a cage-like surface [42]. Finally, the best, most non-biased assessment for motor coordination is the rotarod test, in which mice are placed on a suspended rotating knurled dowel that is turned and slowly accelerated by a machine [43-45]. The time that the mouse is able to keep up with the machine reflects its current motor coordination level.

To evaluate the accumulation of aggregate mutant Htt in cell culture or HD animal models, Western blotting is often used to detect Htt aggregates. In Western blots, mHtt aggregates cannot be dissolved by SDS, so they remain as large

aggregates that can only be visualized in the stacking gel even after running. This allows us to separately evaluate mHtt aggregates from their dissolved forms. If it is necessary to dissolve the aggregates, formic acid may be used to do so [46]. Both normal and polyQ-extended Htt can be easily bound by many commercial antibodies and be detected either via Western Blot or by immunofluorecent microscopy. Additionally, the latter offers the resolution to discern the cellular localization of these aggregates. By examining Htt aggregation in HD animal models, it is possible to assess the therapeutic effects of drugs on HD.

As can be seen from the plethora of efforts and tools for studying HD in the lab, it is apparent that the disease has long been a challenge to humanity and expectedly, well-studied, however, due to its difficulty, still inadequately-understood.

1.3 Huntingtin Aggregation and Huntington's Disease

The pathological hallmark and biological identifier of HD is the accumulation of mHtt in various brain regions, causing loss/degeneration of mass that is most pronounced in the striatum [47-48] (Fig. 1-1). These aggregates are formed from the fragments of broken mHtt proteins. When mutations accumulate 36 or more glutamine repeats on the polyglutamine tract, aberrant protein folding can be seen [49]. While normal folding results in structurally-stable proteins, these misfolded proteins exhibit a structural instability, which can cause fragmentation [50]. These fragmented proteins can interact dynamically with each other and can accumulate in the nucleus and cytoplasm of brain neurons [51-55]. In the beginning, the nascent aggregations are unstable and the particles can freely associate and dissociate [56].

However, once the aggregate structure reaches a certain size and stabilizes, it is very difficult for any particles to leave, and from there, the mega structure will steadily expand [56] (Fig. 1-2). Because the formation of these mHtt aggregates correlates with disease progression, the presence of mHtt aggregates may offer some explanation as to why the disease usually remains asymptomatic until its onset later in life. However, there is still uncertainty as to why the severity of disease increases with an expanding polyglutamine tract. One possibility is that the increased polyQ sizes cause extensive protein misfolding and aggregation, leading the stable structure state to be reached much faster, offering explanation for the phenomenon of juvenile onset HD.

Despite the aggregation of mutant protein fragments being the signature of HD (and thus often used as a molecular indicator of severity of disease), the causal relationship between HD symptoms/pathology and mHtt aggregation remains controversial. Though there seems to be correlation, that more visible mHtt aggregates are seem in more advanced and severe cases, direct causation has yet to be proven. While it is often taken for granted that aggregates are the cause of the disease, a rival theory is that aggregates are actually neuroprotective and represent the body's attempt at diffusing the disease symptoms, and such, the more severe the disease, the greater the effort that the body puts into producing aggregates to protect the brain's neurons. However, this is unlikely, as if this were the case, then treatments that directly target eliminating the aggregates should worsen the symptoms and expedite HD progression. Another possibility is that the

buildup of mHtt aggregates is simply a by-product of the unknown underlying cause (originating from the mutated protein) such that they can serve as relatively accurate indicators but they themselves have no modifying effect on the disease. However, mainstream thought on the issue is that although mHtt aggregate accumulation is the underlying cause of HD, the reason and link between the aggregates and pathology/ symptomology is unclear. One hypothesis is that these aggregates form a pit or trap, entangling several other proteins, occupying them from their functions, which leads to global functional down-regulation [57-59]. When ensnared in mHtt aggregate pits, proteins meant as defenses against some of the body's challenges could lose much of their ability to prevent their targets from causing damage to the body. For example, metal superoxide dismutases could fail to defend the body from excessive ROS damage when trapped by mHtt [60, 61].

To summarize, although there is no conclusive evidence as to whether the mHtt aggregates, which are a key to diagnosing HD, are the causative root, a by-product, or a host-initiated defense against HD neurodegeneration, mainstream thought believes that these aggregate bodies are malevolent and related negatively to HD. Despite the unclear role of mHtt aggregates, there is a general consensus that these aggregates result from the accumulation of misfolded Htt and their formation correlates with disease progression and development.

1.4 Defense Mechanisms Against Protein Aggregation

The body has several biological pathways through which to clear away aggregated accumulations of defunct or harmful proteins. The most notable are the ubiquitin-

mediated proteasome degradation pathway, and the autophagy pathways. The autophagic pathways are united by the use of the autophagosome for degradation but the steps leading up to this divide the autphagic process into three distinct pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy [62]. Of these 4 pathways for clearance of aberrant proteins, each may play a role against the build-up of mHtt in HD.

The uqibuitin-proteasome system (UPS) uses three major enzymes (E1-E3) to activate ubiquitin, transfer it to a holding enzyme, then target it onto the aberrant protein to serve as a tag for destruction [63-64]. This tag serves as a localization signal to be recognized by the proteasome, which then degrades the ubiquitin-tagged protein [65]. The importance of the UPS in combating mHtt aggregation is not well-understood. Because of the relatively small channel for substrate to enter the proteasome, it is not believed to be able to directly degrade large aggregated protein structures, rather it may be limited to degrading soluble mHtt to prevent it from joining an aggregate structure [56]. However, there is also evidence that the proteasome may, at least in some instances, fail to fully degrade the mHtt protein, and as a result, release broken protein fragments into the cytoplasm [66, 67]. Since these fragments can then aggregate, the UPS, in this instance, may serve to expedite the disease, decreasing the time between production of mHtt and its time to fragmentation.

Of the autophagic pathways, macroautophagy is the largest of the trio. This process allows for the engulfment of entire cellular organelles set for destruction and is capable of destroying large mHtt aggregates in addition to soluble mHtt [68, 69]. Briefly, this is

a process in which a double-membraned vacuole starting as a phagopore is formed and begins to elongate around a target designated for destruction until the membranes enclose it in an autophagosome [68, 69]. This process, which converts LC3B I into LC3B II and recruits P62 as an expendable protein escort P62, ultimately ends with fusion into a lysosome for destruction [68, 69]. Macroautophagy is believed to be the workhorse of the autophagic pathways responsible for the majority of protein degradation assignments.

Microautophagy is another form of autophagy that is more associated with starvation response than clearance of defunct or pathogenic proteins [70]. This is a more simplistic pathway that involves lysosome membrane invagination and intake of the substrate (oftentimes lipids during catabolic states) for direct degradation [70]. There is no direct evidence so far that this pathway is of significant consequence in HD.

Finally, chaperone-mediated autophagy is a more complicated and highly selective method of autophagy in which heat shock protein 70 (hsc70) forms a targeting complex with the substrate in order to localize it to the lysosome associated membrane protein 2A (Lamp2A) [71]. There, it can be unfolded and transported across the lysosome membrane into the lysosome for destruction. There is also some evidence that one substrate that the hsc70 may recognize could be Htt/mHtt, since down-regulating hsc70 or Lamp2A causes an acceleration of mHtt aggregation [72-74]. However, one alternative reason for this may be that down-regulation of the chaperone-mediated autophagic pathway created slack that had to be picked up by other protein degradation

pathways, and that caused them to allocate their activities/resources away from mHtt destruction.

Until recently, it was thought that the autophagic pathways and the UPS were independent systems. However, recently, there has been evidence that points to crosstalk between the two. Ubiquitin has risen as a link between autophagy and the UPS, serving as a substrate escort for both functions [75-77]. Indeed, it was also seen that in cases where the UPS was impaired, autophagy would respond by up-regulating to compensate for the slack [78-80]. There is also a great possibility of some overlap in substrate/ target since phenotypes seen in UPS-deficient systems could be rescued by up-regulating autophagy. However, inverse relationships such as UPS up-regulation causing autophagy down-regulation or autophagic impairment leading to UPS acceleration have yet to be seen.

1.5 Berberine

Berberine (BBR) is an organic compound isolated from various herbs such as *Coptis chinensis, Berberis vulgaris, Hydrastis Canadensis, Phellodendron amurense*, etc... For decades, Chinese medicine has used the plants and their extracts to treat diarrhea with no observable negative side-effects or toxicity in patients [81-83]. Modern advances in research, however, allowed us to discover BBR as the active compound and to synthesize it. As a result, BBR was found to be a small molecule, yellow at room temperature (orange when heated in solutions) with a molecular weight of 371.8 Da [81-83] (Fig. 1-3).

One of BBR's main advantages is its safety. Botanically purified, isolated, and chemically-created BBR have been used to treat patients with bacterial diarrhea for many decades in China [82]. It is an over-the-counter medicine that requires no prescription or special precaution. The LD50 for BBR when delivered orally is virtually non-existent [83]. Possibly due to its mechanism for reduction of blood glucose/lipid, a commonly-seen side-effect of BBR (depending on dosage) is weight loss [84]. In the US, BBR is available for purchase from many health-supplement companies such as GNC, either by itself or as part of a plant extract, advertised for general health and/or especially blood-sugar/cholesterol control. The recommended dosage is around 1,500mg daily. When taken orally, BBR is poorly absorbed into the bloodstream and peaks in blood-levels roughly 2-4 hours after use, declining rapidly, thus requiring any BBR regiment to be taken at least daily, possibly more often. Once it enters the body, BBR can easily cross the blood-brain-barrier and shows the highest concentrations in the liver.

The other pillar of BBR's potential is its diversity. BBR has been reported to affect an incredibly vast array of targets, including several of which are of interest to the treatment of neurodegenerative diseases.

1.6 Berberine and Neurodegenerative Diseases

The most encouraging evidence that prompted this research was BBR's potential against neurological diseases, made possible by BBR's ability to quickly cross the blood-brain barrier. One of its most notable successes against neurological disease is its

activity against Alzheimer's Disease (AD) [85-88]. Although no single underlying cause has be established for beta-amyloid plagues-associated AD, a plethora of relatively small imbalances have been found in AD patients and are likely to contribute to the symptoms of the disease. Due to its multi-faceted nature, BBR has been shown to address several of those imbalances in a positive way. These activities include cholesterol reduction, ERK pathway activation, inhibition of MAO-B activity, defense against ROS-induced damage, inhibition of acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and beta-secretase, and reduction in the amyloid-beta genesis [84-86, 88, 89]. This has led to an overall reduction in amyloid plaque aggregation and amelioration of phenotypic pathology in AD mouse models [87].

In 2009, Choi, *et al* ran a number of inhibitory assays to determine the anti-AD effects of several protoberberine alkaloids [88]. The IC50 (50% inhibitory concentration) was determined for each of the six compounds in their ability to stunt an AD-related activity. The inhibitory assays included β-site amyloid precursor protein cleaving enzyme 1 (BACE1), AChE, BChE, and ROS. In addition to total ROS, peroxynitrite ($ONOO^-$) scavenging was given particular attention due to its strong role in amyloid B formation. Through these assays, Choi *et al* found that although BBR was ineffective at inhibition of BACE1 and total ROS, the compound showed promising results against AChE, BChE, and ONOO- (requiring 0.44, 3.44, and 23.06 uM respectively to reach IC50 of a 100 uM target solution) [88].

In 2013, Panahi, *et al* showed that with 50 mg/kg oral BBR taken daily, rabbits with chemically-induced AD-like symptoms showed improvement as compared to the

untreated group. Although weight loss was only moderately abated, survival was significantly improved [85]. Interestingly, the results showed significant inhibition of BACE1 in the treatment group, which stand in contrast to the prior findings of Choi *et al* [88].

Asai, *et al* also discovered that 10 uM BBR could reduce amyloid B levels to 30% of the untreated amount *in vitro* [86]. Furthermore, through Western-blotting of the different components of amyloid precursor protein (APP), they discovered that the mechanism behind the reduction was the result of APP pathway modulation towards a non-amyloid metabolite, as opposed to direct destruction of the protein itself or of its precursor [86].

Using a transgenic AD model mouse strain (CRN8D), Durairajan, *et al* tested the effects of BBR on neurological impairment. The group found that BBR significantly reduced amyloid B plaque aggregation, leading to amelioration of mental and neuronal impairment in the treated group as compared to untreated transgenic controls [87]. Results also showed that these effects were achieved through inhibition of APP phosphorylation via suppression of glycogen synthase kinase (GSK) 3 activation [110]. In addition, the group found that treatment with a lower dosage (25mg/kg) often showed preferential results as compared to treatment with a higher dosage (100mg/kg) [87].

All these studies present consistent information of BBR's effects against the various components of AD pathogenesis. Animal studies also show BBR's amelioration of pathology presentation and retardation of progression of AD. Furthermore, the

dosage study done by the Durairajan [87] suggests that to further develop the therapeutic capabilities of BBR against AD, combination therapies or other advanced methods must be used, since increases in concentration can be counter-productive in treatment.

Another neurodegenerative disease on which BBR's relationship was studied is Parkinson's Disease (PD). Although the pathology of PD has been pinned to the formation of Lewy body protein aggregates and loss of dopaminergic neurons in the brain, the causes that lead up to the pathology are largely unknown [90-96]. Due to our lack of understanding of the true cause of this disease, effective treatment strategies remain to be developed. Thus, current therapies against PD are limited to amelioration of its symptoms, the main focus of which is stemmed around prevention or retardation of further dopaminergic neuronal loss. There has been substantially less research done on the therapeutic potential of BBR against PD than there has been against AD; the mixed results and lack of clear success could be the reason. However, there are at least two documented effects that merit continued consideration and studies to further address the possibility of BBR's use as an anti-PD agent.

In 2014, Kim, *et al* used a chemically-induced mouse model of PD to test the neuro-protective effects of BBR. According to their data, at 50 mg/kg, BBR significantly prevented both memory and balance loss in the PD mice as compared to their untreated counterparts [96]. They found that there was corresponding lack of dopaminergic neuronal loss in the substantia nigra and decreases apoptosis in the hippocampus of the treated group as compared with the untreated control [96]. This recent study showed a

very promising outlook for BBR as a possible select compound for treatment of PD.

However, the results of Kim, *et al* stood in stark contrast to the findings of the Lee group in South Korea, which published two accounts [97, 98] of the neurotoxic effects of BBR when used to treat chemically-induced rat models of PD. In these studies, first, a PC12 cell model of PD showed that when treated with BBR, dopaminergic loss was elevated as compared to the untreated controls [97]. Treatment of a PD rat model with BBR (both at 5mg/kg and 15mg/kg) aggravated depletion of dopamine and norepinephrine as well as degeneration of tyrosine hydroxylase-immunopositive cells *in vivo* [98]. It is important to note that the differences in findings between the Lee and Yang group could possibly be partially attributed to their differences in animal models (mouse vs. rat) and the rate at which the species naturally metabolizes BBR.

As MAO-A and MAO-B are both natural dopamine-degrading agents, their inhibition has always been a therapeutic strategy against PD [99-102]. However, due to the toxic side-effects of MAO inhibitors, this route has usually been reserved as the final effort in a patient's treatment against advancing PD. Castillo, *et al* used direct LED fluorescence to detect changes in MAO-B levels in hopes of using the method as a way to confirm the successes of various MAO-B inhibiting agents in PD treatment [103]. Although it was previously known that BBR could inhibit MAO activity (with an IC50 of 126 uM for MAO-A and 98.4 uM for MAO-B), the results of the Jaimes group put into perspective the usability of BBR as a MAO-inhibitor for PD treatment [89, 103, 104]. Compared to other MAO inhibitors that can often be highly toxic, BBR is highly

preferable for its safety.

Bae, *et al* discovered from *in vitro* studies using human-derived SH-SY5Y cells as an *in vitro* model of dopaminergic neurons that BBR prevented cell death by protecting against ROS damage [105]. The mechanism relied on activation of heme oxygenase-1 and inhibition of caspase-3 activation to parry neuronal apoptosis [105].

Taken together, while these results do show the possibilities of BBR as a therapeutic agent against PD, they also suggest proceeding with caution, as slight species/model-dependent differences in BBR metabolism could account for vastly different results.

BBR has also shown broad neuroprotective effects. General oxidative damage to neurons and neurodegeneration plays an important role in a wide range of neurological disorders. To achieve its neuroprotective effects, BBR has been reported to activate nuclear factor-like 2 (Nrf2), aid in phosphorylation of Protein Kinase B (Akt), and cAMP response element binding protein (CREB), down-regulate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), and enhance phosphoinositide 3-kinase (PI3K) expression via (P55y subunit) increasing promoter activity [106-116].

Using mouse neuroblastoma cell lines, Hsu *et al* determined the *in vitro* neuroprotective effects of BBR. They found that in addition to the known anti-oxidative pathways, BBR was also capable of activating Nrf2, an anti-oxidant factor. Furthermore, they also found evidence that BBR stymied neuronal cell death by bolstering B-cell lymphoma-2 (Bcl-2), an antagonist of apoptosis [106].

In addition, Zhang, *et al* used a rat stroke model to test the abilities of BBR to

protect against stroke. They found BBR to be neuroprotective in the acute phase of the stroke when administered immediately after insult. They also traced the BBR's ability to up-regulate phosphorylation of neuroprotective factors: Akt, and CREB [107]. BBR was also found to down-regulate the inflammatory response factor, NF-kB, to suppress inflammation in the area [107]. Interestingly, they also discovered that BBR can affect the permeability of the blood-brain barrier by up-regulating the integral membrane protein, claudin-5 [107].

In 2011, Hu, *et al* found that in a mouse model with surgically induced stroke, BBR presented a unique way of bypassing a self-insulting down-regulation of the cell growth/proliferation factor, PI3K [108]. In their model, BBR could target the P55y subunit of PI3K and enhance its promoter activity while side-stepping its natural antagonists, Ly294002 and Akti-1/2, which is also an Akt inhibitor [108]. This resulted in significantly enhanced reperfusion to the damaged areas as compared to the untreated controls [108]. Interestingly, this group chose caudal intravenous administration of BBR between 0.002 and 0.2 mg/kg as opposed to the more economic, safer, and much more commonly used oral gavage route.

The multifaceted abilities of BBR to act against several neurodegenerative conditions or afflictions suggest that it may act on the shared pathways of neurodegeneration in these diseases. MAO-B inhibition has been implicated to have positive effects against both Alzheimer's and Parkinson's patients [99-101]. A possible reason could be that the MAO-B-mediated dopamine metabolism pathway leads to H_2O_2 generation and as MAO-B activity is elevated in AD and PD, the excess H_2O_2 can cause

oxidative stress and damage [102]. Damage, as well as proapoptotic stimulation from ROS has also been implicated in a wide array of neurodegenerative afflictions [117, 118] and the ability of BBR to counteract ROS certainly serves to uphold its ability to affect a diverse group of ailments in which neurodegeneration is a major factor. Furthermore, BBR's inhibitory effects on AChE could lead to effects on a great number of neurodegenerative diseases as AChE has exhibited apoptotic induction, in addition to its general role as antagonist to acetylcholine [119]. Because of this, AChE inhibition is already in use as a therapeutic approach to curtailing neurodegeneration [120]. General neuro-protective factors that BBR can enhance or up-regulate include Nrf2, GLP-1 and others [106, 107, 121-123]. Nrf2 has shown neuroprotection through a pathway that defends against ROS damage [124, 125]. GLP-1 up-regulation and receptor stimulation have exhibited neuro-protective effects towards preservation of dopaminergic neurons, which have applications in stroke, AD, PD, Huntington's disease (HD) and more [126-128]. Additionally, BBR's ability to phosphorylate AKT and activate the PI3K-AKT signaling pathway results in the ability to resist apoptosis via inhibition of caspases and cytochrome C [121-131]. CREB is another important protein, the phosphorylation of which can be up-regulated by BBR. Research has shown pCREB to be an essential part of neurons that survive insult, and knocking it down could cause neurodegeneration in the brain [132, 133]. Due to these common pathways that result in neurodegeneration, BBR is able to affect a great number of disorders in which neurodegeneration is key (Fig. 1-4).

BBR has shown great therapeutic potential against neurodegenerative diseases

including AD and PD as well as stroke. Its ability to effectively cross the blood-brain barrier (as well as its small size, which allows for the uncanny ability to act on a number of molecular targets) is a pillar of its merit as an anti-neurodegenerative agent. Because of the extensive range of effects, BBR is likely to address diseases in a positive way by correcting multiple deficiencies and tackling the problem from various angles simultaneously. However, BBR is unlikely to have a very strong effect on any single aspects, which may both limit its effect but also prevent harmful levels of imbalance, which result in toxicity. These facts coupled with its overall favorable effects on brain function suggest a potential myriad of other undiscovered applications on neurodegenerative diseases. The other pillar is its safety, with no documented serious toxic effects even in high doses given orally. Because of this crucial feature, and that it has been safely used in China for decades even for long-term clinical treatment, upon even mild-moderate success on animal models, BBR could quickly advance through clinical trials (for specific ailments) rather than taking the usual several long steps before being deemed safe. On a final note, the therapeutic potential of BBR shows great promise and merits great interest and research investment for future development and exploration into the possibilities of this multi-faceted, non-toxic botanic compound.

1.7 Berberine and Huntington's Disease

To date, no studies have been conducted on the effect of BBR on HD. HD is a highly mysterious and multi-faceted disease and coupled with BBR's diversity, there is a chance that BBR could address one or more of the aspects involved in HD. That AD and

PD are both neurodegenerative diseases characterized by protein aggregate accumulation in the brain via a similar fashion to HD gave additional strong encouragement to explore the possibilities of BBR's effect on the disease course of HD. Among the pathological biomarkers of HD, those which have connections previously established with BBR's abilities include abnormalities and failures of cholesterol metabolism, inactivation of the ERK pathway, abnormally high MAO levels, AChE and BChE deficiences, high glucose levels with resistance to insulin, and increased ROS [134-140]. That BBR has been reported to up-regulate autophagically-induced apoptosis may offer a possible mechanism for enhanced clearance of defunct proteins such as mHtt (in both aggregate and soluble form). BBR may affect autophagy through indirect regulation of transcription factor E-box (TFEB), a master regulator of the autophagic pathways. Two biochemical routes through which BBR may exert its effects on autophagy are 1. BBR phosphorylation of ERK causes up-regulation of TFEB, [84, 141-144] or 2. BBR up-regulates sirtuin 1 (SIRT1), which up-regulates PGC1-α, which up-regulates TFEB [145-150]. While individual parts of these chains have been documented, the entire working process has yet to be evidenced. Nonetheless, they present very interesting possibilities for how BBR may correct some imbalances caused by HD and thus positively modify disease course.

1.8 Summary of Aims and Results of Study

The primary aim of this study was to see if BBR exhibits any significant disease-modifying capability against HD first in an *in vitro* cellular model, then in an animal

model. If the primary objective came back positively, then the secondary aim would be to provide new insight into its broad actions.

The hallmark mHtt aggregation was used solely to assess anti-HD activity *in vivo*. To examine BBR's effect on a HD cellular model, we used HEK-293 cells, a non-polar kidney cell-line, for its ease of culture and transfection. The cells were grown and several concentrations of BBR were used to see if any could diminish mHtt build-up via Western blot and immunofluorescent staining and if so, which concentrations yielded the best results. After transfection with pathogenic 120Q and non-pathogenic 20Q variants of Htt on the HEK-293 cells, BBR concentrations of 5, 25, 50, or 100uM were administered and the results showed a pronounced mHtt-reducing effect that became apparent at the 50-100uM range. No significant changes were observed in non-pathogenic mHtt expression, and since the expression of both normal and mutant Htt was under the same promoter, this could temper the factor of BBR interfering with promoter activity. After this, the question was how quickly BBR had to be introduced in order to have an effect. Since the previous experiment was carried out with BBR added at the time of transfection, there was the concern that if BBR could not act on pre-formed aggregates, then it would not be effective as a drug since that meant it would have to be given to patients long before symptoms arose while most HD is only diagnosed when symptoms appear and mid-late life. So a time-trial was carried out using 50uM BBr to treat HD-120Q transfected cells at different times, ranging from immediately after transfection to 12 and 24 hours later. The results were detected by Western blot, then confirmed by immunofluorescent cell-counting, taking into account

both the number of aggregates and the number of cells. Both cases showed that while waiting 24 hours usually resulted in negligible benefits from BBR treatment, waiting 12 hours still yielded noticeable improvement over untreated samples. So these 2 experiments showed that BBR treatment could reduce mHtt build-up in a dose and time-dependent maner.

Next, we wanted to see what the underlying mechanism to BBR's anti-mHtt activity was. Since defunct protein clearance is done in the body mainly by the ubiquitin-proteasome system (UPS) and autophagy pathway, these would be of interest. The effect of the UPS on HD is unclear, with some people suggesting that the UPS may be incapable of completely degrading mHtt, instead, releasing polyQ fragments which contribute to aggregation. Also, BBR has already been reported to increase autophagy in cancer cells to induce apoptosis. Thus, BBR's effect on autophagy became the main hypothesis and focus of this study. We first wanted to see if our previous experiments, in which BBR diminished mHtt aggregation, could show a correlation between mHtt aggregate reduction and increase in autophagic flux. Autophagic markers P62 and LC3B were used as indicators of autophagy, and in both cases, they showed that decreases in mHtt aggregation from BBR treatment were correlated with increases in autophagy. However, this showed only correlation; causation still needed to be established. Thus, HEK-293 cells were transfected with HD-120Q and left untreated, or treated with BBR, Bafilomycin-A (BFA), or both. (BFA was selected as an autophagy inhibitor.) The results showed that when BBR was used, mHtt aggregation was

diminished, but when BFA was added along with BBR, the effect could no longer be seen. This suggested that BBR indeed acted through autophagy to clear mHtt.

Our next aim was to see if this could all be replicated in an animal model. N171 82Q transgenic HD model mice from Jackson Labs were used. These mice expressed a 171-amino acid fragment of htt with 82 CAG repeats in the polyQ tract. These mice and their WT siblings were separated into groups and given either vehicle or BBR orally every day at a dose of 40mg/kg for BBR. Throughout the 5-month study, the mice were weighed and tested on the rotarod for motor coordination and given the grip strength test for power. The results showed the obvious superiority of the BBR-treated mutants as compared to their untreated mutant siblings, although there was still a widening gap when compared to WT siblings. The differences in rotarod performance became apparent and statistically significant (between the treated and untreated mutant groups) much earlier in the trial than the differences in grip strength, pointing to neurological degeneration proceeding muscular atrophy. An additional balance beam test showed similar results, with the 2 WT groups roughly even, the treated mutant group taking a few seconds longer, and the untreated mutant group taking the longest by a statistically significant margin. In the end, the average lifespan of the treated mutant group was roughly 10% or 14 days longer than their untreated mutant siblings, however, statistically, this is only suggestive, not strictly significant (P=0.052).

Pathology of the brain samples was evaluated by Western blotting and immunohisto fluorescent staining. Immunohisto fluorescent staining indicated that through evaluation of 3 mutant brains for both untreated and treated groups, on

average, there was greater mHtt aggregation in the untreated brains. This was confirmed through Western blotting, which also revealed through P62 staining that on average, autophagy was increased in the BBR-treated groups (both mutant and WT). RT PCR was performed on the 6 previously-evaluated mutants (3 treated and 3 untreated) to ensure that differences in mHtt aggregation were not due to variations in expression levels of transgenic mice and the results showed nearly identical average mHtt expression between the 2 mutant groups.

Tab. 1-1

Table. Summary of clinical findings for Huntington's disease (HD) patients.

Clinical findings	Group HD	
	Juvenile and childhood onset (n=4)	Adult and late onset (n=46)
Rigidity	100%	86%
Bradykinesia	100%	33.5%
Ataxia	100%	67%
Dystonia	75%	48%
Chorea	75%	86%
Dysarthria	100%	76%
Pyramidal signs	100%	76%
Dysmetria	50%	38%

Ruocco, Heloísa H., et al. "Clinical presentation of juvenile Huntington disease." Arquivos de neuro-psiquiatria 64.1 (2006): 5-9.

Tab. 1-1 HD manifests in several commonly-seen symptoms.

Frequency of most commonly presented HD symptoms among juvenile and late onset HD patients

Tab. 1-2

Median Age at Onset

CAG Repeat Size	Median Age at Onset[a] (95% CI) (years)
39	66 (72–59)
40	59 (61–56)
41	54 (56–52)
42	49 (50–48)
43	44 (45–42)
44	42 (43–40)
45	37 (39–36)
46	36 (37–35)
47	33 (35–31)
48	32 (34–30)
49	28 (32–25)
50	27 (30–24)

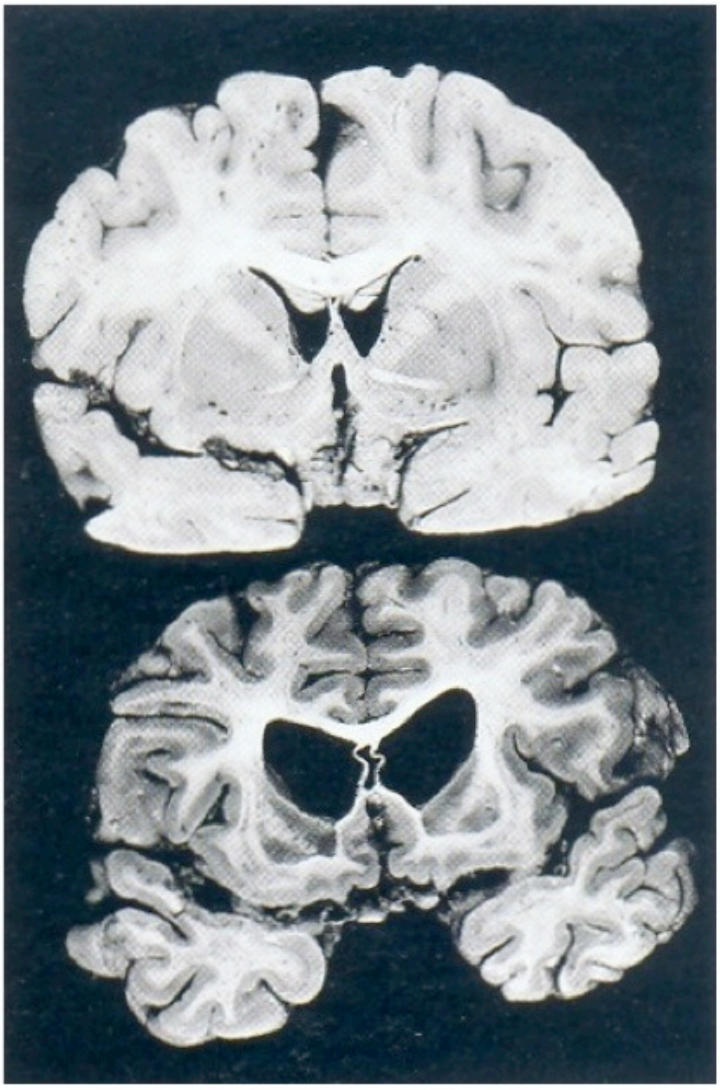
[a] Age by which 50% of individuals will be affected.

Brinkman RR, Mezei MM, Theilmann J, Almquist E, Hayden MR. The Likelihood of being affected with Huntington Disease by a particular age, for a specific CAG size Am. J. Hum. Genet. 60 (1997) 1202-1210.

Tab. 1-2 Polyglutamine tract length increases hasten HD onset.

Polyglutamine tract length of Htt protein as correlated to average expected age of onset in HD patients

Fig. 1-1



Association des paralysés de France. 1996. Déficiences motrices et handicaps, Aspects sociaux, psychologiques, médicaux, techniques et législatifs, troubles associés. Paris : Association des paralysés de France. 505 p.

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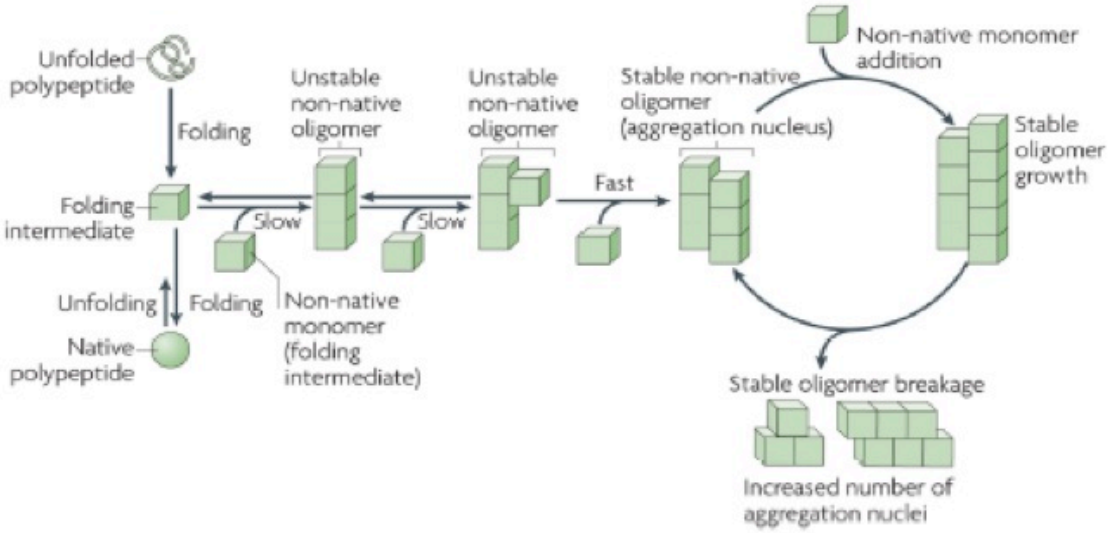
Fig. 1-1 HD causes degeneration of the brain.

Clinical presentation of HD-caused cortical, hippocampal degeneration with enlarged ventricles (top) as compared to normal brain (bottom) cross section.

Fig. 1-2



Brundin, Patrik, Ronald Melki, and Ron Kopito. "Prion-like transmission of protein aggregates in neurodegenerative diseases." *Nature Reviews Molecular Cell Biology* 11.4 (2010): 301-307.

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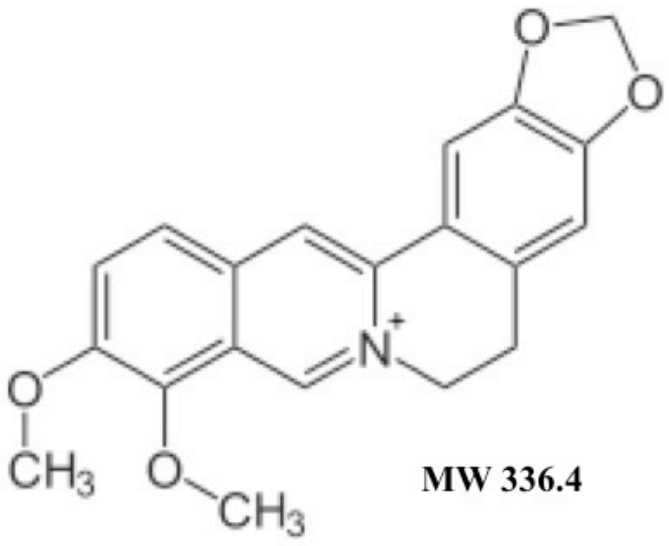
Fig. 1-2 HD is characterized by mHtt aggregate accumulation.

Theoretical model for dynamics of protein aggregate formation and accumulation with oligomer stabilization as a key contributing event.

Fig. 1-3



MW 336.4

Fig. 1-3 BBR is a stable organic compound.

Natural structure of BBR

Fig. 1-4

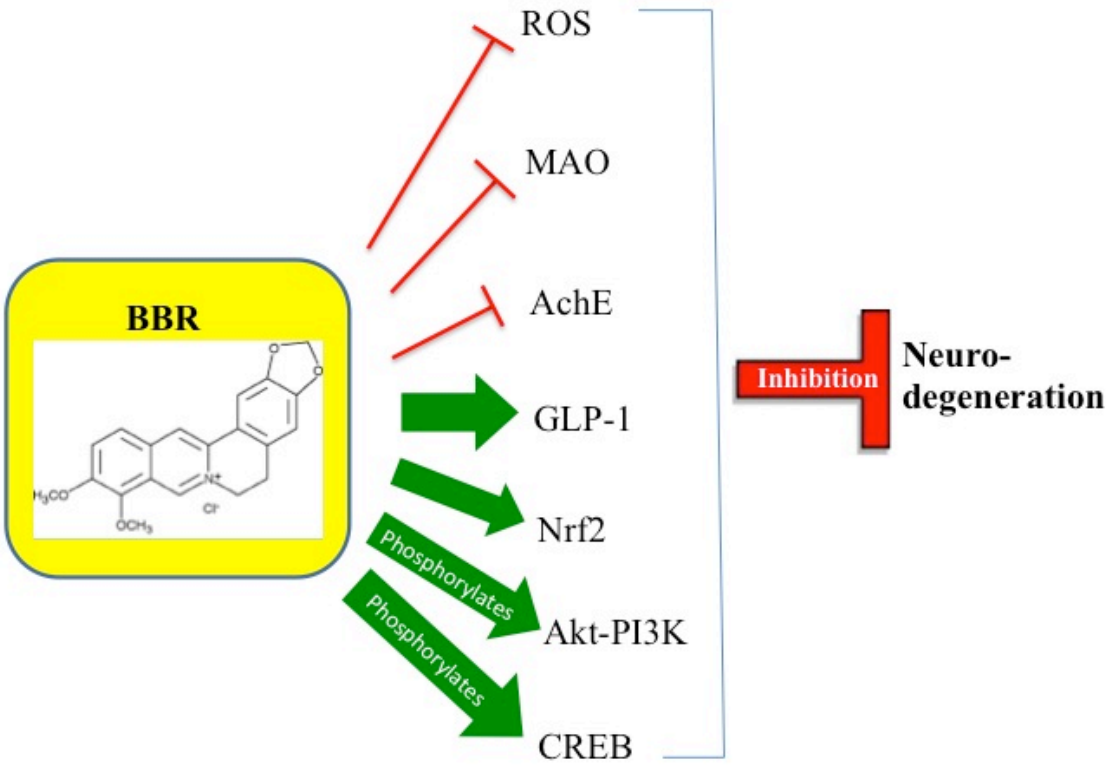

BBR
ROS
MAO
AchE
GLP-1
Nrf2
Phosphorylates
Akt-PI3K
Phosphorylates
CREB
Inhibition
Neuro-
degeneration

Fig. 1-4 BBR exhibits several neuroprotective activities.

Known activities of BBR as pertinent to neurological disorders and defense against neurodegeneration

Chapter II:

Therapeutic potential of Berberine Against Neurodegenerative Diseases

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Jiang, WenXiao, ShiHua Li, and XiaoJiang Li. "Therapeutic potential of berberine against neurodegenerative diseases." *Science China Life Sciences* (2015): 1-6.

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AUTHOR CONTRIBUTIONS

Wenxiao Jiang authored the manuscript while Shihua Li and XiaoJiang Li edited the paper for content.

2.1 Abstract

Berberine (BBR) is an organic small molecule isolated from various plants that have been used in traditional Chinese medicine. Isolation of this compound was its induction into modern medicine, and its usefulness became quickly apparent as seen in its ability to combat bacterial diarrhea, type 2 diabetes, hypercholesterolemia, inflammation, heart diseases, and more. However, BBR's effects on neurodegenerative diseases remained relatively unexplored until its ability to stunt Alzheimer's disease (AD) progression was characterized. In this review, we will delve into the multi-faceted defensive capabilities and bio-molecular pathways of BBR against AD, Parkinson's disease (PD), and trauma-induced neurodegeneration. The multiple effects of BBR, some of which enhance neuro-protective factors/ pathways and others counteract targets that induce neurodegeneration, suggest that there are many more branches to the diverse capabilities of BBR that have yet to be uncovered. The promising results seen provide a convincing and substantial basis to support further scientific exploration and development of the therapeutic potential of BBR against neurodegenerative diseases.

Key Points

- BBR has a positive effect on AD, PD, and general neurodegeneration
- BBR merits exploration of therapeutic potential against neurodegenerative diseases

2.2 Introduction

Berberine (BBR) is an organic compound isolated from various herbs such as *Coptis chinensis, Berberis vulgaris, Hydrastis Canadensis, Phellodendron amurense.* For decades, Chinese medicine has used the plants and their extracts to treat diarrhea with no observable negative side-effects or toxicity in patients [1-3]. Modern advances in research, however, allowed us to discover BBR as the active compound and to synthesize it. As a result, BBR was found to be a small molecule with a molecular weight of only 371.8 Da [3] (Fig.1).

BBR has been used clinically to treat bacterial diarrhea, hypercholesterolemia, type 2 diabetes, cardiac disease, cancer, and more [3-14]. Although studies in rodents have shown that BBR can cross the blood brain barrier with positive effects on brain function, the mechanism remains unclear [15]. This factor points to the possibility that BBR may have pronounced effects on the brain and central nervous system. Additionally, in animal trials, BBR has shown itself to have positive effects on Alzheimer's and Parkinson's models [16, 17]. Although we possess only a nascent understanding of BBR's effects and mechanisms on the brain and nervous system, its effects on Alzheimer's models (achieving phenotypical amelioration), Parkinson's models, and its uncanny ability to act with robust diversity, begin to shed light on BBR's abilities to positively effect the outcome in diseases of the central nervous system.

According to the Center of Disease Control in the United States, an estimated 5 million people suffer from Alzheimer's disease (AD) [18]. AD is a late-onset disease, typically presenting after age 60 that is characterized by memory loss and handicapped

daily functions. To this day, the exact cause of AD has not been pinpointed, with scientists currently believing the disease to arise from multiple contributing factors including genetic and environmental influences. However, end diagnosis always rests with beta-amyloid plaque build-up in the brain [19]. Despite the prevalence and severity of AD, its complexity has left modern science without answer, but in dire need of treatment options.

Parkinson's disease (PD) is another common form of neurological disease that presents classically with resting tremor, rigidity, bradykinesia, postural instability and oftentimes, senile dementia [20]. PD is highly prominent especially in western European populations where the prevalence rate is estimated at 160 per 100,000 and as high as 4% amongst persons over the age of 80 [20]. Although the direct pathological cause of PD has been stemmed to protein aggregations (called Lewy bodies) and loss of dopaminergic cells in the substantia nigra, the etiology behind PD is believed to be highly diverse [20, 21] and the exact pathogenic mechanism is still unclear. Although current treatments for PD include dopamine agonists and monoamine oxidase B (MAO-B) inhibitors (to reduce breakdown of dopamine), they are symptom-targeted and produce serious side-effects [22]. To this day, a cure or non-toxic treatment for PD has eluded modern science.

2.3 The therapeutic effect of BBR on AD

Although no single underlying cause has be established for beta-amyloid plagues-associated AD, a plethora of relatively small imbalances have been found in AD patients

and are likely to contribute to the symptoms of the disease. Due to its multi-faceted nature, BBR has been shown to address several of those imbalances in a positive way. These activities include cholesterol reduction, ERK pathway activation, inhibition of MAO-B activity, defense against damage from reactive oxygen species (ROS), inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity, and beta-secretase, and reduction in the amyloid-beta genesis [3, 23-26]. This has lead to an overall reduction in amyloid plaque aggregation and reduction in phenotypic pathology of AD in the mouse model [27].

In 2009, Jung, *et al* ran a number of inhibitory assays to determine the anti-AD effects of several protoberberine alkaloids [24]. The IC50 (50% inhibitory concentration) was determined for each of the six compounds in their ability to stunt an AD-related activity. The inhibitory assays included β-site amyloid precursor protein cleaving enzyme 1 (BACE1), AChE, BChE, and reactive oxygen species (ROS). In addition to total ROS, peroxynitrite ($ONOO^-$) scavenging was given particular attention due to its strong role in amyloid B formation. Through these assays, Jung *et al* found that although BBR was ineffective at inhibition of BACE1 and total ROS, the compound showed promising results against AChE, BChE, and ONOO- (requiring 0.44, 3.44, and 23.06 uM respectively to reach IC50 of a 100 uM target solution) [24].

In 2013, Panahi, *et al* showed that with 50 mg/kg oral BBR taken daily, rabbits with chemically-induced AD-like symptoms showed improvement as compared to the untreated group. Although weight loss was only moderately abated, survival was significantly improved [25]. Interestingly, the results showed significant inhibition of

BACE1 in the treatment group, which stand in contrast to the prior findings of Jung *et al* [24].

Asai, *et al* also discovered that 10 uM BBR could reduce amyloid B levels to 30% of the untreated amount *in vitro* [26]. Furthermore, through Western-blotting of the different components of amyloid precursor protein (APP), they discovered that the mechanism behind the reduction was the result of APP pathway modulation towards a non-amyloid metabolite, as opposed to direct destruction of the protein itself or of its precursor [26].

Using a transgenic AD model mouse strain (CRN8D), Durairajan, *et al* tested the effects of BBR on neurological impairment. The group found that BBR significantly reduced amyloid B plaque aggregation, leading to amelioration of mental and neuronal impairment in the treated group as compared to untreated transgenic control [27]. Results also showed that these effects were achieved through inhibition of APP phosphorylation via suppression of glycogen synthase kinase (GSK) 3 activation [27]. In addition, the group found that treatment with a lower dosage (25mg/kg) often showed preferential results as compared to treatment with a higher dosage (100mg/kg) [27].

All these studies present consistent information of BBR's effects against the various components of AD pathogenesis and its end treatment results as manifested in the amelioration of disease and pathology presentation as well as retardation of progression of AD. Furthermore, the dosage study done by the Li's group [27] suggests that to further develop the therapeutic capabilities of BBR against AD, biochemical

approaches must be elicited, as increases in concentration can be counter-productive in treatment.

2.4 The therapeutic effect of BBR on PD

Although the pathology of Parkinson's Disease (PD) has been pinned to the formation of Lewy body protein aggregates and loss of dopaminergic neurons in the brain, the causes that lead up to the pathology are largely unknown. Due to our lack of understanding of the true cause of this disease, effective treatment strategies remain to be developed. Thus, current therapies against PD are limited to amelioration of its symptoms, the main focus of which is stemmed around prevention or retardation of further dopaminergic neuronal loss. There has been substantially less research done on the therapeutic potential of BBR against PD than there has been against AD, and the mixed results and lack of clear success could be the reason. However, there are at least two documented effects that merit BBR or at least some derivative or metabolite in consideration for continued studies to further address the possibility of its use as an anti-PD agent.

In 2014, Kim, *et al* used a chemically-induced mouse model of PD to test the neuro-protective effect of BBR. According to their data, at 50 mg/kg, BBR significantly prevented both memory and balance loss in those PD mice as compared to their untreated counterparts [28]. They found that there was corresponding lack of dopaminergic neuronal loss in the substantia nigra and decreases apoptosis in the hippocampus of the treated group as compared with the untreated control [28]. This

recent study showed a very promising outlook for BBR for consideration as a select compound for treatment against PD.

However, the results of Kim, *et al* stand in stark contrast to the findings of the Lee group in South Korea, which published two accounts [29, 30] of the neurotoxic effects of BBR when used to treat chemically-induced rat models of PD. In these studies, first, a PC12 cell model of PD showed that when treated with BBR, dopaminergic loss was elevated as compared to the untreated controls [29]. Treatment of a PD rat model with BBR (both at 5mg/kg and 15mg/kg) aggravated depletion of dopamine and norepinephrine as well as degeneration of tyrosine hydroxylase-immunopositive cells *in vivo* [30]. It is important to note that the differences in findings between the Lee and Yang group could possibly be partially attributed to their differences in animal models (mouse vs. rat) and the rate at which the species naturally metabolizes BBR.

As MAO-A and MAO-B are both natural dopamine-degrading agents, their inhibition has always been therapeutic strategy against PD. However, due to the toxic side-effects of MAO inhibitors, this route has usually been reserved as the final effort in a patient's treatment against advancing PD. Castillo, *et al* used direct LED fluorescence to detect changes in MAO-B levels in hopes of using the method as a way to confirm to successes of various MAO-B inhibiting agents in PD treatment [31]. Although it was previously known that BBR could inhibit MAO activity (with an IC50 of 126 uM for MAO-A and 98.4 uM for MAO-B), the results of the Jaimes group put into perspective the usability of BBR as a MAO-inhibitor for PD treatment [23, 31, 32]. Compared to

other MAO inhibitors that can often be highly toxic, BBR is highly preferable for its safety.

Bae, *et al* discovered from *in vitro* studies using the human-derived SH-SY5Y cells as an *in vitro* model of dopaminergic neurons that BBR prevented cell death by protecting against ROS damage [33]. The mechanism relied on activation of heme oxygenase-1 and inhibition of caspase-3 activation to parry neuronal apoptosis [33].

Taken together, while these results do show the possibilities of BBR as a therapeutic agent against PD, they also suggest proceeding with caution, as slight species-dependent differences in BBR metabolism could account for different efficacy of BBR.

2.5 General neuroprotective effects of BBR

BBR has also shown broaden neuroprotective effects. General oxidative damage to neurons and neurodegeneration plays an important role in a wide range of neurological disorders. To achieve its neuroprotective effects, BBR has been reported to activate nuclear factor-like 2 (Nrf2), aids in phosphorylation of Protein Kinase B (Akt), and cAMP response element binding protein (CREB), down-regulates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), and enhances phosphoinositide 3-kinase (PI3K) expression via (P55y subunit) increasing promoter activity [34-44].

Using mouse neuroblastoma cell lines, Hsu *et al* determined the *in vitro* neuroprotective effects of BBR. They found that in addition to the known anti-oxidative

pathways, BBR was also capable of activating Nrf2, an anti-oxidant factor. Furthermore, they also found evidence that BBR stymied neuronal cell death by bolstering B-cell lymphoma-2 (Bcl-2), an antagonist of apoptosis [34].

In addition, Zhang, *et al* used a rat stroke model to test the abilities of BBR to protect against stroke. They found BBR to be neuroprotective in the acute phase of the stroke when administered immediately after insult. They also traced the BBR's ability to up-regulate phosphorylation of neuroprotective factors: Akt, and CREB [35]. BBR was also found to down-regulate the inflammatory response factor, NF-kB, to suppress inflammation in the area [35]. Interestingly, they also discovered that BBR can affect the permeability of the blood-brain barrier by up-regulating the integral membrane protein, claudin-5 [35].

In 2011, Hu, *et al* found that in a mouse model with surgically induced stroke, BBR presented a unique way of bypassing a self-insulting down-regulation of the cell growth/proliferation factor, PI3K [36]. In their model, BBR could target the P55y subunit of PI3K and enhance its promoter activity while side-stepping its natural antagonists, Ly294002 and Akti-1/2, which is also an Akt inhibitor [36]. This resulted in significantly enhanced reperfusion to the damaged areas as compared to the untreated controls [36]. Interestingly, this group chose caudal intravenous administration of BBR between 0.002 and 0.2 mg/kg as opposed to the more economic, safer, and much more commonly used oral gavage route.

2.6 Effects of BBR on the common pathways in neurodegeneration

The multifaceted abilities of BBR to act against several neurodegenerative conditions or afflictions suggests that it may act on the shared pathways of neurodegeneration in these diseases. MAO-B inhibition has been implicated to have positive effects against both Alzheimer's and Parkinson's patients [45-6]. A possible reason could be that the MAO-B-mediated dopamine metabolism pathway leads to H_2O_2 generation and as MAO-B activity is elevated in AD and PD, the excess H_2O_2 can cause oxidative stress and damage [47]. Damage, as well as proapoptotic stimulation from reactive oxygen species (ROS) has also been implicated in a wide array of neurodegenerative afflictions [48-9] and the ability of BBR to counteract ROS certainly serves to uphold its ability to affect a diverse group of ailments in which neurodegeneration is a major factor. Furthermore, BBR's inhibitory effects on AChE could lead to effects on a great number of neurodegenerative diseases as AChE has exhibited apoptotic induction, in addition to its general role as antagonist to acetylcholine [50]. Because of this, AChE inhibition is already in use as a therapeutic approach to curtailing neurodegeneration [51]. General neuro-protective factors that BBR can enhance or up-regulate include Nrf2, GLP-1 and others. Nrf2 has shown neuroprotection through a pathway that defends against ROS damage [52-3]. GLP-1 up-regulation and receptor stimulation has exhibited neuro-protective effects to preserve dopaminergic neurons, which have applications in stroke, AD, PD, Huntington's disease (HD) and more [54-6]. Additionally, BBR's ability to phosphorylate AKT and activate the PI3K-AKT signaling pathway results in the ability to resist apoptosis via inhibition of caspases and cytochrome C [57-9]. CREB is another

important protein, the phosphorylation of which can be up-regulated by BBR. Research has shown pCREB to be an essential part of neurons that survive insult, and knocking it down could cause neurodegeneration in the brain [60-1]. Due to these common pathways that result in neurodegeneration, BBR is able to affect a great number of disorders in which neurodegeneration is key (Fig.2).

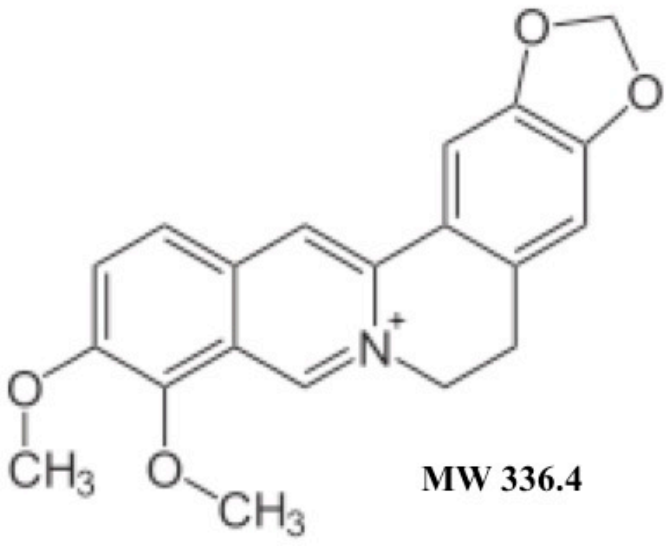
To summarize, BBR has exhibited promising neuroprotective properties going through a multitude of known bio-molecular pathways and undoubtedly a vast number of unknown ones to not only defend against neuronal damage and loss but also to aid in reperfusion of previously damaged tissue. Furthermore, due to the multi-faceted nature of BBR, there are sure to be more that await discovery, some of which could hold the key to some of the stubborn neurodegenerative diseases that we struggle with today.

2.7 Conclusion

BBR has shown great therapeutic potential against neurodegenerative diseases including AD and PD as well as stroke. Its ability to effectively cross the blood-brain barrier as well as its small size, which allows for the uncanny ability to act on a number of molecular targets, is a pillar of its merit as an anti-neurodegenerative agent. Because of the extensive range of effects, BBR is likely to address diseases in a positive way by correcting multiple deficiencies and tackling the problem from multiple angles simultaneously. However, BBR is unlikely to have a very strong effect on any single one of these aspects, which may both limit its effect but also prevent harmful levels of imbalance. These facts coupled with its overall favorable effects on brain function

suggest a potential myriad of other undiscovered applications on neurodegenerative diseases. The other pillar is its safety, with no documented serious toxic effects even in high doses given orally. Because of this crucial feature, and that it has been safely clinically used in China for decades even for long-term treatment, upon even mild-moderate success on animal models, BBR could quickly advance through clinical trials (for specific ailments) rather than taking the usual several long steps before being deemed safe. On a final note, the therapeutic potential of BBR shows great promise and merits great interest and research investment for future development and exploration into the possibilities of this multi-faceted, non-toxic botanic compound.

Fig. 1



MW 336.4

Fig.1 Molecular structure of Berberine

Fig. 2

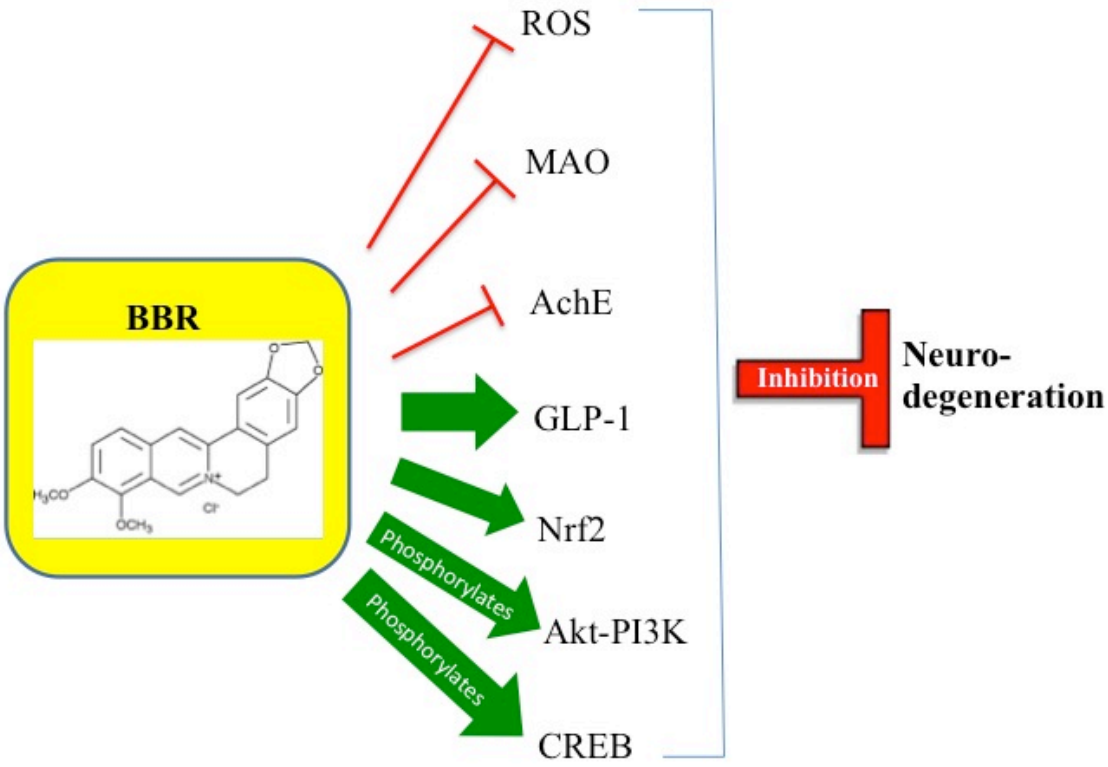

BBR
ROS
MAO
AchE
GLP-1
Nrf2
Phosphorylates
Akt-PI3K
Phosphorylates
CREB
Inhibition
Neuro-
degeneration

Fig.2 BBR interacts with many pathways that are affiliated with neurodegeneration or neuroprotection.

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Chapter III:

Therapeutic Effect of Berberine on Huntington's Disease Transgenic Mouse Model

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Wenxiao Jiang authored the manuscript, analyzed data, and performed experiments other than qRT-PCR. Wenjie Wei performed qRT-PCR. Marta A. Gaertig helped with maintaining mice. Li XiaoJiang and Li Shihua edited the manuscript

3.1 Abstract

Huntington disease (HD) represents a family of neurodegenerative diseases that are caused by misfolded proteins. The misfolded proteins accumulate in the affected brain regions in an age-dependent manner to cause late-onset neurodegeneration. Transgenic mouse models expressing the HD protein, huntingtin, have been widely used to identify therapeutics that may retard disease progression. Here we report that Berberine (BBR), an organic small molecule isolated from plants, has protective effects on transgenic HD (N171-82Q) mice. We found that BBR can reduce the accumulation of mutant huntingtin in cultured cells. More importantly, when given orally, BBR could effectively alleviate motor dysfunction and prolong the survival of transgenic N171-82Q HD mice. We found that BBR could promote the degradation of mutant huntingtin by enhancing autophagic function. Since BBR is an orally-taken drug that has been safely used to treat a number of diseases, our findings suggest that BBR can be tested on different HD animal models and HD patients to further evaluate its therapeutic effects.

Key words: Berberine, Huntington, autophagy, therapy

3.2 Introduction

Huntington's disease (HD) is a severe neurodegenerative disease that is characterized by chorea, dystonia, motor coordination loss, and mental deterioration. Age of HD onset is usually mid to late life, but in rare cases, may be seen in juveniles. The HD gene encodes huntingtin (Htt), a 350kDa protein with a variable-length polyglutamine (polyQ) tract encoded in exon 1 of the HD gene [1]. Expansion of the polyQ repeat tract (>36Q) results in HD, and increases of over 55Q lead to fast progression juvenile-onset HD [2, 3]. The reason for this threshold is most likely that expanded polyQ repeats cause N-terminal Htt fragments to misfold, leading to abnormal protein interactions and aggregation of mutant Htt [4, 5]. Although the role of Htt aggregates in HD remains controversial, they result from the accumulation of mutant Htt and have been used to assess the therapeutic effects of drugs on HD.

Currently, there is no effective treatment for HD, though some drugs, such as tetrabenazine and haloperidol, have been used in clinical studies for controlling symptoms of HD [6, 7]. One candidate that has shown promise through relatively recent discoveries is the plant-derived protoberberine alkaloid called Berberine (BBR). This small molecule with a molecular weight of 336.4 g/mol is derived from the roots and bark of various plants, such as *Coptis chinenses, Berberis sp.,* and has been used for over six decades in Chinese medicine as an over-the-counter for treatment of bacterial diarrhea [8, 9]. However, recent discoveries have unearthed a bounty of additional uses for this botanical compound, including its ability to combat hypercholesterolemia, diabetes, cardiac disease, inflammation, and the side-effects of radiotherapy [10-17].

Due to the high tolerance for orally-taken doses (LD50>5g/kg), BBR has been proven safe for long-term use, is readily available in the bloodstream 2 hours after oral intake and, more importantly, is able to freely cross the blood-brain-barrier [14, 18, 19], which make it an ideal drug candidate to test its protective effects on chronic neurological disorders.

The most encouraging and relevant discovery to HD is BBR's ability to ameliorate the symptoms and pathology of Alzheimer's Disease (AD) and Parkinson's disease (PD) animal models [16, 20-23]. The ability of BBR to reduce b-amyloid aggregation and accumulation in AD mice bears promising hope that it could do the same against HD, as both diseases are caused by the accumulation of misfolded proteins. Here, we examined the effects of BBR on mutant Htt's accumulation and toxicity in cellular and animal models of HD.

3.3 Materials and Methods

Ethics statement

All procedures were performed in accordance with the U.S. Public Health Service Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Emory University.

Mice

The N171-82Q mice were obtained from The Jackson Laboratory (strain 003627), which are a transgenic HD mouse model expressing 171 amino acids of the N-terminal Htt containing 82 glutamine repeats in the PolyQ tract under the mouse prion promoter [28]. Male N171-82Q mice were mated with wild type female mice to produce offspring mice on a B6 x C3H background. Genotyping was performed using PCR on DNA obtained from tail samples. Primers for genotyping N171 82Q mice are as follows: sense S26: 5'-CTA CGA GTC CCT CAA GTC CTT CCA GC-3', antisense A151: 5'-GAC GCA GCA GCG GCT GTG CCT G-3'. Wild type littermates and N171-82Q mice were used for investigation of their behaviors and the expression of transgenic mutant Htt. N171-82Q mice become increasingly symptomatic from 6-8 weeks with a short lifespan of 16-22 weeks. All mice were housed in groups of no more than 5 under a 12 h light/dark cycle with ad libitum access to normal chow diet at normal room temperature (22-25°C). 4 groups were created: mutant group treated with BBR, mutant group treated with vehicle, WT group treated with BBR, and WT group treat with vehicle. Each group consisted of 7 animals: 5 males and 2 females. The groups were created by separating the mutant and WT siblings of several litters all birthed within 4 days of each other. The fathers of these litters were N171 82Q mutants from a single bloodline and the mothers were unrelated WT females.

Antibodies and Reagents

Mouse monoclonal antibody to Htt (mEM48) is as described [24]. Rabbit anti-LC3B and mouse anti-b-actin were purchased from ABGENT; mouse anti-P62 was

purchased from Sigma. Green fluorescent Alexa488 donkey anti-mouse and all secondary antibodies were purchased from Jackson Labs.

Berberine-chloride and Bafilomycin-A were purchased from Sigma. Htt plasmids expressing exon1 Htt containing 120Q (HD120Q-GFP) or 20Q (HD20Q-GFP), which is fused with GFP, were generated in our previous studies [25].

Cell culture and transfection

HEK293 cells were cultured in 12-well plates in DMEM/F12 (Invitrogen) containing 10% (vol/vol) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 250 μg/μL Fungizone amphotericin B. Cells were maintained at 37°C in 5% CO_2 incubators. At roughly 50% confluence, cells were transfected with 0.25 μg of plasmid DNA per well using Lipofectamine 2000 (2mg/mL from Invitrogen) for 6 h in serum free medium. Within the next 24-48 h, the following were added: BBR (dissolved in methanol to final concentration of 5-100 μM), BFA (Bafilomycin A dissolved in DMSO to final concentration of 200 nM). In each case, the dilution was made such that 1 μL of the dissolved BBR or BFA was added to the well with 1 mL culture media. One μL of the vehicle was added to the control wells. The cells were then harvested at 48 h after BBR treatment or 24 h after BFA treatment.

Oral gavage of BBR

Four mg BBR were dissolved in 1 mL ddH_2O and mixed in a 2 mL tissue emulsifier (Wheaton). The solution of BBR (40 mg per kg or 10ml per gram of body

weight) was gavaged daily to each mouse (without anesthesia due to the dangers of daily anesthesia and the need for alert feedback from the mouse in case of abnormal occurrence). The same volume of ddH2O was gavaged into control mouse groups. Oral gavage was performed with 24G-1" 1.25 mm ball-tip stainless steel feeding needles slightly bent inserted along the back of the throat in mice. One mL syringes were used for the injection of BBR solution, which was done slowly without excessive pressure. If any mouse struggling was noticed, needle was withdrawn, and the mouse was given a brief rest before the needle was reinserted to administer the remaining solution. Mice were observed for roughly 15 minutes post-gavage to ensure normal and alert behavior. Oral administration of BBR started from 4 weeks of age until the death or sacrifice of animals.

Behavioral studies

Behavioral tests were performed in a room that was set to minimize external sound or visual disturbance. Rotarod performance was examined using Rotamex from Columbus Instruments. Bottom position of drop pan was well-padded with tissue paper to diminish chance of injury from fall. Mice were first trained once a day for 3 consecutive days, allowing them to run freely for 10 min per training session. After training, each mouse was run in triplicate per test and the average was taken. If any value presented at less than half of other values, a retrial was given and counted only if it was higher than the initial value.

Grip strength was tested using Chatillon-Ametek 2lbf force meter mounted to Columbus Instruments grip force meter stand. Mice were allowed to grip the grid with all 4 appendages and were pulled with a single constant force by the tail until removed from the machine. Each mouse was tested in triplicate and the average was taken.

Balance beam test was run using a 0.6 cm thick meter stick suspended from a platform on both sides by metal grips. The total running distance was roughly 0.8 m. There is a bright light at the starting point and a dark box at the endpoint. Prior to data-collection, each mouse was trained for 3 consecutive days with 3 runs per day. After training, mice were allowed to run only 1 time, since consecutive runs always yielded increasingly inferior results due to lack of will/curiosity, and the time taken to cross the beam was collected.

Sacrifice

The bodyweights of mice were at first monitored weekly, but increased to bi-weekly near the final weeks of the experiment as the mice atrophy. Mice were monitored daily before each gavage for excessive trembling, loss of coordination and muscle weakness. The endpoint was reached when a sudden weight drop of ~20% over a week was coupled with severe tremor, weakness, or abnormal gait. When the mice reached this point, they were sacrificed for their brain tissue. They would be put into an isofluorane chamber until knocked out, then swiftly decapitated with a scissor and the brains harvested with one half stored in an 1.5ml Eppendorf tude at -80°C and the other half preserved in Tissue Plus Optimum Cutting Temperature Compound (Fisher

Healthcare) and stored at -80°C as well. However, despite these efforts, 6 out of 14 mutant mice died of disease without being sacrificed. (There were no unexpected losses in WT mice.) In these cases, they died without reaching the described endpoint as sometimes N171 82Q mice may pass without obvious debilitation. Care was taken not to over-assess symptoms and sacrifice too early in order to provide more accurate survival data.

Fluorescent microscopy

Cultured cells were fixed with 4% paraformahdehyde (Electron Microscopy Sciences) for 10 min at room temperature and then treated with 0.5% saponin (Fluka). They were then stained with 1:3,000 Hoechst solution for nuclear labeling. The plates were then used directly for fluorescent microscopy to examine the expression of Htt-120Q-GFP and Htt-20Q-GFP.

For immunostaining of mouse brain sections, the freshly-isolated brain tissues were preserved in Tissue Plus Optimum Cutting Temperature Compound and transferred from -80°C for cutting to 10 micron-slices with the Leica CM1850 cryo-cutting machine set at -20°C. The sections were mounted onto slides and preserved in 4% paraformaldehyde for 10 min and washed 3 times in PBS before blocking in 3% bovine serum albumin (BSA), 0.3% Triton X-100 for 2 h at room temperature. Primary antibody mEM48 in 3% BSA was incubated with brain sections for 16 h at 4°C. After washing in PBS, the secondary antibody (green fluorescent Alexa488 donkey anti-

mouse) was added at 1:5,000 for incubation at 4°C for 1-2 h. The slides were then washed again in PBS 3 times before microscopic examination.

Western Blotting

Cultured cells (in 12-well plates) were dissolved in SDS-1% Triton 100-X /PBS at 125 mL per well. The cells were collected into Eppendorf tubes and sonicated (QSonica, LLC XL-2000 at setting 1) for 10 sec on ice. After boiling for 10 min, the samples were loaded into a 4-20% polyacrylamide SDS gel. When brain tissues were analyzed, homogenization buffer was first made freshly by adding 1% Triton 100X, protease inhibitor Pierce 78430 1:100, phenylmethanesulfonyl fluoride 100 mm/mg (Sigma), 2 mM sodium orthovanadate 1:200 (Sigma), and sodium fluoride 100 mM (Sigma) to PBS. The mouse brain cortex was homogenized in 100 volumes of the chilled homogenization buffer. The tissue extracts were then sonicated for 10 sec on ice and rocked (Boekel Scientific Orbitron Rotator II Model 260250 at max rpm) for 30 min at 4°C before being spun down at 16,000 RPM for 15 min at 4°C. The supernatant was then taken and combined with 0.25 volumes 5x loading dye, followed by boiling for 10 min and sonicated again for 5 sec. The samples were resolved by SDS gel, which was transferred to a nitrocellulose membrane (GE Healthcare Life Science) for 1 hr, which was then blocked for 1 hr in 5% Nestle/Carnation brand powder nonfat bovine milk/PBS. After washing 3 times in PBS for 10 min, each primary antibody in 3% BSA was used to probe the blots for 16 h at 4°C while rocking. Secondary antibodies (donkey anti-mouse, goat anti-rabbit) were used at 1:5,000 for 2 h while rocking at room

temperature, and the HyGLO QuickSpray kit (Denville Scientific) and the Konica Minolta SRX-101A film developer were used to detect immunostaining signals.

RT-PCR

Total RNA was isolated from the brain cortex tissues of 3 BBR-treated N171-82Q transgenic mice and 3 control mice using the RNeasy Lipid Tissue Mini Kit (Qiagen). Reverse transcription reactions were performed with 1.5 μg of total RNA using the Superscript III First-Strand Synthesis System (Invitrogen, 18080-051). cDNA (100 ng) was combined with 10 μl SYBR Select Master Mix (Applied Biosystems, 4472908) and 1 μl of each primer in a 20-μl reaction. The reaction was performed in the Eppendorf, Realplex Mastercycler thermocycler. The sequences of the primers are as follows:

Htt sense: 5′-ATGGCGACCCTGGAAAAGCT-3’; Htt antisense: 5-TGCTGCTGGAAGGACTTGAG-3′.

GAPDH sense: 5’-AACTTTGTCAAGCTCATTTCCTGGT-3’; GAPDH antisense: 5’-GGTTTCTTACTCCTTGGAGGCCATG-3’

Statistical analysis

Differences between two groups were evaluated by 2-tailed Student's t-test. One-way analysis of variance (ANOVA) with the Bonferroni post-hoc correction was performed to determine pairwise comparisons amongst multiple data sets. For behavioral analysis, at least 7 mice per group were examined, and the results were expressed as the mean± SEM. A P-value of <0.05 was considered significant.

3.4 Results

BBR reduces Htt aggregation in transfected HEK293 cells

First, we wanted to see whether BBR would produce any effect on the accumulation of mutant Htt in transfected cells. BBR was added to the culture medium at concentrations of 0, 5, 25, 50, and 100 mM immediately after transfection of HEK293 cells with GFP-exon1 Htt containing 120Q (Htt-120Q). After 48 h of incubation with BBR, the cells were examined via fluorescent microscopy. The results showed a dose-dependent decrease of Htt-120Q aggregates, which were presented as puncta, with notable reduction at 50 mM BBR (Fig. 1A). However, HEK293 cells transfected with the control GFP-exon1 Htt containing 20Q (Htt-20Q) did not exhibit any significant GFP signal reduction with even the highest concentration of BBR (100 mM). The effect of BBR on reducing Htt aggregation was also shown by Western blotting that revealed aggregated Htt in the stacking gel. Quantification of the ratios of aggregated Htt to actin via densitometry also verified the reduction of Htt aggregates by BBR (Fig. 1B, C). These results suggest that BBR can suppress the aggregate formation or the accumulation of mutant Htt (Fig. 1A-C).

Mutant Htt aggregates can become stabilized after they are formed in cells. We wanted to see whether BBR could reduce mutant Htt aggregation after HEK293 cells had expressed mutant Htt for different times. Thus, 50 mm BBR was added to Htt-

120Q transfected cells at 0, 12, or 24 h post transfection and then incubated with the cells for 48 hours. Immunofluorescent staining images showed time-dependent effects on Htt aggregates, with the greatest reduction of Htt aggregates when HEK293 cells were treated immediately with BBR after transfection and diminishing effect as the treatment was delayed. However, adding BBR at 12-hours post transfection still produced a pronounced inhibitory effect (Fig. 1D). The percentage of cells containing Htt aggregates relative to total cell numbers, which were revealed by the nuclear DAPI staining, was also significantly reduced by BBR (Fig. 1E). Western blotting analysis of aggregated proteins and quantification of the relative levels of aggregated Htt confirmed that the earlier BBR treatment yielded the greater reduction in Htt aggregation (Fig. 1F,G).

BBR activates the autophagy pathway

The effect of BBR to reduce Htt aggregates led us to investigate whether BBR can increase the ability of cells to clear misfolded proteins. Since BBR has been found to up-regulate autophagy in cancer cells [26, 27], the autophagy pathway was selected as the primary candidate for investigation.

To determine autophagic flux, we examined LC3B, which converts from form I to from II to serve as the recruiter of autophagosome substrate P62 during the activation of autophagy. Non-transfected or Htt-transfected HEK293 cells were treated with 0, 5, 25, 50, or 100 mM BBR for 48 h. Western blotting analysis of LC3B-I and LC3B-II (Fig 2A) and densitometry were done to compare LC3-I/LC3-II ratio (Fig. 2B). A lower ratio

indicates more conversion of LC3-I to LC3-II or activation of autophagy. This ratio was decreased as the concentration of BBR was increased, suggesting that BBR could increase autophagic function (Fig 2B). Increased autophagic activity was also observed in both non-transfected cells and cells transfected with the control Htt-20Q (Fig.2B). To establish a causative relationship between autophagic increases and Htt aggregate reduction, we inhibited autophagy in Htt-120Q-transfected HEK293 cells using bafilomycin A (BFA) and treated these cells with 50 mM BBR. Western blotting showed that BBR reduced Htt aggregation and also antagonized the effect of BFA on increasing Htt aggregation (Fig. 2C). P62 was used as an autophagic indicator, as it is an expendable substrate that decreases with autophagic up-regulation. We also saw a decrease in P62 after BBR treatment and its increase after BFA treatment, verifying BFA's activity to inhibit autophagy. The opposite effects of BBR and BFA on Htt aggregation and P62 were further confirmed by densitometry quantifying of the Western blot results (Fig 2D).

BBR ameliorates the neurological phenotypes in transgenic N171-82Q mice

Next, we wanted to see if the inhibitory effect of BBR on the accumulation of mutant Htt in cultured cells could be mirrored in an HD animal model and result in phenotypical improvements. The N171-82Q mice, which express N-terminal mutant Htt with 82Q and show progressive neurological phenotypes [28], were divided into BBR and vehicle-treated groups (n=7 mice per group). These mice were orally gavaged daily with ddH_2O or 40 mg/kg BBR that was dissolved in ddH_2O. WT siblings were also set

up into groups (n=7 per group) treated with BBR or ddH_2O. BBR treatment was started at 4 weeks of age and continued until sacrifice or death of mice, and mouse behaviors were tested at 6 weeks of age.

The rotarod performance was one of the key tests for assessing motor coordination in a variety of HD mouse models. Rotarod tests, which were conducted weekly from week 6 to 21, indicated that HD mice treated with BBR showed significant improvements on their rotarod performance as compared to HD siblings without BBR treatment ($P<0.001$) (Fig. 3A). When comparing the rotarod test scores of the BBR-treated and untreated HD mice, one can see an almost linear relative decline of rotarod performance for the untreated group as compared to the BBR treated group (Fig. 3B).

Muscular weakness is another key feature of HD mice, which can be assessed by the grip strength test. Grip strength tests conducted weekly from week 6 to 21 also indicated that the average muscle strength of HD mice was markedly increased after BBR treatment as compared with untreated HD siblings ($P<0.05$) (Fig. 3C, D). The difference between the untreated and treated HD mice in grip strength became obvious after the poor rotarod performance became apparent (Figs. 3A, C), suggesting that muscular weakness followed neuronal dysfunction in HD mice.

We also used the balance beam test to examine motor coordination function and balance. HD mice were given a balance beam assessment in which the mice walked across a 0.6cm thick, 0.8m long beam (Fig. 3E). The shorter time for the mouse to successfully get across the beam is correlated with the greater motor coordination and balance. The results showed that while BBR-treated HD mice did not exhibit the same

dexterity as WT siblings, they were faster than their untreated HD siblings (mean values: 8.69 s for BBR-treated, and 12.65s for untreated HD mice) (Fig. 3E).

Life span is another critical criterion for evaluating treatment efficacy, and we also saw that BBR improved the life span of N171-82Q mice. In general, untreated HD mice began to die before their BBR-treated siblings (life span range: untreated HD mice=112-156 days; BBR-treated HD mice=146-173 days) (Fig. 3G,H). The average life span was 140.86 days for the untreated HD mice and 154.86 days for the BBR-treated HD mice, which is roughly a 10% difference. Despite their prolonged life span, BBR-treated HD mice did not show any significant alteration in body weight compared with the untreated HD group (Fig. 3F). This could possibly be because N171-82Q mice have metabolic defects mediated by mutant Htt in both CNS and peripheral tissues [29, 30] and that BBR is more likely to alleviate motor deficits caused by mutant Htt in the brain. Also, BBR may induce weight loss via cholesterol reduction [9] so this effect may mask any rescuing effects on body weight.

BBR treatment reduced Htt aggregation and increased autophagy in HD mice

Based on the results from cultured cells, we wanted to examine whether BBR also reduced mutant Htt accumulation and aggregation in the HD mouse brain. Immunofluorescent staining of cerebellar brain slices revealed a drastic reduction of mutant Htt aggregates in the BBR-treated HD mouse brains as compared to the untreated HD mouse brains (Fig. 4A). Western blotting of brain cortex samples from 3

separate mice per group was done and quantified by densitometry, revealing nearly 50% reduction in mutant Htt aggregate formations in the BBR-treated HD mice as compared to their untreated HD siblings (Fig 4B, C). To investigate whether this reduction occurred at the transcriptional level, RT-qPCR was performed on the HD mouse brain samples that were treated with or without BBR. The results show that BBR did not alter the transcriptional levels of transgenic mutant Htt (Fig. 4D). Thus, BBR is likely to inhibit the accumulation of mutant Htt at the protein level. To examine whether BBR stimulates autophagy to clear mutant Htt, we also probed the western blots with an antibody to P62 and found a moderate decrease in P62 in the BBR-treated group (Fig. 4B,C). Because the BBR-treated HD mice were examined for their behaviors and then sacrificed when they were near death, the most pronounced effects of BBR on autophagy in the HD mouse brain might not have been detected. Thus, the WT control group, which was gavaged with BBR daily at the same time as their HD siblings, was given their last gavage and sacrificed 4 hours later. The brain cortex samples of BBR-treated and untreated mice were then collected for western blot analysis with anti-P62. Densitometry analysis revealed a much more marked decrease in P62 in the BBR-treated mouse brain (Fig 4E,F), which also supports the idea that BBR treatment can increase autophagic activity in the mouse brain.

Discussion

HD shares many pathological features with other neurodegenerative diseases that are caused by protein misfolding. These pathological features include age-dependent

accumulation of misfolded proteins and selective degeneration of neuronal cells. Since HD is a monogenetic disorder caused by polyQ expansion in Htt, HD provides an ideal model for us to find therapeutics for neurodegenerative diseases. However, there is still lack of effective treatments for HD, despite large efforts being made to identify its therapeutics [31, 32].

In the current study, we found that BBR can effectively improve motor function of N171-82Q mice. This improvement is evident by the significant increase in performance on the rotarod test by N171-82Q mice after BBR treatment. The increased grip strength of HD mice by BBR treatment also indicates the alleviation of muscle weakness caused by mutant Htt. Also, balance beam test results support the improved motor function and movement coordination of HD mice after BBR treatment. All these effects could contribute to the prolonged life span of BBR-treated HD mice. The dosage of BBR used in this study was 40mg/kg. We also tried two higher doses (150 and 250mg/kg) but both did not yield greater effects than the dosage of 40mg/kg. It remains to be seen if other doses of BBR would produce a greater protective effect on HD mouse symptoms.

BBR has been found to have protective effects on a wide range of pathological events [10-17]. Such diverse effects could account for the protective effect of BBR on HD mice. How BBR can mediate broad beneficial effects against different pathological events remains unknown. It is possible that BBR may act on some common pathological pathways shared by many disease conditions. With potential effects against neurodegenerative diseases, BBR has also exhibited several known effects such as

inhibition of monoamine oxidase B (MAO-B), acetylcholinesterase (AChE), up-regulation of nuclear factor erythroid 2 (Nrf2), (glycagon-like protein 1) GLP-1, as well as phosphorylation of Protein kinase B (AKT) and CREB [40-44]. inhibition of MAO-B and up-regulation of Nrf2 are both able to defend against damage from reactive oxygen species (ROS) while inhibition of AChE as well as activation of phosphoinositide 3-kinase(PI3K)-AKT pathways defend against apoptosis. GLP-1 plays a role in preservation of dopaminergic neurons and phosphorylated cyclic adenosine monophosphate response element-binding protein (CREB) may be related to the ability of neurons to survive damage. Our findings suggest that BBR can activate autophagic function to reduce the accumulation and aggregation of mutant Htt. This therapeutic effect is different from the previous findings for other therapeutics, which rely on improving transcriptional function [33], metabolic function [34, 35], neurotrophic factor signaling pathways [36], mutant Htt conformation, and its interactions with other proteins [24, 37]. The increased autophagic function by BBR could also be beneficial for other pathological conditions in which the accumulation of misfolded proteins leads to neurodegeneration. Consistent with this idea, BBR has been found to reduce the neuropathology in AD transgenic mice [16, 20- 23]. It is also known that the accumulation of toxic forms of peptides and misfolded proteins can lead to a variety of pathological events such as inflammation and altered cellular signaling [38, 39], which could explain the broad protective effects of BBR on a variety of pathological events.

Although our findings suggest that BBR may increase autophagic function to reduce mutant Htt accumulation and the associated neurological phenotypes in HD

mice, it remains to be investigated how BBR can up-regulate the autophagy function. Such investigation would require more in-depth studies with sophisticated tools that can explore whether BBR directly associates with autophagic proteins to modulate their function. Despite this, there is a need to report the beneficial effects of BBR on HD, because BBR is an over-the-counter drug that has been safely used to treat a number of diseases. Discovery of its protective effects in N171-82Q mice may promote further exploration of the use of BBR in other HD mouse models as well as patients and perhaps other neurodegenerative diseases that are also caused by misfolded proteins.

Fig. 1

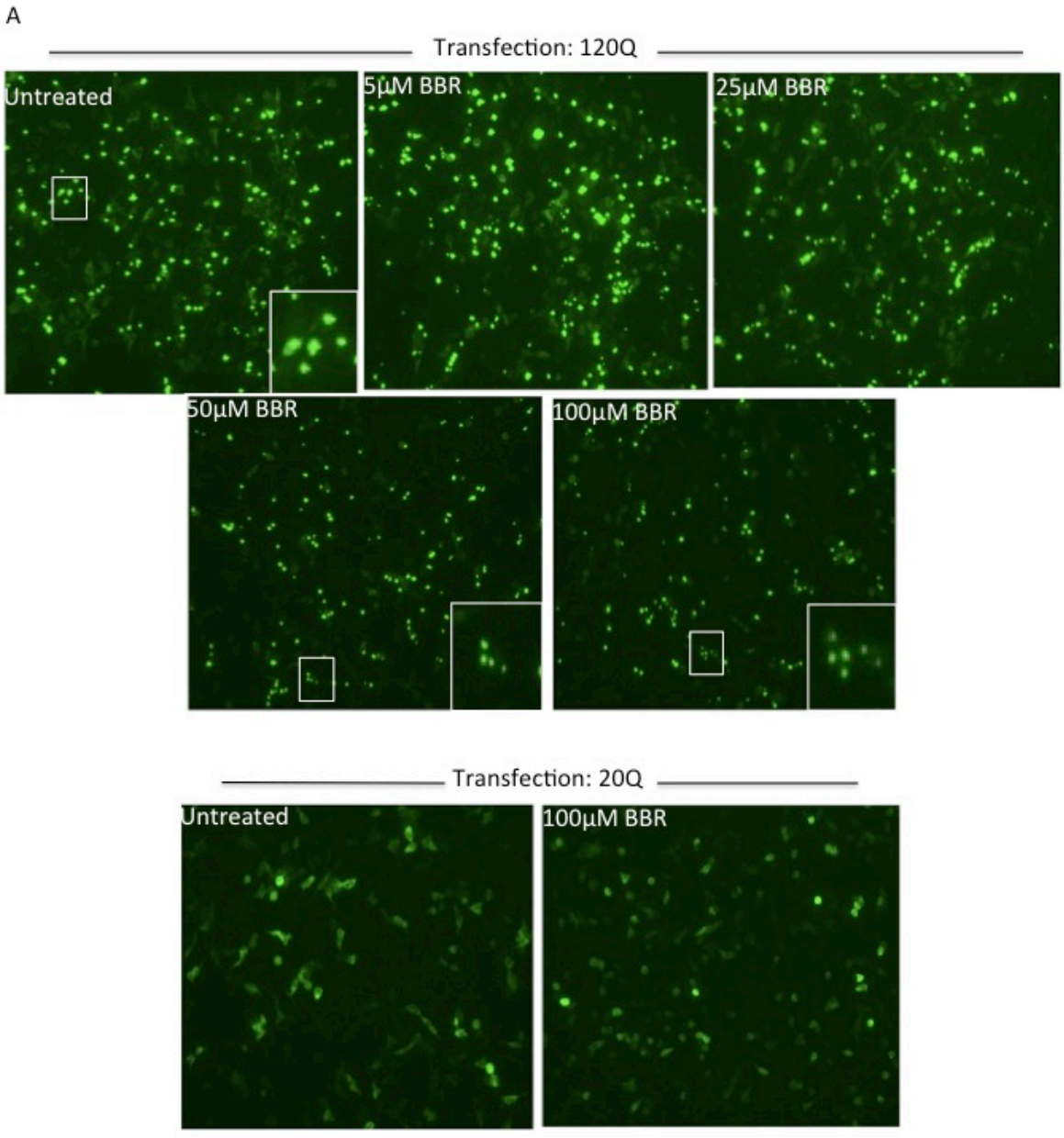

A
Transfection: 120Q
Untreated
5μM BBR
25μM BBR
50μM BBR
100μM BBR
Transfection: 20Q
Untreated
100μM BBR

Fig. 1 (Continued)

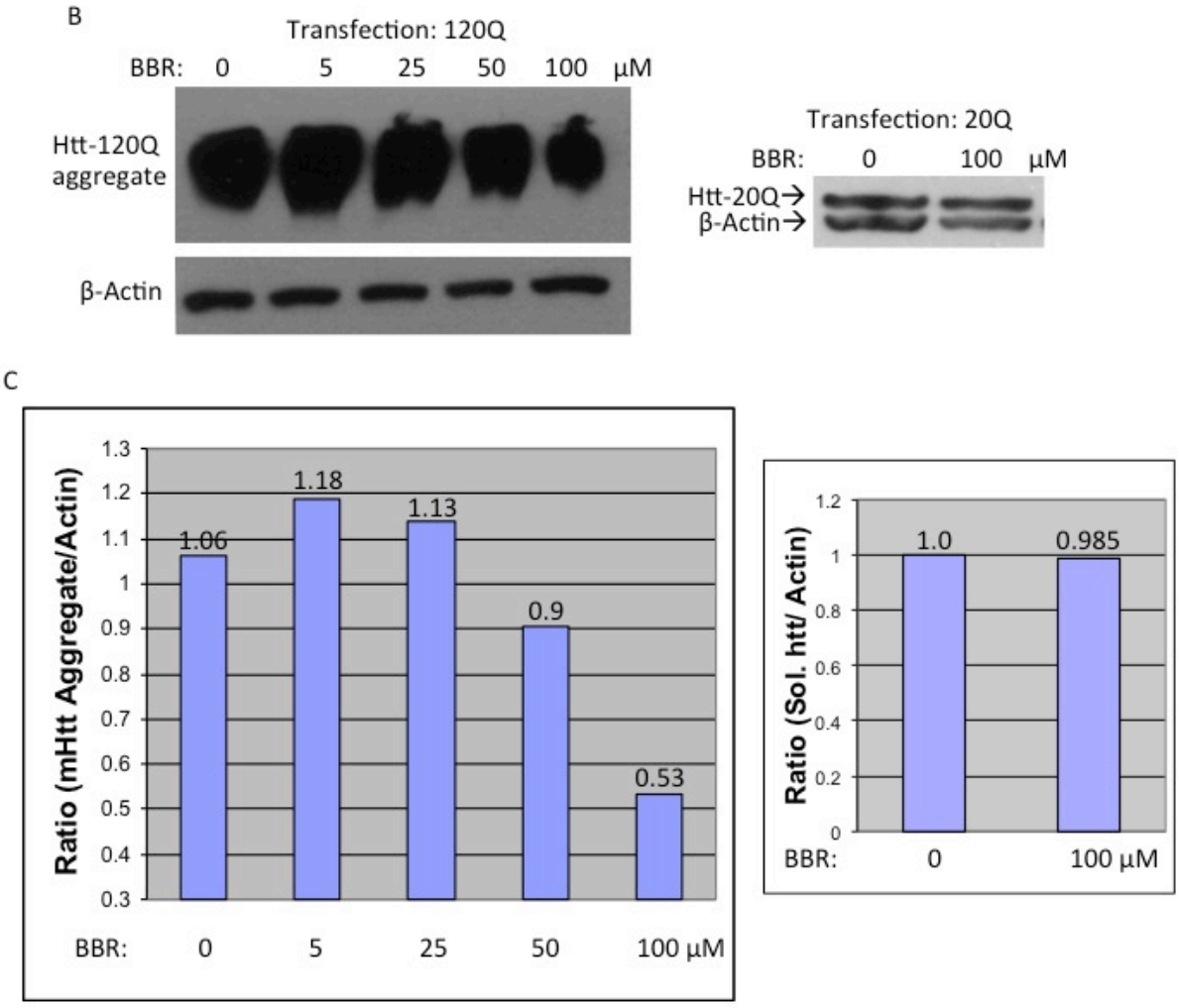
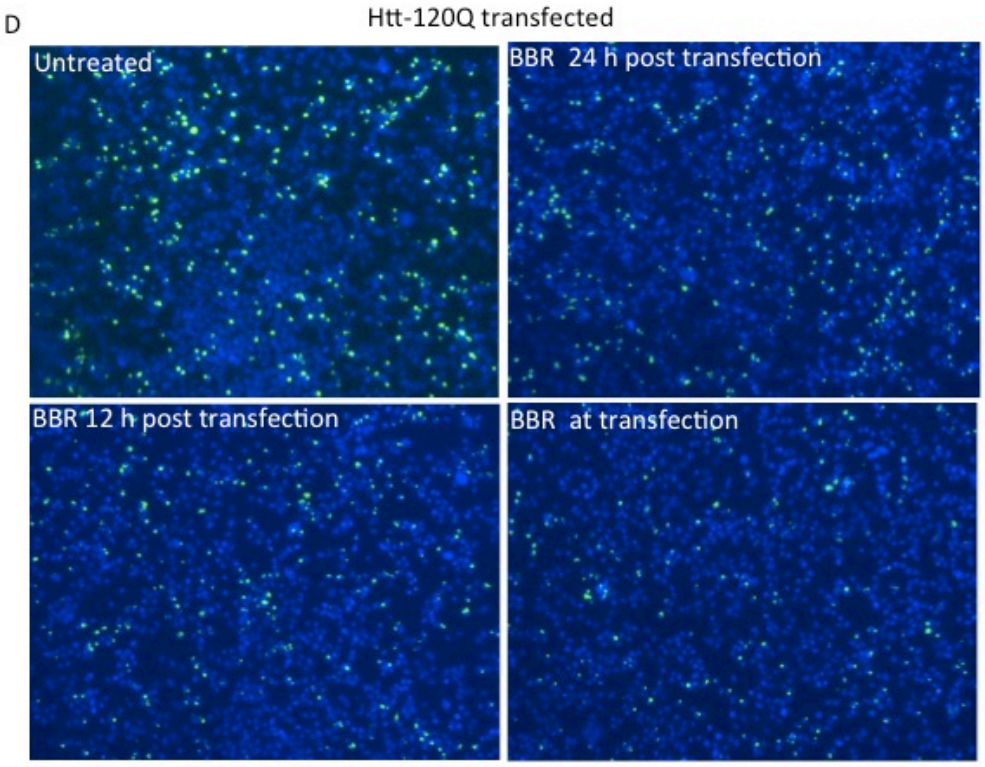


Fig. 1


D
Htt-120Q transfected
Untreated
BBR 24 h post transfection
BBR 12 h post transfection
BBR at transfection

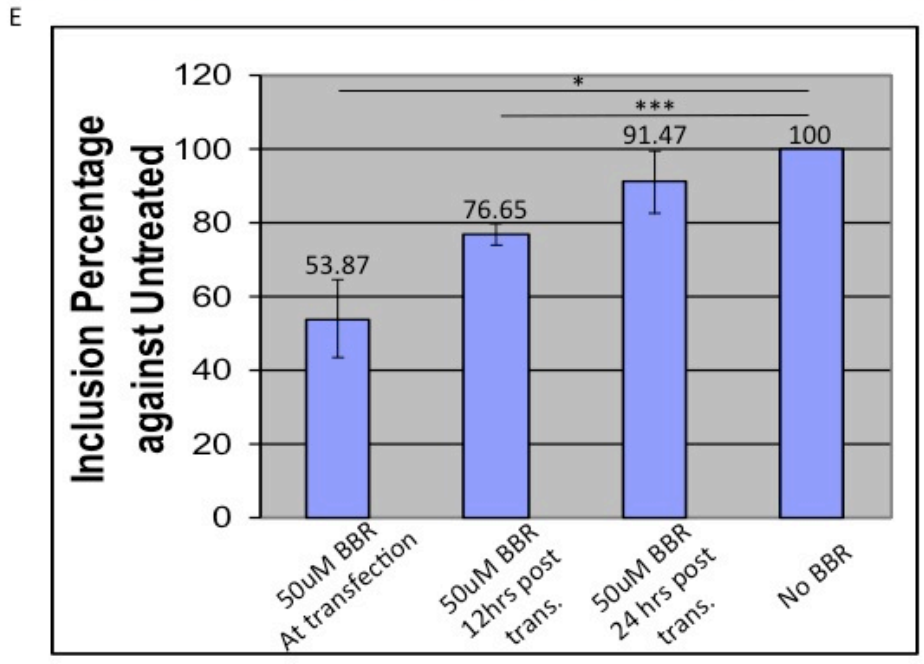
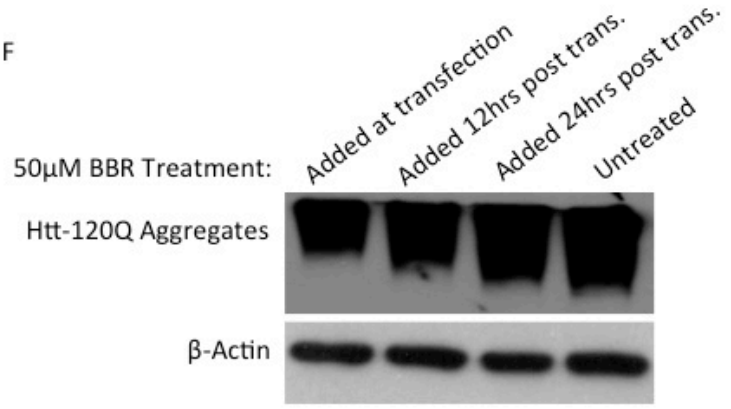

E
Inclusion Percentage against Untreated
120
100
80
60
40
20
0
*
***
53.87
76.65
91.47
100
50uM BBR At transfection
50uM BBR 12hrs post trans.
50uM BBR 24 hrs post trans.
No BBR

Fig. 1 (Continued)

F



G

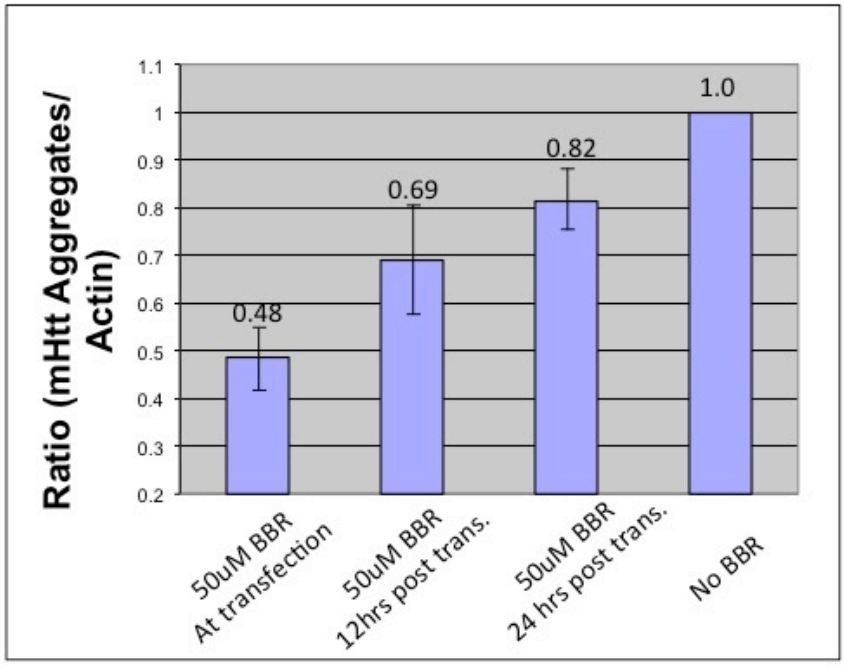


Figure. 1 BBR reduced Htt aggregation *in vitro* in a dose and time-dependent manner. (A) Immunocytostaining images (10 X) of Htt-120Q- or Htt-20Q-transfected HEK293 cells that were treated with different concentrations of BBR (0, 5, 25, 50, 100 mM). **(B)** Western blot analysis of Htt-transfected cells treated with or without BBR at different concentrations. Aggregated Htt in the stacking gel was detected by mEM48 antibody. **(C)** Densitometry analysis of the ratios of aggregated Htt to b-actin on western blots in (B). **(D)** Fluorescent microscopic images of Htt-120Q-transfected HEK293 cells that were treated with 50 mM BBR at 0 h, 12 h or 24 h post-transfection. **(E)** Cell-counting analysis of images obtained in (D) showing the percentage of aggregates relative to the total cells revealed by DAPI nuclear staining. **(F)** Western blotting of Htt-120Q transfected HEK293 cells treated with 50 mm BBR for different times showing aggregated Htt in the stacking gel. **(G)** Densitometry analysis of the ratios of mutant Htt to b-actin on western blots in (F).

Fig. 2

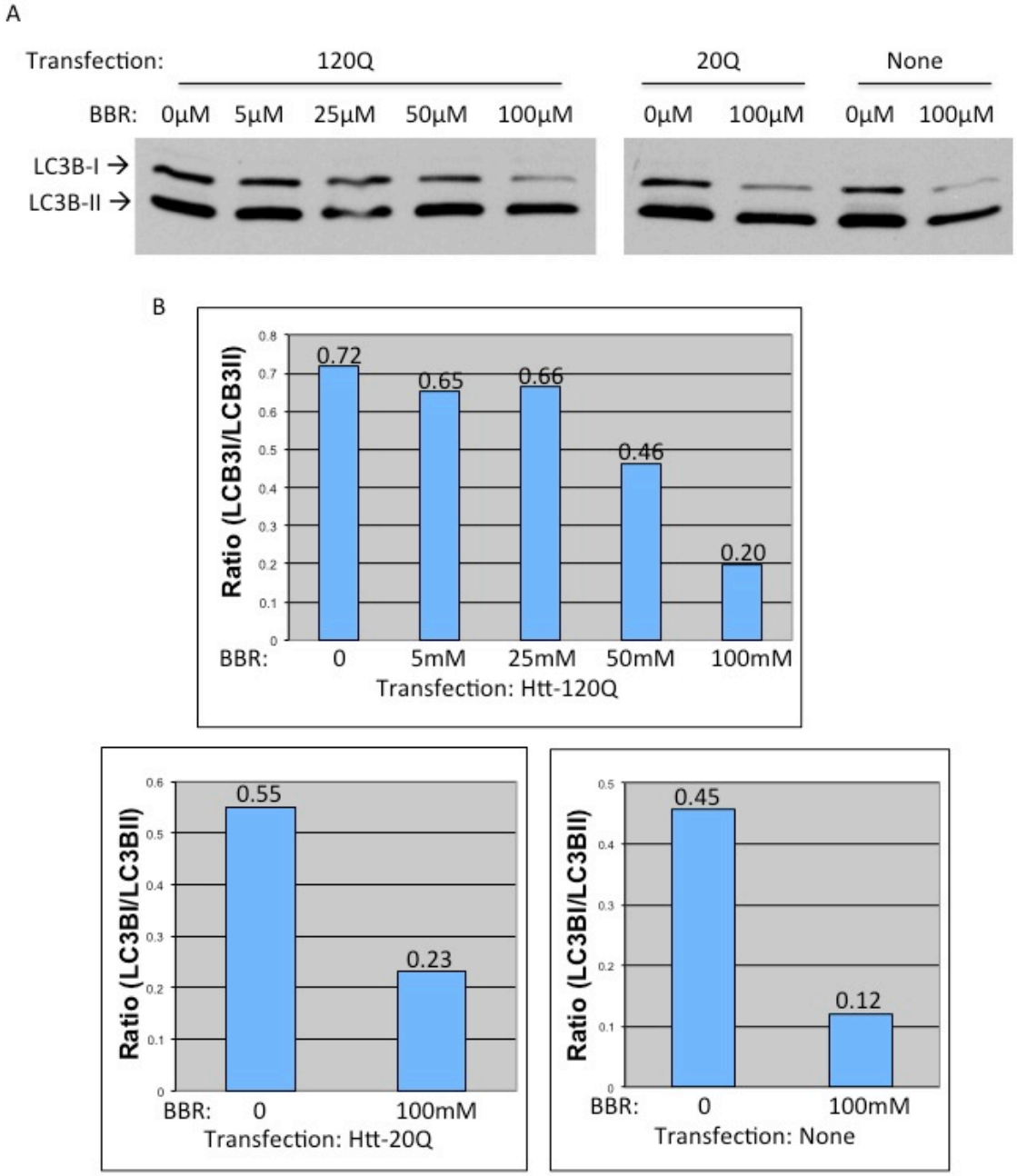

A
Transfection:
120Q
20Q
None
BBR:
0μM
5μM
25μM
50μM
100μM
0μM
100μM
0μM
100μM
LC3B-I
LC3B-II
B
Ratio (LCB3I/LCB3II)
0.72
0.65
0.66
0.46
0.20
BBR:
0
5mM
25mM
50mM
100mM
Transfection: Htt-120Q
Ratio (LC3BI/LC3BII)
0.55
0.23
BBR:
0
100mM
Transfection: Htt-20Q
Ratio (LC3BI/LC3BII)
0.45
0.12
BBR:
0
100mM
Transfection: None

Fig. 2
(Continued)

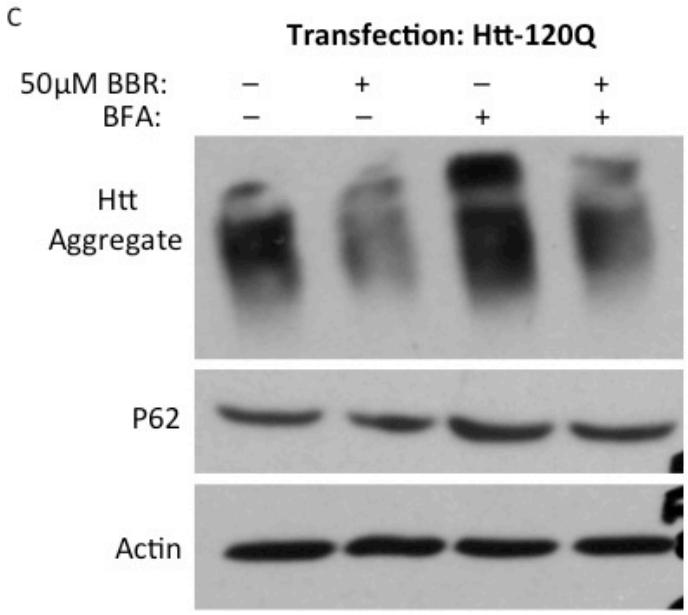


Fig. 2 (Continued)

D

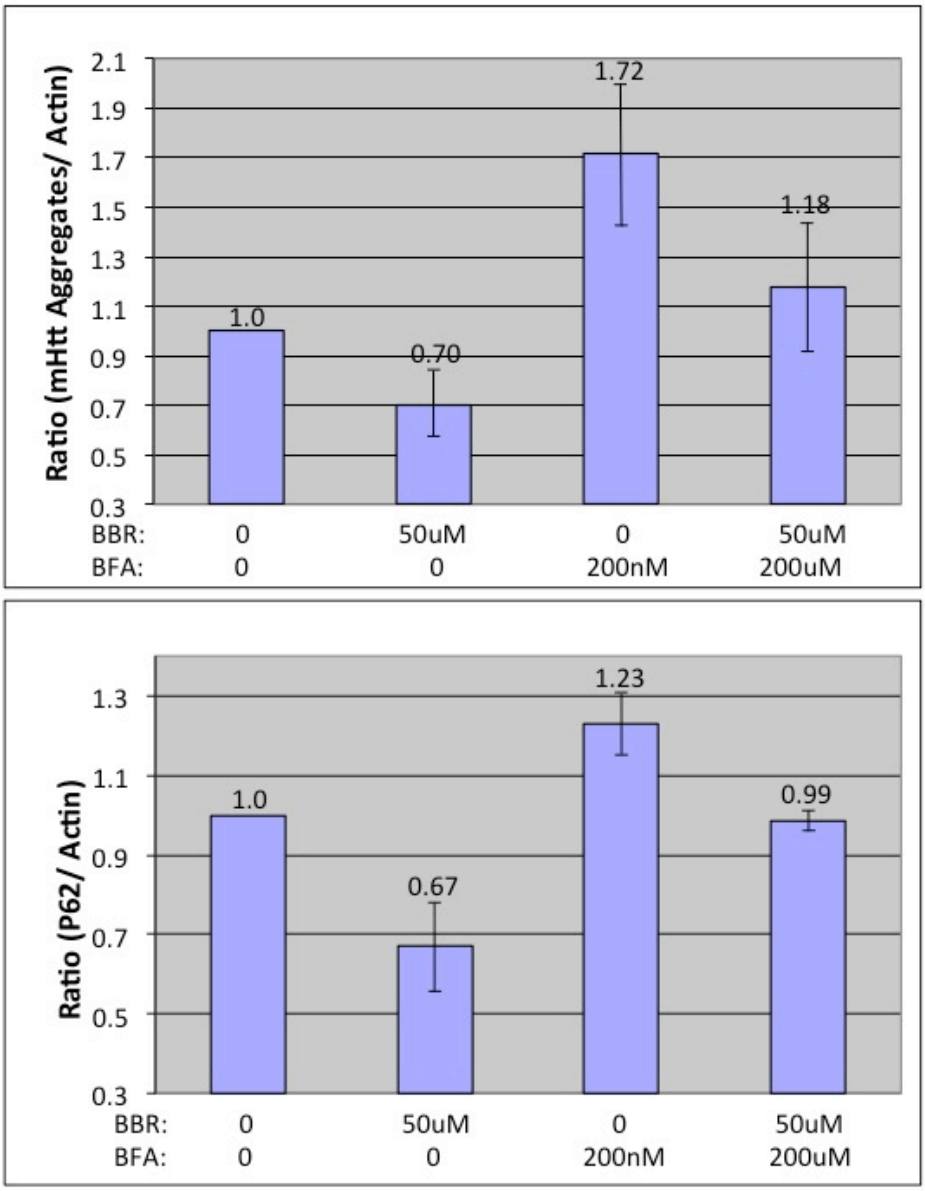
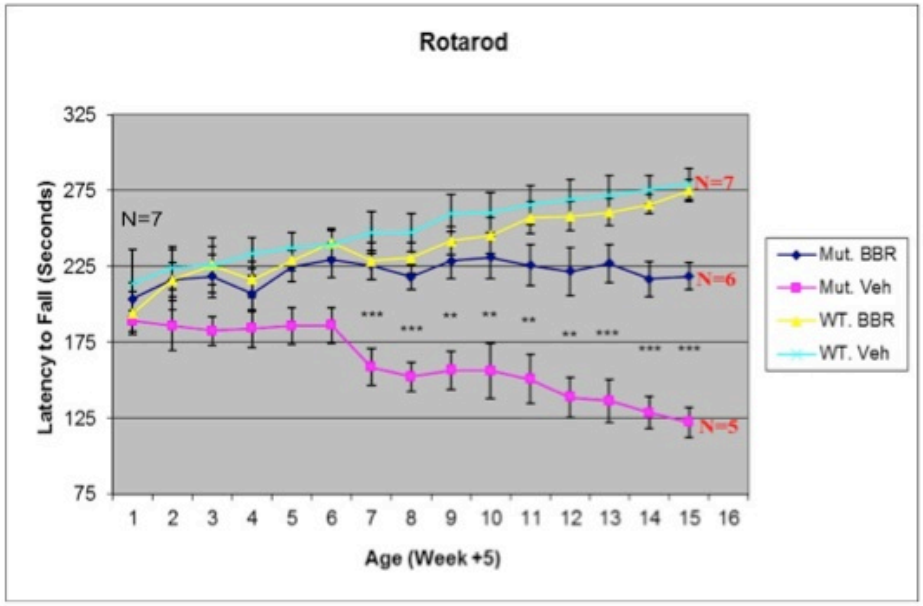


Figure 2. BBR increases autophagic activity in cultured cells. (A) Western blotting of LC3B in HEK293 cells transfected with or without Htt and treated with BBR (0, 5, 25, 50, or 100 mM). **(B)** Densitometry analysis of the ratios of LC3-I to LC3-II in above Western blots. **(C)** Western blotting of Htt-transfected HEK293 cells treated with or without 50 mM BBR or bafilomycin (BFA), an autophagy inhibitor. Antibody to P62, which decreases when autophagy activates, was used to confirm the altered activity of autophagy. **(D)** Densitometry analysis of the ratios of aggregated Htt or P62 to actin in Western blots in (C).

Fig. 3

A


Rotarod
Latency to Fall (Seconds)
325
275
225
175
125
75
N=7
N=7
N=6
N=5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Age (Week +5)
Mut. BBR
Mut. Veh
WT. BBR
WT. Veh

B

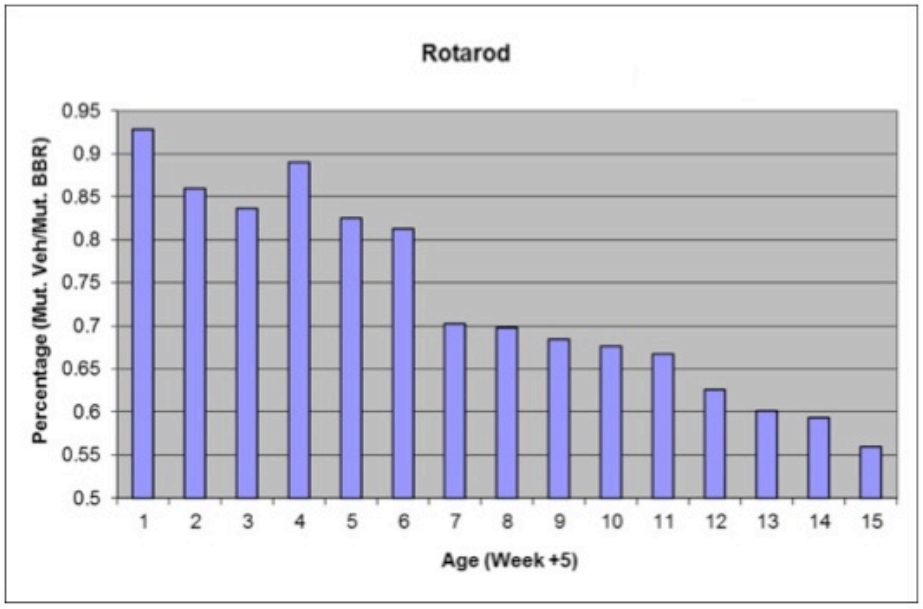
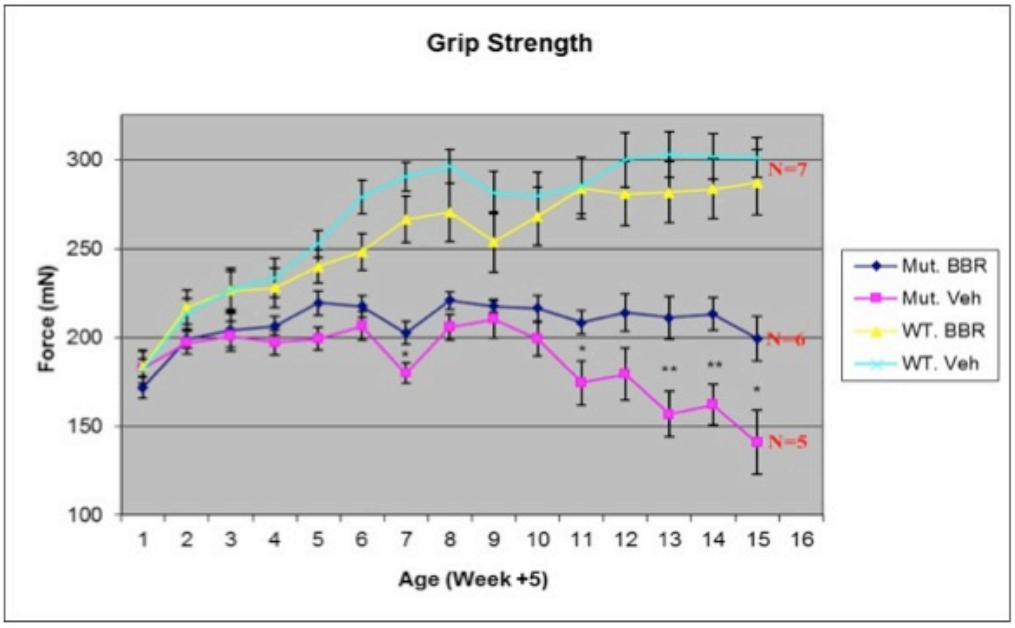

Rotarod
Percentage (Mut. Veh/Mut. BBR)
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
0.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Age (Week +5)

Fig. 3 (Continued)

C



D

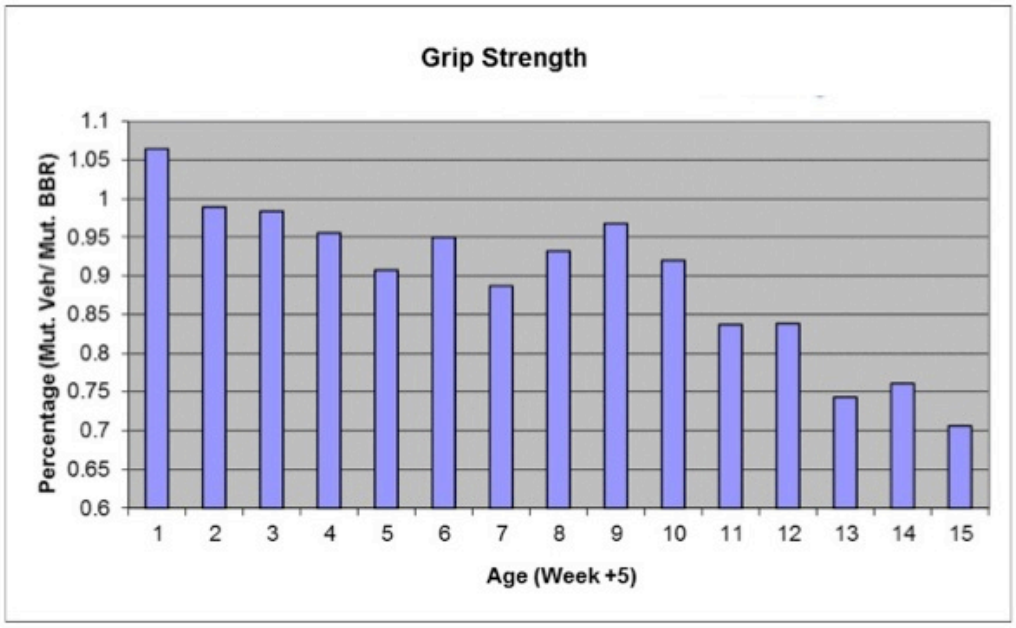


Fig. 3 (Continued)

E

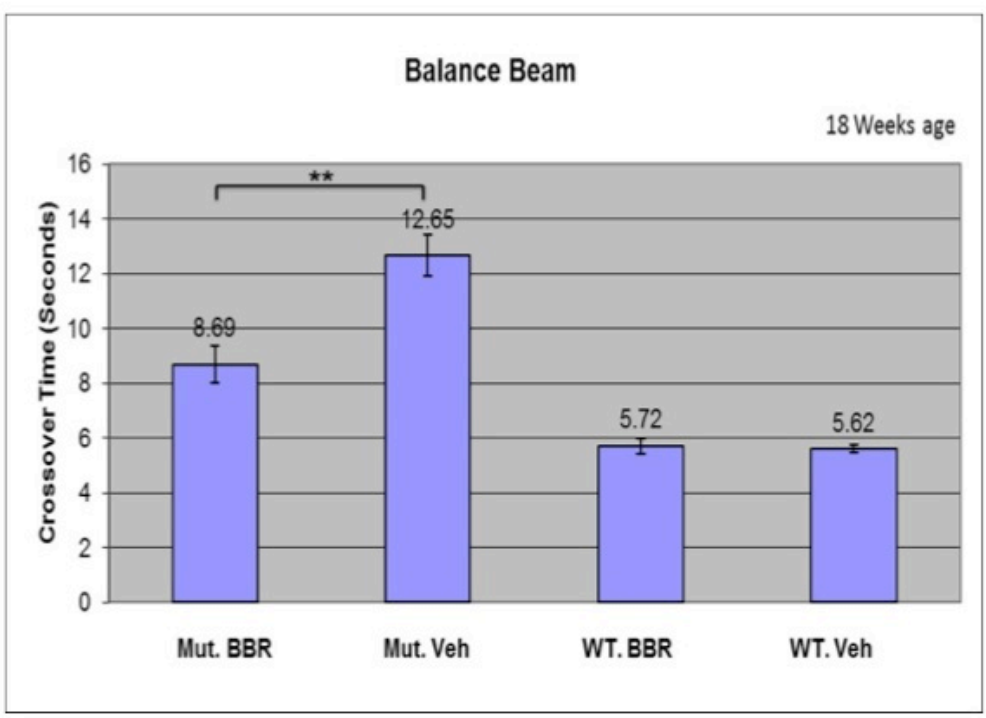


Fig. 3 (Continued)

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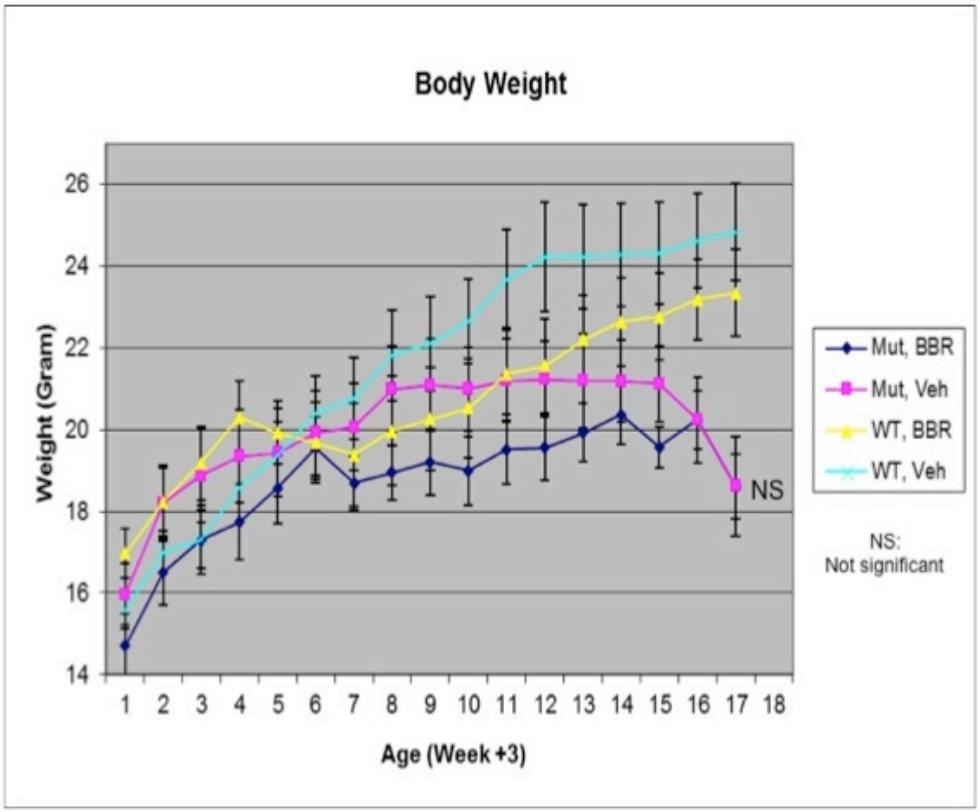
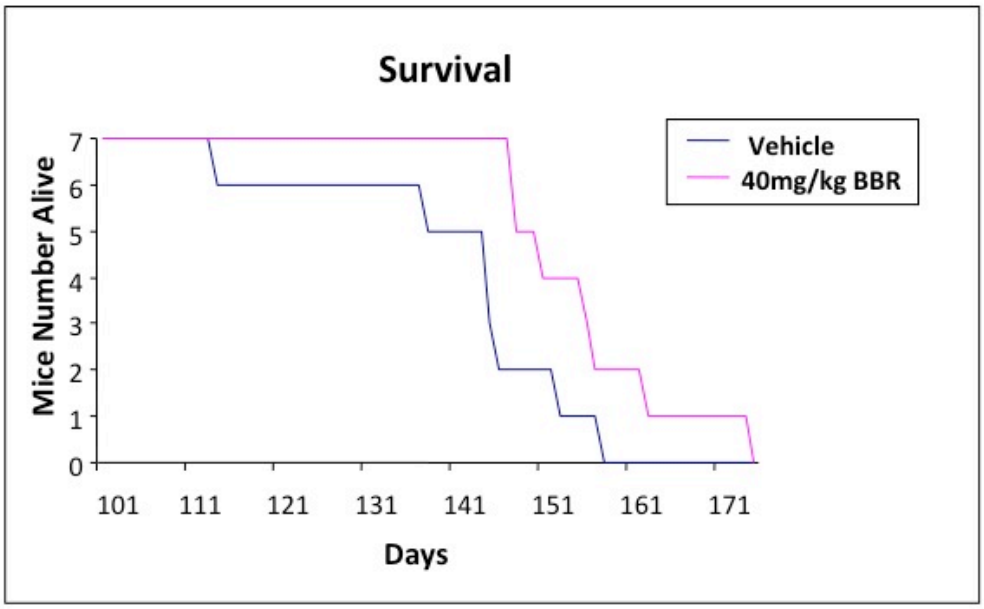


Fig. 3 (Continued)

G



H

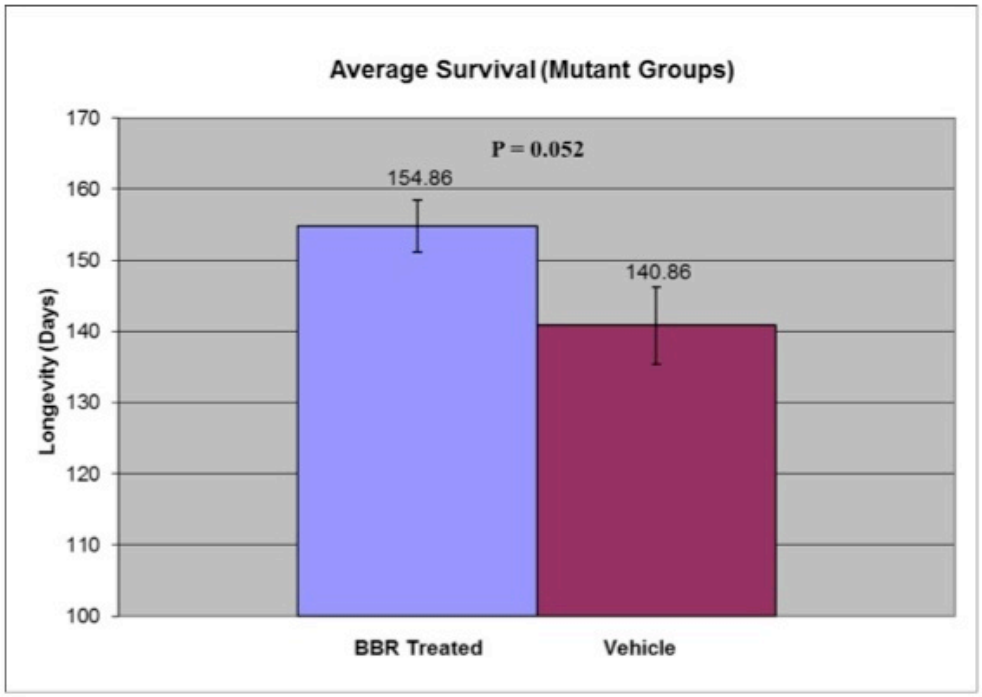
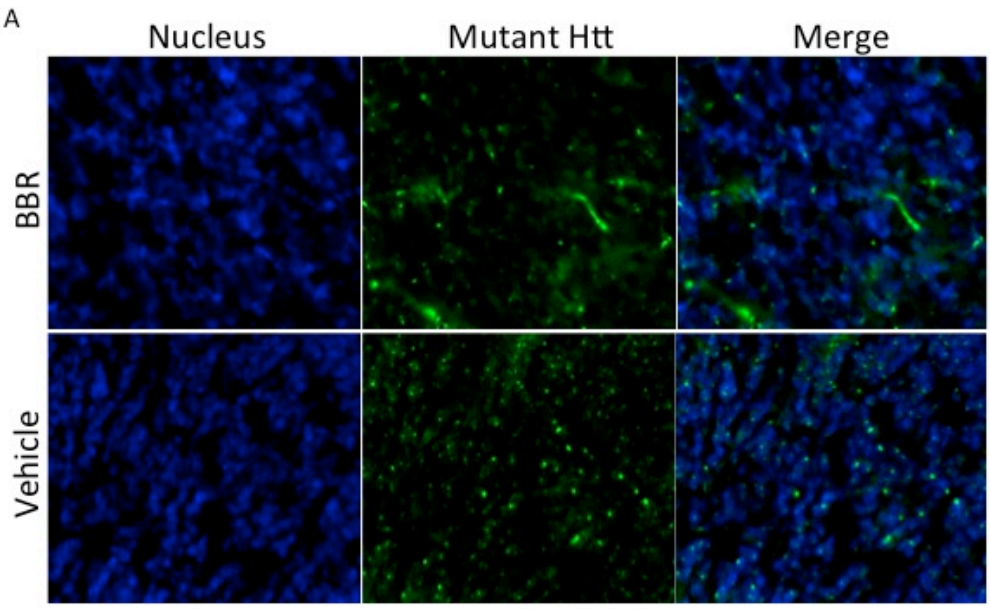


Figure 3. Oral administration of BBR ameliorates neurological symptoms in transgenic N171-82Q mice. **(A, B)** Rotarod test of motor coordination of N171-82Q transgenic mice and WT siblings, orally gavaged with 40 mg/kg BBR or vehicle daily. **(C, D)** Grip strength test on transgenic N171-82Q mice and WT siblings treated with BBR or vehicle. **(E)** Balance beam test on 18-week-old N171-82Q and WT mice that had been treated with BBR or vehicle. **(F)** Body weight of N171-82Q mice and WT siblings treated with or without BBR. **(G)** Survival curve for N171-82Q mice and WT siblings treated with BBR or vehicle. (**H**) The BBR-treated N171-82Q group survived 15 days longer than their untreated HD siblings. *p<0.05. **p<0.01. ***p<0.001, n=7 mice per group.

Fig. 4


A
Nucleus
Mutant Htt
Merge
BBR
Vehicle

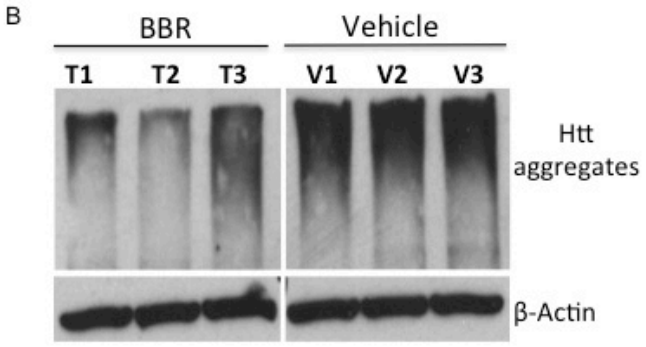
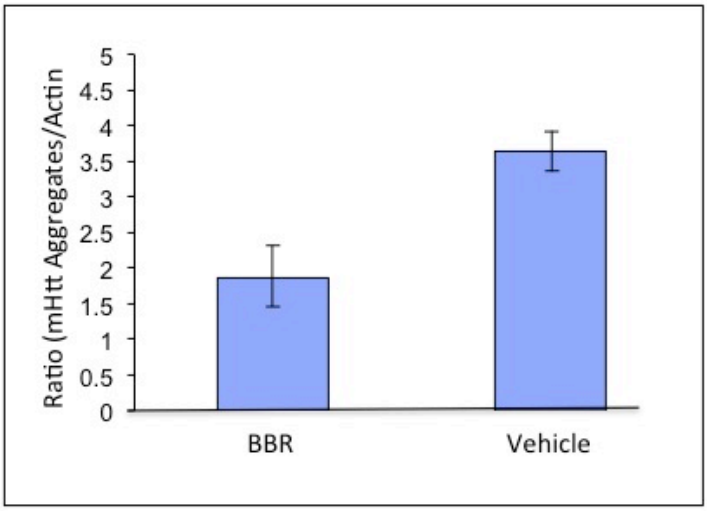

B
BBR
Vehicle
T1
T2
T3
V1
V2
V3
Htt aggregates
β-Actin

Fig. 4 (Continued)

C



D

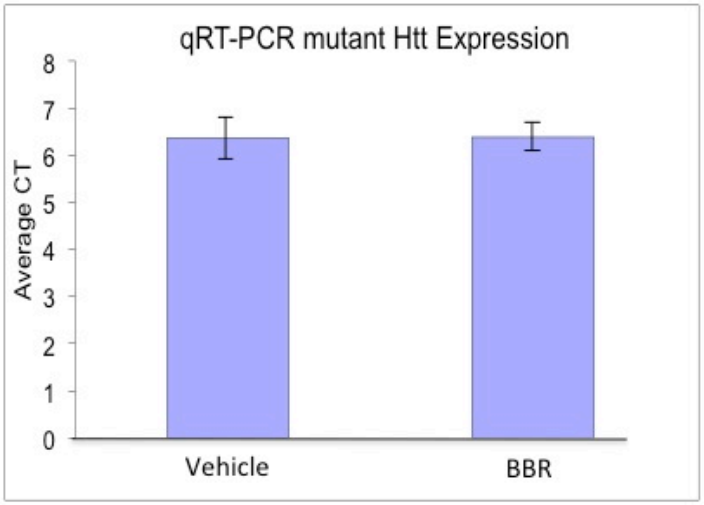


Fig. 4 (Continued)

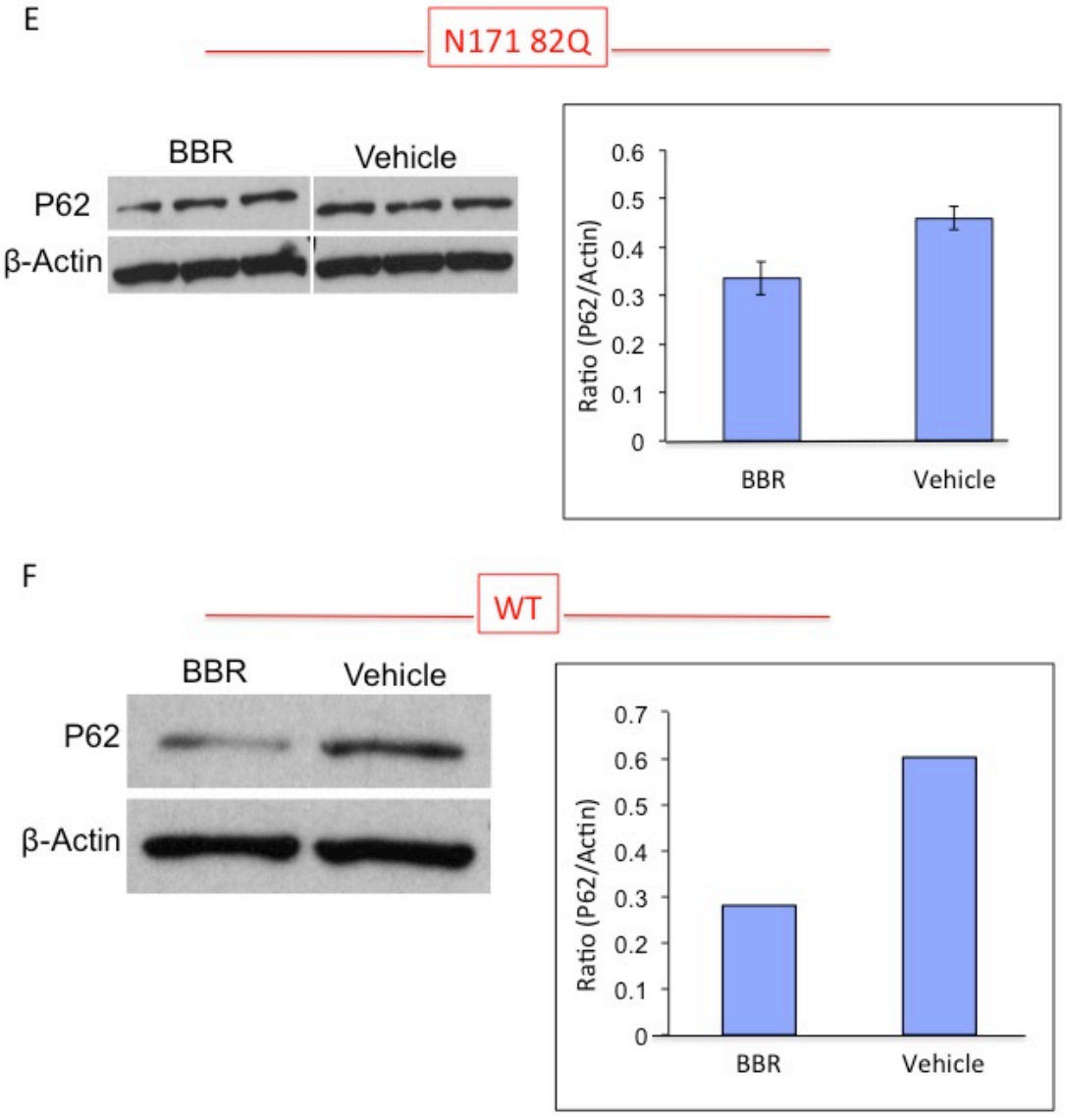


Figure 4. Oral administration of BBR reduced mutant Htt aggregation and increased autophagy in transgenic N171-82Q mice. (A) Fluorescent immunohistostaining of mouse brain slices from transgenic N171-82Q HD mice and WT siblings, orally gavaged with 40 mg/kg BBR or vehicle daily. **(B)** Western blotting of the cortex samples of 3 mice per group (HD+BBR vs. HD+vehicle) showing mutant Htt aggregates in the stacking gel and P62. Anti-actin was used to show the internal control protein on western blot. **(C)** Densitometry analysis of above Western blots. The data are mean±SE (n=3). **(D)** Quantitative RT-PCR analysis of transgenic mutant Htt mRNA in the cortex of the BBR-treated or untreated N171-82Q mice. n=3 mice per group. Data are presented as mean CT±SEM. **(E)** Western blotting of WT mouse cortex samples (3-mouse pool per group) after the mice had been orally gavaged with 40 mg/kg BBR or vehicle daily for 24 weeks and sacrificed 4 h after the last BBR administration. **(F)** Densitometry analysis of Western blots in (E) showing the ratio of P62 to actin.

Acknowledgements

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Chapter IV:

Final Discussion

4.1 Summary

In this study, we showed that BBR, a botanically-derived small molecule, has the ability to reduce mHtt aggregation and accumulation both *in vitro* and *in vivo*. In HEK-293 transfected HD cellular models, BBR acted in a dose-dependent manner to reduce mHtt aggregates (up to 100uM). BBR also acted in a time-dependent manner; a fixed concentration of BBR produced greater aggregate-reduction results when administered closer to transfection time, though pronounced results could still be seen when administered 12 hours past. Mechanistic studies showed that autophagic flux corresponded inversely to mHtt aggregation levels when treated with varying amounts of BBR. Also, inhibition of autophagy antagonized the ability of BBR to reduce mHtt aggregation, implicating autophagic increase as a facilitator in this pathway.

In the N171 82Q transgenic HD mouse model, we found that daily oral administration of 40mg/kg BBR starting from 1 month of age significantly reduced the rate of decline in motor coordination, balance, and strength as compared to untreated littermates. Furthermore, lifespan was increased by about 10%, though statistically, the difference was considered highly suggestive, but not strictly significant (P=0.052). Immunohistological studies revealed that BBR-treated mice showed an average reduction in mHtt aggregation of 48.77% over untreated siblings. Autophagic flux was also increased in BBR-treated mice, though it was more apparent in the WT controls due to more precisely controlled times of sacrifice (as mutant mice were sacrificed according to health conditions in line with previously established end points). RT-PCR

revealed that between the treated and untreated N171 82Q mouse groups, there was no significant difference in mHtt expression levels (99.89% similarity, SE=7.64%), ruling out uneven expression levels leading to the obtained results.

These results suggest a model in which BBR up-regulates autophagy, which may enhance the rate of degradation of misfolded, fragmented, and aggregated mHtt protein particles, thus decreasing the incorporation of free particles into the aggregates while eliminating some aggregates outright (Fig. 4-1). Indeed, it seems that BBR has untapped potential when it comes to protection again neurological disease, particularly against HD. Its effectiveness at reducing the hallmark mHtt aggregate accumulations of HD models and its excellent clinical safety (no known LD50 when taken orally) merit further research into the possibilities of clinical use of BBR in HD therapy. Despite all the questions that still remain pertaining to BBR's optimum dosage and frequency as well as its in-depth mechanism, there is compelling need to quickly report the potential benefits to translational medicine that were discovered in this study.

4.2 Interpretation and significance of results

BBR successfully diminished mHtt aggregate accumulation in both a dose and time-dependent manner. In transfected HEK-293 cells, the treatment concentration was taken as high as 100uM, with increasing effect as the dosage increased. The concentration was not taken further because normally, the cells would be grown for 48 hours post transfection and at higher concentrations, oftentimes, the entire plate of cells would not survive for 48 hours. The reason is not understood but

could be due to BBR's alkaline properties changing the pH of the medium. BBR is not known to be toxic in humans when ingested even in great quantities, however, when taken intravenously, the LD50 is 9.0386 mg/kg in mice. This may be due to the body's poor ability to absorb orally-taken BBR as a limiting factor to the drug's bioavailability when taken orally, which serves as a built-in safety mechanism. Despite this, how a large amount of BBR is harmful when presented to cells either *in vitro* or *in vivo* is not fully understood.

That even the highest tested concentration (100uM) BBR did not reduce htt expression levels in HD20Q-GFP-transfected cells showed two things. First, because both HD20Q-GFP and HD120Q-GFP were expressed under the same cytomegalovirus (CMV) promoter, BBR did not achieve its mHtt aggregate-lowering effects by blocking the transcription of transfected htt because it did not lower the expression of non-pathogenic htt. Secondly, it shows that the autophagic pathway that was activated was one that preferentially clears mutant proteins.

The time trial assay was of particular importance in this study because it allowed us to see how quickly the treatment must be administered in order to be effective. In the final application to patients, it would be unrealistic to assume that a treatment could be undertaken long before HD symptoms manifested. Therefore, it is paramount that BBR have some effect even if treatment were to be delayed as expected in patients. On the bio-molecular front, mHtt forms unstable aggregates early on. In this stage, soluble mHtt particles freely associate and dissociate, building up very slowly. However, when the aggregates reach a critical stage, they become stable, at which point association is

largely unchanged; by then, the ability to dissociate drops dramatically, causing aggregates to enlarge at a much faster rate. Typically, symptoms do not arise until this stage has arrived, making it difficult to catch HD before. Therefore, since mHtt aggregates accumulate in this manner, it is important to know if BBR can still facilitate their clearance even after mHtt aggregates have gotten a foothold.

The mice of the untreated N171 82Q group were sacrificed (upon reaching endpoint for each mouse) at an average of 140.86 days and the treated mutant brethren were sacrificed at an average of 154.86 days; the difference in lifespan was roughly 10%. Since the death of N171 82Q mice can result from multiple and systemic failures in CNS and peripheral function, the mild increase in lifespan does suggest that protective effect of BBR is mainly to improve motor function or neuronal function that regulate motor activities.

When comparing performance between the groups across different tests, it is apparent that the difference in performance between the treated and untreated mutant groups became significant much earlier in the motor coordination test (rotarod) than it did in the strength test (grip strength apparatus). This in concurrent with the general assumption that in neurodegenerative diseases such as HD, neurological damage precedes muscular atrophy. It is also important to note that until the end of the experiment when mice were near their endpoints, the BBR-treated mutant group exhibited lower average bodyweight than their untreated counterparts. This seems to suggest that BBR does not protect against physical atrophy, which is a symptom of HD mostly towards the advanced phases of disease. However, since the grip strength test

suggested reduction in muscular atrophy, there seems to be some discrepancy. This may be explained by the fact that since BBR is a cholesterol and blood glucose-lowering agent, it also exhibits weight loss potential. As such, BBR may have caused fat loss in the treated mice while preventing muscular atrophy, which would explain why the treated group exhibited greater grip strength despite lower bodyweight for most of the trial. Thus, in effect, BBR's fat loss effect may have masked its protective effects against physical atrophy in this study.

5.3 Future studies

Upon the foundation created with the base knowledge that BBR is able to reduce mHtt aggregate accumulation, many more questions arise and much more knowledge needs to be obtained. Some of these directions include finding the detailed biochemical pathway(s) that allow(s) BBR to up-regulate autophagic activity, finding other ways in which BBR may modify HD progression, determining the optimum treatment regiment using BBR for HD, using BBR as a chemical probe to gain better understanding to the inner workings and factors of HD.

The proposed possible mechanisms that facilitate BBR's reduction of mHtt aggregation represent only a small fraction of what may be the underlying pathways. We know TFEB is a master regulator of autophagic flux and we know, from multiple different accounts pieced together, that BBR can phosphorylate ERK, which can up-regulate TFEB [84, 141-144]. We also believe that since BBR can up-regulate SIRT-1, SIRT1 can up-regulate PGC1-α, and PGC1-α can cause an increase in TFEB activity [145-

150], that represents a secondary pathway through which BBR can affect autophagy. However, both of these hypotheses have never been tested in their entirety as functioning chains. In order to test these two possible pathways, the proteins that form the steps of the path must be analyzed for up-regulation (or increase in phosphorylated form) after BBR administration. Then, blocking the expression of any of them should hinder their supposed downstream targets and ultimately render ineffective BBR's ability to elicit autophagic increase. However, this may be difficult to detect, as a single blocked pathway may result in obstruction of only a fraction of the total effect, making precise autophagic quantification paramount to determining the weight of each pathway. This is further mired by the possibility of certain pathways being up-regulated to compensate for loss experienced in other pathways. Despite these efforts, these hypothesized pathways represent only a very small scope of possibilities, limited by the protein-to-protein interactions that have already been reported. In order to break free of that limit and to include the possible discovery of pathways that were completely unknown, microarrays or RNA sequencing must be employed to find all (or many) of the proteins that were up-regulated by uptake of BBR. Although this method would open up many leads as to the proteins that BBR effects or works through, the difficulty will inevitably arise from screening all of them to determine what is relevant to HD and/or neurodegeneration. This method, however, has a great range of additional benefits as well. By RNA sequencing, in addition to finding more possible ways that BBR can upregulate autophagy, we can also see changes in expression that represent other ways through which BBR may regulate HD that are independent of the autophagic pathway

(if there are any). If the BBR-treated HD sample group can be compared with a normal sample pool and then an untreated HD sample pool, we can determine if BBR corrected or ameliorated any imbalances seen in the untreated HD group. Among those found, the genes known to be involved in autophagic regulation could reveal other pathways through which BBR may affect autophagic flux. The favorably altered expression of non-autophagy-related genes would give us some clues and leads into the other ways that BBR could modify HD independently from the autophagy pathway. The findings may be of consequence even to the treatment of other neurodegenerative diseases as well.

Through these studies, BBR may also allow us to gain a better understanding of HD as a disease. Since it is known that BBR can abate progression of HD, understanding all the channels through which BBR exerts its effects could allow us to use BBR as a chemical probe to understand which of these corrections led to retardation of HD progression. Simply knowing all of the imbalances that HD sample pools exhibit in comparison to their WT counterparts is not enough to truly gain a better understanding of disease mechanism since it is unclear which of these imbalances create an HD-related problem and which are simply a (benign) by-product. However, if we see improvement when BBR counteracts certain imbalances, we flag them for further research to discover their part in HD in detail.

Given the dose dependency of BRR *in vitro*, it is likely that the effects of BBR on multiple cellular functions *in vivo* also depend on its concentrations. It is imperative to determine the optimal concentration of BBR *in vivo* that is specific to up-regulation of

autophagic function involved in removing misfolded proteins such as mHtt. Determination of such an optimal concentration or drug treatment regiment may also be helpful for applying BBR to other neurodegenerative diseases caused by misfolded proteins.

Durairajan's work previously showed that increasing BBR levels beyond a certain point *in vivo* produced a counter effect and becoming less productive than a limited treatment. In the early phases of this study, two smaller experiments were conducted with N171 82Q mice using higher concentrations of BBR (150 and 250mg/kg, data not shown) and both studies resulted in less aggressive tempering of HD symptoms than the data shown (treatment using 40mg/kg BBR). However, we have yet to explore the effectiveness of BBR in even lower dosages such as 20mg/kg against HD models. Without reaching a lower tipping point when BBR begins to lose effect, we still lack an optimal range of efficiency in terms of dosage, which is crucial to better-understanding BBR's ability to counteract HD progression and developing a refined therapeutic regiment. A second issue is the administration frequency; as we saw in our *in vivo* P62 autophagy Western blots, the effect of BBR (on autophagy) may wear thin near the end of the 24 hour time-frame before the next gavage (as seen in HD mice), whereas the effect was very pronounced 4 hours after gavage (as seen in WT mice). Therefore, in order to produce more consistent and uniform effects on physiology, dosage frequency of less than 24 hours may be more effective. Further studies could evaluate the efficacy of a therapeutic regiment of BBR using concentrations less than 40mg/kg per day split between 2 feedings, 12 hours apart or even 3 feedings, 8 hours apart. Such studies on

refining therapeutic regiment, as well as possible tests in animal models with more similarity to humans can take BBR closer to clinical trials and inception into medicine as an agent against HD and possibly other neurodegenerative diseases.

4.4 Conclusion

In conclusion, using HD cellular and animal models, we found that BBR can activate the autophagic pathway to remove mHtt and reduce aggregate Htt-mediated neurological phenotypes in a HD mouse model. Although BBR is likely to act on multiple cellular pathways to elicit its protective effects, its safety and ability to pass through the blood-brain barrier makes it an ideal candidate for further investigation. Its novel function in enhancing the clearance of mHtt via autophagic activation is highly promising to translational medicine. The findings of this study and others suggest that BBR possesses versatile functions, which may depend on its dosage and target cell type. Deeper understanding of BBR's mechanisms will help us verify its potential for treatment of neurodegenerative diseases.

Fig. 4-1

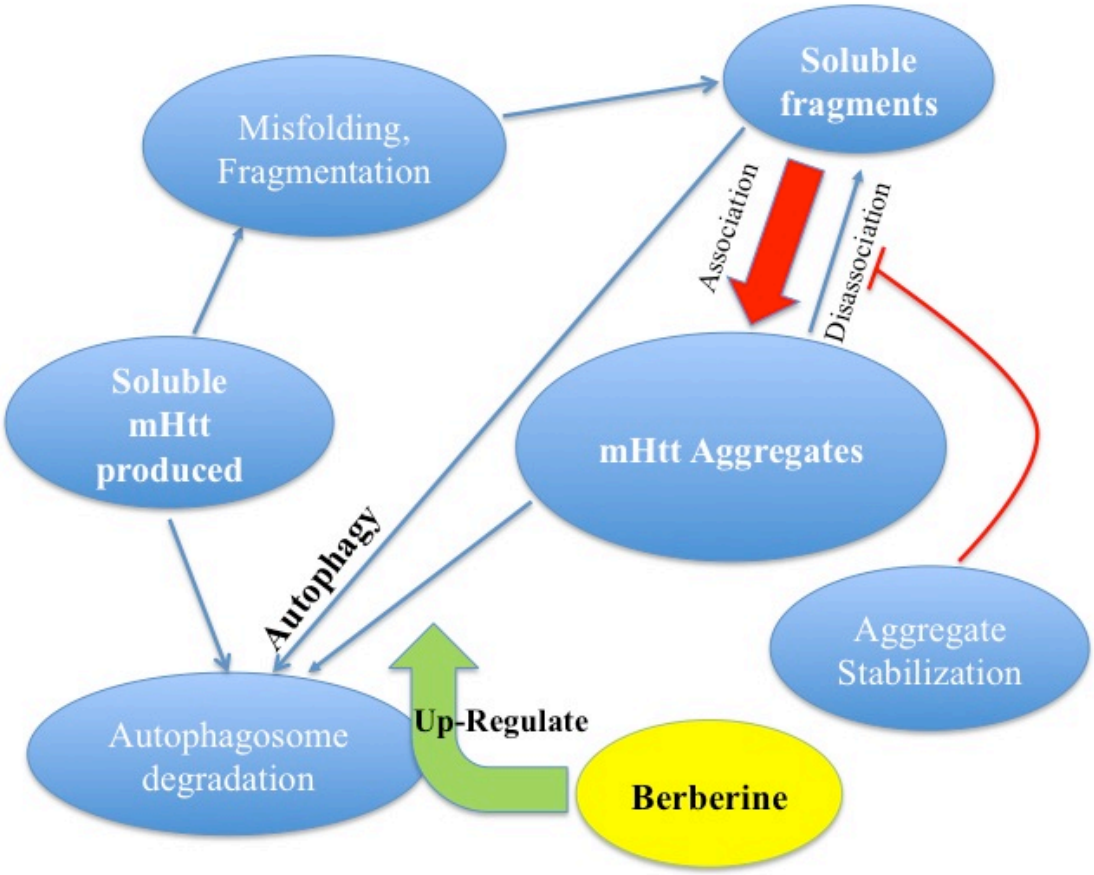
Soluble fragments
Misfolding, Fragmentation
Association
Disassociation
Soluble mHtt produced
mHtt Aggregates
Autophagy
Aggregate Stabilization
Autophagosome degradation
Up-Regulate
Berberine

Fig. 4-1 BBR up-regulates autophagic activity, resulting in an increased natural clearance of mHtt.

Theoretical model of BBR's role in HD as up-regulator of autophagic breakdown of all forms of mHtt

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