mGluR5 antagonism increases autophagy and prevents disease progression in the zQ175 mouse model of Huntington’s disease

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Huntington’s disease (HD) is a neurodegenerative disease caused by an expansion in the huntingtin protein (also called Htt) that induces neuronal cell death with age. We found that the treatment of 12-month-old symptomatic heterozygous and homozygous zQ175 huntingtin knockin mice for 12 weeks with CTEP, a negative allosteric modulator of metabotropic glutamate receptor 5 (mGluR5), reduced the size and number of huntingtin aggregates, attenuated caspase-3 activity, and reduced both neuronal apoptosis and neuronal loss in brain tissue. Both motor and cognitive impairments were improved in CTEP-treated zQ175 mice. The reduction in huntingtin protein aggregate burden by CTEP correlated with the activation of an autophagy pathway mediated by the kinase GSK3β, the transcription factor ZBTB16, and the autophagy factor ATG14. Inhibition of mGluR5 with CTEP also reduced the inhibitory phosphorylation of the autophagosome biogenesis–related kinase ULK1, increased the phosphorylation of the autophagy factor ATG13, and increased the abundance of the autophagy-related protein Beclin1 in homozygous zQ175 mice. The findings suggest that mGluR5 antagonism may activate autophagy through convergent mechanisms to promote the clearance of mutant huntingtin aggregates and might be therapeutically in HD patients.

INTRODUCTION

Huntington’s disease (HD) is an inherited autosomal dominant neurodegenerative disorder that is characterized by progressive motor, cognitive, and psychiatric impairment and ultimately results in patient death within 15 to 20 years (1, 2). HD results from a polyglutamine (CAG) expansion/repeat in the N terminus of the huntingtin protein (3, 4), which causes early loss of medium spiny neurons in the striatum, impairing both motor and cognitive functions (2, 5). Cleavage of the polyglutamine-expanded huntingtin protein N terminus results in the formation of intranuclear and cytoplasmic aggregates that are strongly correlated with HD symptoms and severity (4, 6, 7). Despite the well-characterized etiology and the availability of early genetic diagnosis, there are no disease-modifying treatments for HD.

Metabotropic glutamate receptor 5 (mGluR5) is a member of the heterotrimeric guanine nucleotide–binding protein–coupled receptor (GPCR) superfamily. mGluR5 is highly expressed in regions of the brain that are most affected in HD, including the striatum and cortex (8). Previously, we have demonstrated that mutant huntingtin interacts with and regulates mGluR5 signaling as part of a protein complex that includes both autophagy adaptor protein optineurin and the small guanosine triphosphatase Rab8 (9, 10). Moreover, wild-type huntingtin has been demonstrated to contribute to the regulation of autophagy (11). GPCR signaling is also implicated in attenuating the autophagic removal of huntingtin aggregates through the glycosgen synthase kinase 3β (GSK3β)–mediated suppression of an E3 ubiquitin ligase complex composed of the transcription factor ZBTB16, the scaffold Cullin3, and the RING finger protein Roc1, which results in the ubiquitin-dependent degradation of the autophagy adaptor ATG14 (autophagy-related protein 14) (12). However, the specific GPCR involved in regulating this autophagic clearance of huntingtin aggregates has yet to be identified.

We have shown that the deletion of mGluR5 reduces disease pathology in a Q111 mutant huntingtin knockin (Q111) mouse model, thereby implicating mGluR5 as a potential drug target for the treatment of HD (13). Moreover, the injection of mGluR5 antagonist into either the dorsal striatum or dorsal hippocampus results in increased locomotor activity (14). Thus, there is a rationale that targeted antagonism of mGluR5 signaling may be effective for the treatment of HD by altering the autophagic removal of mutant huntingtin aggregates. Several mGluR5 antagonists have undergone phase 2 clinical trials for the treatment of major depressive disorder, fragile X mental retardation, and Parkinson’s disease (15). Here, we investigated whether mGluR5 blockade prevents disease progression in a zQ175 huntingtin knockin (zQ175) mouse model. We found that prolonged treatment of 12-month-old zQ175 mice prevents neuronal cell death and reduces the size and number of huntingtin aggregates as a consequence of autophagy activation through a newly identified signaling pathway involving GSK3β and ZBTB16 and ultimately results in improved motor and cognitive function in both heterozygous and homozygous zQ175 mice. We also found that mGluR5 antagonism activated a classical autophagic pathway for autophagosomal biogenesis in this HD mouse model, indicating that increased mGluR5-mediated suppression of autophagy may contribute to neurodegenerative pathology associated with HD.

RESULTS

Chronic mGluR5 antagonism improves motor deficits in zQ175 mice

Wild-type, heterozygous, and homozygous zQ175 mice 12 months of age were treated with vehicle or CTEP (2-chloro-4-[2-[2,5-dimethyl-1-[4-(trifluoromethoxy)phenyl]imidazol-4-yl]ethynyl]pyridine)....
(2 mg/kg) every 48 hours and tested for improved motor performance 1 week (acute) and 3 months (chronic) after the initiation of drug treatment. The highly potent mGluR5-specific negative allosteric modulator (NAM) CTEP was chosen for these studies because it is orally bioavailable, crosses the blood-brain barrier, and has a half-life of 18 hours, and its analog basimglurant has proven to be well tolerated in phase 2 trials for major depressive disorder (16–18). When assessed for grip strength, we found a gene dosage–dependent impairment of grip strength in both heterozygous and homozygous zQ175 mice when compared to wild-type littermate controls at 12 months of age (Fig. 1A). Acute treatment (1 week) of the mice with CTEP did not alter grip strength (Fig. 1A), but a significant improvement in grip strength was observed for both heterozygous and homozygous zQ175 mice that were chronically treated with CTEP (12 weeks) when compared to vehicle-treated mice (Fig. 1B). We also observed a gene dosage–dependent impairment in the latency to fall in the accelerated rotarod test at 12 months of age in both heterozygous and homozygous zQ175 mice (Fig. 1C). After acute treatment with CTEP, the performance of heterozygous zQ715 mouse on the rotarod became indistinguishable from wild-type mice (Fig. 1C). However, after chronic CTEP treatment, the performance of heterozygous zQ175 mice was equivalent to that observed for wild-type mice, and the performance of CTEP-treated homozygous mice was significantly improved as compared with vehicle-treated mice (Fig. 1D). When mice were assessed for locomotor activity in open field, we observed a reduction in locomotor activity (velocity and distance) of 15-month-old homozygous zQ175 mice, and chronic mGluR5 inhibition resulted in the return of homozygous zQ175 mouse locomotor activity to levels that were indistinguishable from that of wild-type mice (Fig. 1E and F).

When mice were tested for step errors on a horizontal ladder rung walking test, only homozygous zQ175 mice scored significantly fewer errors than wild-type mice, and both acute (1 week) and chronic (12 weeks) CTEP treatment reduced the number of errors to wild-type levels (Fig. 2, A and B). Notably, none of the zQ175 mouse groups exhibited differences in the time it took to complete the task at 12 months of age when acutely treated with vehicle, but at 15 months of age, the time it took vehicle-treated homozygous zQ175 mice to complete the task was significantly increased, and chronic mGluR5 antagonism completely prevented the development of this impairment (Fig. 2, C and D). Together, these results indicate that antagonism of mGluR5 with an mGluR5-specific NAM could prevent and treat the progression of motor dysfunction in zQ175 mice.

**Chronic antagonism of mGluR5 improves cognitive impairment in zQ175 mice**

HD has been associated with cognitive impairment that affects patients before the onset of motor symptoms (19). Therefore, we tested whether heterozygous and homozygous zQ175 mice exhibited impairments in the novel object recognition test of memory and whether acute or chronic mGluR5 antagonism with CTEP could alleviate impairments. We found that vehicle-treated 12- and 15-month-old wild-type mice discriminated between novel and familiar objects, whereas 12- and 15-month-old heterozygous and homozygous zQ175 mice did not exhibit the ability to discriminate between familiar and novel objects. (Fig. 3, A and B). CTEP treatments ameliorated the cognitive deficits in heterozygous zQ175 mice (Fig. 3, A and B).

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**Fig. 1. Chronic administration of CTEP improves motor impairments in zQ175 mice.** (A and B) Mean ± SEM of grip strength (gram-force (gf)) after acute (1 week) and chronic (12 weeks) treatment with vehicle or CTEP (2-chloro-4-[2-[2,5-dimethyl-1-[4-(trifluoromethoxy)phenyl]imidazol-4-yl]ethynyl]pyridine) (2 mg/kg) in heterozygous zQ175 (Q175/+), homozygous zQ175 (Q175/Q175), and wild-type (+/+; n = 12 for all groups). (C and D) Mean ± SEM of latency to fall from accelerating rotarod after acute (1 week) and chronic (12 weeks) treatment with vehicle or CTEP in Q175/+ , Q175/Q175, and wild-type mice (n = 12 for all groups). (E and F) Mean ± SEM of velocity (E) and distance traveled (F) in open field after acute (12 weeks) treatment with vehicle or CTEP in Q175/+ , Q175/Q175, and wild-type mice (n = 12 for all groups). *P < 0.05, **P < 0.01, and ***P < 0.001 by two-way analysis of variance (ANOVA) and Fisher’s least significant difference (LSD) comparisons.
after CTEP treatment (Fig. 4, G to I). Together, these results indicate that the size and number of these aggregates decreased significantly after CTEP treatment. Consistent with the observation that huntingtin aggregates in heterozygous and homozygous zQ175 mice were reduced after 3 months of CTEP treatment since the age of 12 months, we examined whether both the number and size of huntingtin aggregates in heterozygous and homozygous zQ175 mice were reduced after 3 months of CTEP treatment. Therefore, we assessed whether chronic inhibition of mGluR5 by CTEP was capable of activating autophagy through this GPCR signaling pathway. We found that GSK3β and stabilization of ZBTB16 abundance, a key component of the ZBTB16-Cullin3-Roc1 E3 ubiquitin ligase, which promoted the degradation of autophagy protein ATG14. GPCRs represent the largest family of membrane-bound receptor proteins, and they regulate a myriad of cellular processes, making the indiscriminate activation and inhibition GPCR activity undesirable. However, identification and targeting of specific GPCRs that regulate neuronal huntingtin autophagy represent a potentially exciting novel therapeutic approach.

**mGluR5 inhibition initiates the clearance of huntingtin aggregates in zQ175 mice**

One of the distinguishing features of HD pathology has been the deposition of insoluble huntingtin aggregates in the striatum (4). We have previously demonstrated that the deletion of GRM5, which encodes mGluR5, resulted in a significant reduction in the number of huntingtin aggregates in Q111 huntingtin knockin mice (13). Therefore, we examined whether both the number and size of huntingtin aggregates in the brains of heterozygous and homozygous zQ175 mice were reduced after 3 months of CTEP treatment since the age of 12 months. We found that the deposition (size and number) of huntingtin aggregates in heterozygous and homozygous zQ175 striatal (Fig. 4, A to C) and cortical (Fig. 4, D to F) brain slices was significantly reduced after CTEP treatment. Consistent with the observation that zQ175 mice were cognitively impaired, we observed that huntingtin aggregates in the hippocampus of heterozygous and homozygous zQ175 mice and that the size and number of these aggregates decreased after CTEP treatment (Fig. 4, G to I). Together, these results indicate that chronic mGluR5 antagonism with CTEP resulted in reduced huntingtin pathology.

**Chronic mGluR5 inhibition reduces ERK1/2 phosphorylation, caspase-3 activity, and cell death**

Activation of extracellular signal–regulated protein kinases 1 and 2 (ERK1/2) contributes to cell death upstream of caspase-3 in many cell types and animal models of brain injury, and we previously reported an mGluR5-dependent increase in ERK1/2 activity in homzygous Q111 mice (20, 21). We found that ERK1/2 phosphorylation in brain lysates derived from vehicle-treated mice was significantly increased in both heterozygous and homozygous zQ175 mice (Fig. 5A). Chronic CTEP treatment reduced the amount of ERK1/2 phosphorylation in both heterozygous and homozygous zQ175 mice (Fig. 5A). We also observed that caspase-3 activity was significantly increased in homozygous zQ175 brain samples and that chronic CTEP treatment reduced caspase-3 activity to levels that were equivalent to CTEP-treated control animals (Fig. 5B). Consistent with the increase in caspase-3 activity, we observed a gene-dosage increase in terminal deoxynucleotidyl transferase–mediated deoxuridine triphosphate nick end labeling (TUNEL) staining (a marker of apoptosis) in striatal sections from heterozygous and homozygous zQ175 mice, which was also attenuated by chronic CTEP treatment (Fig. 5, C and D). We also observed an increase in the number of striatal neurons after staining for neuronal nuclei (NeuN) in CTEP-treated heterozygous and homozygous zQ175 mice (Fig. 5E). Thus, mGluR5 antagonism reduced neuronal cell death that could be associated with the manifestation of both motor and cognitive impairments in zQ175 mice.

**Chronic mGluR5 blockade activates autophagy by a ZBTB16/ATG14-regulated pathway**

Aggregated mutant huntingtin protein is a hallmark of HD, and it has been proposed that up-regulation of autophagy is effective in clearing these protein aggregates (22). Recently, GPCR signaling was reported to reduce autophagy initiation (12). Specifically, GPCR signaling inhibited autophagy by promoting inhibitory phosphorylation of GSK3β and stabilization of ZBTB16 abundance, a key component of the ZBTB16-Cullin3-Roc1 E3 ubiquitin ligase, which promoted the degradation of autophagy protein ATG14. GPCRs represent the largest family of membrane-bound receptor proteins, and they regulate a myriad of cellular processes, making the indiscriminate activation and inhibition GPCR activity undesirable. However, identification and targeting of specific GPCRs that regulate neuronal huntingtin autophagy represent a potentially exciting novel therapeutic approach. Therefore, we assessed whether chronic inhibition of mGluR5 by CTEP was capable of activating autophagy through this GPCR signaling pathway. We found that GSK3β phosphorylation at Ser9 was...
Phosphorylation of ATG13 at Ser\textsuperscript{355} leads to the recruitment of ATG13, enabling efficient autophagy (26). The kinase mammalian target of rapamycin (mTOR) phosphorylates ULK1 at Ser\textsuperscript{757}, thereby suppressing ULK1 kinase activity and autophagy initiation (27). Because mGluR5 is known to activate the mTOR pathway (28), we tested whether blocking mGluR5 with CTEP could induce autophagy by activating ULK1. Chronic blockade of mGluR5 reduced the inhibitory phosphorylation of ULK1 at Ser\textsuperscript{757} observed in zQ175 mice (Fig. 7, A and B) and was accompanied by an increase in ATG13-Ser\textsuperscript{355} phosphorylation, indicating that ULK1 activity was increased upon CTEP treatment (Fig. 7, A and C). The abundance of Beclin1, a critical regulator of autophagy, was also increased in CTEP-treated zQ175 mice, reflecting an induction of autophagy (Fig. 7, A and D). Together, these findings suggest that mGluR5 antagonism can potentially enhance autophagy by multiple convergent mechanisms to increase the clearance of mutant huntingtin aggregates.

**DISCUSSION**

To date, there are no disease-modifying drugs for the treatment of HD. Our data indicate that mGluR5 antagonism using a highly selective NAM significantly reduced significantly increased in brain lysates derived from vehicle-treated heterozygous and homozygous zQ175 mice; CTEP significantly reduced GSK3β phosphorylation, resulting in activation of GSK3β (Fig. 6, A and B). We also found that ZBTB16 protein expression was reduced after chronic treatment with CTEP in both heterozygous and homozygous zQ175 mice (Fig. 6, A and C), which was associated with increased ATG14 protein expression (Fig. 6, A and D) and a reduction in the abundance of p62 (Fig. 6, A and E), a protein that recognizes and associates with toxic cellular waste and is scavenged upon initiation of autophagy. We also found that p62 protein aggregates were predominantly colocalized with huntingtin aggregates in striatal brain sections from vehicle-treated homozygous zQ175 mice, indicating a blockage in aggregate autophagy (Fig. 6, F and G). Moreover, we observed that chronic CTEP treatment reduced the number of p62 aggregates in homozygous zQ175 mice, indicating an increase in the autophagic clearance of huntingtin aggregates (Fig. 6H). Together, these data indicate that targeted inhibition of mGluR5 was sufficient to promote huntingtin clearance by autophagic induction.

**Chronic mGluR5 antagonism activates a canonical autophagy pathway required for autophagosome biogenesis**

ULK1 and ULK2 (Unc-51–like kinase) are ubiquitously expressed kinases that localize to phagophore membrane upon nutrient starvation to promote autophagosome formation (23, 24). ULK1/2 forms a complex with ATG13 along with FIP200 at the autophagic isolation membranes to regulate autophagosome biogenesis (25). ULK1-dependent phos-
their ability to complete the ladder rung task, but by 15 months of age, the homozygous zQ175 mice took significantly longer to complete the task than wild-type mice, and mGluR5 antagonism prevented the development of this deficit. This provides additional evidence that mGluR5 inhibition represents an excellent therapeutic target to slow and/or prevent the progression of the HD process.

Several HD models have been developed in an attempt to model the disease process, and they each present with varying levels of phenotypic disease severity, pathology, and onset (32). The zQ175 HD mouse model is the first to demonstrate an HD phenotype on a heterozygous genetic background, which has allowed us to assess the effects of gene dosage on the penetrance of the HD phenotype in the zQ175
mice and the relative effectiveness of mGluR5 antagonism treatment to affect motor function (33, 34). We observed that homozygous mice are far more severely impaired in motor function when compared to either wild-type or heterozygous mice and that heterozygous mice display an intermediary phenotype. This is paralleled by the effectiveness of chronic mGluR5 blockade to reduce the ob-
served motor deficits. Specifically, we found that, although chronic antagonist treatment significantly improved grip strength and rotarod performance in homozygous zQ175 mice, it did not completely ameliorate the loss of motor function of these severely impaired mice in this task. In contrast, we found that mGluR5 acute NAM treatment of heterozygous mice returns open-field locomotor activity to wild-type levels. However, chronic antagonism completely reversed the reduction of locomotion in the open-field box observed for homozygous zQ175 mice and significantly improved motor performance on the ladder rung test, suggesting that disease progression can be arrested or even reversed with drug treatment.

Cognitive impairment is an often disregarded consequence of HD, and mGluR5 signaling is known to be intimately linked to processes associated with memory and learning (19, 35–37). Similar to what we have observed previously in Alzheimer’s disease mouse models (38), chronic mGluR5 antagonist treatment reversed the cognitive impairment observed in both heterozygous and homozygous zQ175 mice. However, unlike what we previously reported (38) for Alzheimer’s disease mice, acute (1 week) antagonist treatment effectively improved the memory of heterozygous, but not homozygous, zQ175 mice. We find evidence for huntingtin aggregates in the hippocampus of zQ175 mice suggesting that, similar to what we propose for Alzheimer’s disease mice, mGluR5 antagonism potentially results in the removal of both soluble and insoluble misfolded mutant huntingtin protein, which results in improved cognitive function.

Our findings support a critical/central contribution of pathological mGluR5 signaling to the pathophysiology underlying HD. mGluR5 is highly expressed in the striatum, a major region affected in HD (8), and we have previously shown that mGluR5 interacts with mutant huntingtin to antagonize mGluR5 signaling in a protein kinase C (PKC)-dependent manner but that this PKC-dependent regulation of mGluR5 signaling is lost with age (9, 21). We show that the number and size of huntingtin aggregates are affected by huntingtin gene copy number and that mGluR5 antagonism results in a reduction in the number and size of huntingtin aggregates in both heterozygous and homozygous zQ175 mice. This suggests that the loss of the PKC-dependent attenuation of mGluR5 signaling with age contributes to the deposition of huntingtin aggregates and/or antagonizes the clearance of misfolded

**Fig. 5. Treatment with CTEP reduces ERK1/2 phosphorylation, caspase-3 activity, and cell death.** (A) Representative Western blots and mean ± SD showing the fold change in pERK1/2 as a percent of ERK1/2 extracellular signal–regulated protein kinases 1 and 2) in brain lysates from wild-type (+/+), heterozygous zQ175 (Q175+/+), and homozygous zQ175 (Q175/Q175) mice after 12 weeks of treatment with either vehicle or CTEP (2 mg/kg) expressed as the fraction of the vehicle-treated wild-type control (n = 5 to 6). (B) Caspase-3 activity measured in brain lysates from vehicle- and CTEP-treated wild-type, Q175+/+, and Q175/Q175 mice (n = 3). RFU, relative fluorescence unit. (C and D) Representative confocal microscopy images of in situ BrdU-Red DNA Fragmentation [terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL)] staining (C) and quantification of TUNEL-positive cells counterstained with Hoechst (D) in striatal slices from vehicle- and CTEP-treated wild-type, Q175+/+, and Q175/Q175 mice (n = 6). Scale bar, 50 μm. (E) Quantification of the number of neuronal nuclei (NeuN)–positive neurons in striatal brain slices from vehicle- and CTEP-treated wild-type, Q175+/+, and Q175/Q175 mice (n = 6). Data are quantified from five different 300-µm² regions from five to six independent mouse brains for each group. Data are means ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 by two-way ANOVA and Fisher’s LSD comparisons.
huntingtin protein with age. The observation that mGluR5 antagonism reduces the size and number of huntingtin aggregates indicates that these aggregates themselves are neurotoxic and/or that neurotoxic misfolded mutant huntingtin species normally sequestered into aggregates are cleared from neurons as a consequence of mGluR5 inhibition.

It has been previously shown that the overexpression of genes implicated in autophagy enhances the clearance of mutant huntingtin and reduces aggregate formation in Caenorhabditis elegans and that the activation of autophagy reduces the formation of huntingtin aggregates in animal models of HD (12, 39–44). Previous methodologies to pharmacologically enhance the autophagy pathway have focused on rapalogs, derivatives of the mTOR inhibitor rapamycin. Two limitations with the use of rapalogs are the poor uptake across the blood-brain barrier and immunosuppressive properties (45, 46). In contrast, CTEP exhibits superior transfer across the blood-brain barrier and exhibited no major side effects, indicating that CTEP treatment may represent a safer and more effective method of activating autophagy, suggesting that mGluR5 antagonists will be effective therapeutic tools to treat HD patients.

We have shown here that the prolonged antagonism of mGluR5 results in the dephosphorylation at Ser9 and activation of GSK3β in heterozygous and homozygous zQ175 mice, which has been previously shown to phosphorylate and promote the autoubiquitination and degradation of ZBTB16 (12). We also found that the loss of ZBTB16 expression is associated with stabilization of the autophagy adaptor ATG14 and a reduction in p62 protein levels, indicating an

Fig. 6. Chronic CTEP treatment activates an autophagy pathway in zQ175 mice. (A) Representative Western blots of pGSK3β-Ser9, ZBTB16, ATG14, and p62 with the corresponding loading controls in brain lysates from heterozygous zQ175 (Q175+/+), homozygous zQ175 (Q175/Q175), and wild-type (+/-) mice after chronic treatment with either vehicle or CTEP (2 mg/kg). GSK3β, glycogen synthase kinase 3β. (B to E) Quantification of blots represented in (A), presented as fold change in pGSK3β-Ser9 (B), ZBTB16 (C), ATG14 (D), and p62 (E) band intensity relative to each in vehicle-treated wild-type samples (n = 5 to 6). Data are means ± SD. (F and G) Representative confocal images of EM48 (green) and p62 (red) colocalization in striatal slices from vehicle-treated (F) and CTEP-treated (G) Q175/Q175 mice. Images are representative of three independent experiments. Scale bars, 50 μm. (H) Quantification of p62 immunostaining shown as mean ± SD of the number of fluorescent p62 aggregates in five 900-μm² regions from six brain slices from three different vehicle- and CTEP-treated wild-type, Q175/+, and Q175/Q175 mice. *P < 0.05, **P < 0.01, and ***P < 0.001 by two-way ANOVA and Fisher’s LSD comparisons.
that mGluR5-targeted treatments of neurodegenerative disease can be achieved through multiple mechanisms that converge downstream of the receptor.  

Associated with the clearance of mutant aggregates is a reduction in caspase-3 activation and neuronal apoptosis, suggesting that mGluR5 antagonism also results in the preservation of neurons in the striatum of zQ175 mice. ERK1/2 activation has been shown to precede caspase-3 activation (20, 48, 49), and consistent with this observation, we find that the activation of caspase-3 in zQ175 mice is correlated with an increase in ERK1/2 activation. Chronic CTEP treatment of wild-type mice increases ERK1/2 phosphorylation, which is opposite to the effect of drug treatment of heterozygous and homozygous zQ175 mice. However, this increase in ERK1/2 phosphorylation did not translate into a change in caspase activity in wild-type mice. Mutant huntingtin is also a substrate for caspase-3, and the proteolytic cleavage of huntingtin releases the polyglutamine toxic fragments, resulting in the cellular dysfunction and death that correlates with HD progression (50, 51). Thus, it is possible that mGluR5 antagonism may reduce the formation of huntingtin aggregates, in part, by reducing the cleavage of the mutant huntingtin protein.

In summary, we find that mGluR5 antagonism is effective in reducing behavioral impairments, pathological hallmarks, and cell death associated with HD in zQ175 mice. Moreover, we provide evidence that antagonism of mGluR5 signaling promotes the clearance of toxic misfolded protein species and suggest that this may occur as the consequence of the up-regulation of autophagy. There are now a number of highly selective mGluR5 NAMs that are in clinical trials for the treatment of neurodevelopmental, psychiatric, and neurodegenerative diseases such as fragile X mental retardation disorder, major depressive disorder, and Parkinson’s disease (15, 18, 52). Our preclinical data suggest that the use of mGluR5 NAMs may be effectively extended to the treatment of HD.

**MATERIALS AND METHODS**

**Reagents**

CTEP was purchased from Axon Medchem. Horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G secondary antibody was from Bio-Rad (1662408EDU). HRP-conjugated anti-mouse secondary and rabbit anti–phospho-p44/42 ERK1/2 (Thr202/Tyr204); 9101), ERK1/2 (9102), phospho-ULK1 (Ser757; 14202), ULK1 (8054), phospho-ATG13 (Ser355; 43533), ATG13 (13273), phospho-GSK3β (Ser9; 9323), Beclin1 (3495), and mouse anti-GSK3β (9832) antibodies were from Cell Signaling Technology. Rabbit anti–actin (CL2810AP), ATG14L (9832), and consistent with other observations, we find that the observed reduction of huntingtin aggregates after antagonist treatment may occur as the consequence of increased autophagy. To our knowledge, this study represents the first evidence to suggest that pharmacologically targeting a single GPCR will be effective in clearing neurotoxic aggregates by drugs that are tolerated by patient populations.

There is also evidence that an mGluR5 positive allosteric modulator [3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB)] may also modestly improve motor function in BACHD mice by a mechanism that has yet to be delineated (47). However, it is possible that the CDPPB may bias endogenous glutamate signaling such that mGluR5 activation does not lead to the GSK3β-mediated attenuation of ATG14-dependent autophagy. Nevertheless, our current study extends our previous work, demonstrating that mGluR5 knockout improves the performance of Q111 mice and indicates that this improved performance was not the consequence of compensatory developmental adaptation (13). It is likely that mGluR5-targeted treatments of neurodegenerative disease can be achieved through multiple mechanisms that converge downstream of the receptor.

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**Fig. 7. Chronic CTEP treatment activates classical autophagic pathways that regulate autophagosome biogenesis.** (A to D) Representative Western blots (A) and mean ± SD for fold change in pULK1-Ser757 (B), pATG13-Ser355 (C), and Beclin1 (D) with the corresponding loading controls in brain lysates from heterozygous zQ175 (Q175/+), homozygous zQ175 (Q175/Q175), and wild-type (+/+ ) mice after chronic treatment with either vehicle or CTEP (2 mg/kg) and expressed as a fraction of the vehicle-treated wild-type samples (n = 5 to 6). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. *P < 0.05, **P < 0.01, and ***P < 0.001 by two-way ANOVA and Fisher’s LSD comparisons.
controlled HD mice were obtained courtesy of CHDI Foundation from The Jackson Laboratory (stock #370476) and bred to establish littermate-controlled male wild-type, heterozygous zQ175 (Q175/+), knockin, and homozygous zQ175 (Q175/Q175) knockin mice. zQ175 knockin mice carry ~188 CAG repeat expansions. Groups of 24 male wild-type, Q175/+, and Q175/Q175 mice were aged to 12 months of age, and 12 mice from each group were treated every 48 hours with either vehicle [dimethyl sulfoxide (DMSO) in chocolate pudding] or CTEP (2 mg/kg; dissolved in 10% DMSO and then mixed with chocolate pudding; final DMSO concentration was 0.1%) for 12 weeks. This drug dose was calculated weekly on the basis of weight and is consistent with the dose given to fragile X syndrome mice [25)]. Mice were returned to the maze for 5 min and allowed to explore. The time spent exploring each object was recorded, and mice were considered to be exploring an object if their snout was within 1 cm of the object. Twenty-four hours after first exposure, the experiment was repeated with one object replaced with a novel object. The time spent exploring each object was recorded. Data were interpreted using the recognition index (time spent exploring the familiar object or the novel object over the total time spent exploring both objects multiplied by 100) and was used to measure the recognition memory [\( TA/(TA + TB) \) * 100, where \( T \) represents the time, \( A \) represents a familiar object, and \( B \) represents a novel object.

### Western blotting

The brains were dissected, and one hemisphere was lysed in ice-cold lysis buffer [50 mM tris (pH 8.0), 150 mM NaCl, and 1% Triton X-100] containing protease inhibitors [1 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride], leupeptin (10 \( \mu \)g/ml), and apro tin (2.5 \( \mu \)g/ml)] and phosphatase inhibitors (10 mM NaF and 500 \( \mu \)M Na\(_3\)VO\(_4\)) and centrifuged at 15,000 rpm at 4°C for 15 min. The supernatant was collected, and the total protein levels were quantified using Bradford protein assay (Thermo Fisher Scientific). Homogenates were diluted in a mix of lysis buffer and \( \beta \)-mercaptoethanol containing 3X loading buffer and boiled for 10 min at 95°C. Aliquots containing 30 \( \mu \)g of total proteins were resolved by electrophoresis on a 7.5% SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad). Blots were blocked in tris-buffered saline (pH 7.6) containing 0.05% Tween 20 (TBST) and 5% nonfat dry milk for 2 hours at room temperature and then incubated overnight at 4°C with primary antibodies diluted (1:1000) in TBST containing 1% nonfat dry milk. Immunodetection was performed by incubating with secondary antibodies (anti-rabbit/mouse) diluted (1:5000) in TBST containing 1% nonfat dry milk for 1 hour. Membranes were washed in TBST, and then, bands were detected and quantified using a Bio-Rad chemiluminescence system.

### EM48 immunohistochemistry

Staining for aggregated huntingtin was performed using an EM48 monoclonal antibody that recognizes mutant huntingtin in both humans and computer in a separate room and analyzed using the Noldus EthoVision 10 software. The distance traveled and velocity of each mouse in the open box were determined.
and transgenic mice has been shown to react with 82 to 150 repeats in transgenic mice and is believed to recognize multiple forms of mutant huntingtin, as described previously (13). Briefly, the brains were coronally sectioned through the striatum and hippocampus, and staining was performed on 40-μm free-floating sections using a peroxidase-based immunostaining protocol. Sections were incubated in primary antibody for huntingtin overnight (1:100); anti-huntingtin protein, mouse EM48 monoclonal antibody) at 4°C, washed, incubated in biotinylated antibody (1:400; biotinylated horse anti-mouse, Vector Elite ABC kit (mouse), Vector Laboratories), and then incubated in an avidin/ biotin enzyme reagent [PK-6102, Vector Elite ABC kit (mouse), Vector Laboratories]. Immunostaining was visualized using a chromogen (Vector SG substrate). Sections were mounted on slides and visualized with a Zeiss Axio Observer epifluorescent microscope with a Zeiss 20× lens, using representative 900-μm² areas of the striatum, cortex, and hippocampus.

**p62 and NeuN immunofluorescence**

Coronal brain sections, as described above, were fixed using 4% paraformaldehyde, after which membranes were permeabilized using 0.2% Triton X-100. Nonspecific binding was blocked using 2.5% normal donkey serum and 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), followed by incubation in p62 primary antibody (1:100) or NeuN primary antibody (1:100) overnight at 4°C. After a 3× 5-min wash in PBS, sections were incubated in donkey anti-mouse Alexa Fluor 488 (1:400) for 1 hour at room temperature. Sections were mounted on slides and visualized with a Zeiss Axio Observer epifluorescent microscope with a Zeiss 20× lens, using representative 900-μm² areas of the striatum.

**Huntingtin and p62 double immunofluorescence**

Coronal brain sections were fixed using 4% paraformaldehyde, after which membranes were permeabilized using 0.2% Triton X-100. Nonspecific binding was blocked using 2.5% normal goat serum and 1% BSA in PBS, followed by incubation in primary antibody for huntingtin overnight (1:100) at 4°C. After a 3× 5-min wash in PBS, sections were incubated in goat anti-mouse Alexa Fluor 647 (1:400) for 1 hour at room temperature. Sections were again washed three times for 5 min in PBS, and then, nonspecific binding was blocked using 2.5% normal donkey serum and 1% BSA in PBS, followed by incubation in p62 primary antibody (1:100) overnight at 4°C. After a 3× 5-min wash in PBS, sections were incubated in donkey anti-mouse Alexa Fluor 488 (1:400) for 1 hour at room temperature. Sections were mounted on slides and visualized with a Zeiss LSM 800 microscope with a Zeiss 40× lens, using representative 900-μm² areas of the striatum.

**Caspase-3 activity assay**

Caspase-3 activity was measured using the caspase-3 assay kit from Abcam (ab39383), as per the manufacturer’s instructions. Briefly, brain hemispheres were homogenized in cell lysis buffer and centrifuged at 15,000 rpm at 4°C for 15 min. The supernatant was collected, and the total protein levels were quantified using Bradford protein assay kit. Reaction buffer containing dithiothreitol and the DEVD-AFC substrate was added to 50 μg of total proteins, then the mixture was incubated at 37°C for 2 hours, and the samples were read on a Synergy Neo multimode reader using a 400-nm excitation and 505-nm emission filter.

**In situ BrdU-Red DNA Fragmentation (TUNEL) assay**

Apoptosis was measured using the in situ BrdU-Red DNA Fragmentation (TUNEL) assay kit from Abcam (ab66110) according to the manufacturer’s instructions. Briefly, fixed coronal brain slices (in 4% formaldehyde) were washed with PBS, followed by the addition of proteinase K solution. Slices were then washed with wash buffer, followed by the addition of DNA labeling solution. Anti-BrdU (Red) antibody was then added, followed by Hoechst staining, and slides were imaged using a Zeiss LSM 800 confocal microscope.

**Statistical analysis**

Means ± SD shown for each independent experiment are shown in the various figure legends. GraphPad Prism software was used to analyze the data for statistical significance. Statistical significance was determined by a series of 3 (strain) × 2 (drug treatment) analyses of variance (ANOVA), followed by Fisher’s least significant difference comparisons for the significant main effects or interactions.

**References and notes**

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mGluR5 antagonism increases autophagy and prevents disease progression in the zQ175 mouse model of Huntington's disease

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A treatment for Huntington's disease?

There are currently no therapies that stop or reverse Huntington's disease (HD), a progressive neurodegenerative disease caused by an expansion in the huntingtin protein that promotes its toxic accumulation in the central nervous system. Impaired autophagy, the process of breaking down and removing cellular debris and dysfunctional proteins, is implicated in HD. Blocking the kinase mTOR therapeutically can activate autophagy, but these drugs have serious side effects and poor penetrance into the brain. Abd-Elrahman et al. found that inhibiting the neuronal cell-surface glutamate receptor mGluR5 with the drug CTEP stimulated autophagy of huntingtin aggregates and improved motor and cognitive performance in HD model mice, without any apparent side effects. Given that CTEP analog is already in clinical trials for other neuronal-associated disorders, these findings suggest that the trials ought to be expanded to include HD patients.