1521-0103/379/1/74–84$35.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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https://doi.org/10.1124/jpet.121.000735
J Pharmacol Exp Ther 379:74–84, September 2021

The Journal of PHARMACOLOGY And Experimental Therapeutics

Metabotropic Glutamate Receptor 2/3 Activation Improves Motor Performance and Reduces Pathology in Heterozygous zQ175 Huntington Disease Mice

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Received May 17, 2021; accepted July 20, 2021

ABSTRACT

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease that leads to progressive motor impairments with no available disease-modifying treatment. Current evidence indicates that exacerbated postsynaptic glutamate signaling in the striatum plays a key role in the pathophysiology of HD. However, it remains unclear whether reducing glutamate release can be an effective approach to slow the progression of HD. Here, we show that the activation of metabotropic glutamate receptors 2 and 3 (mGluR2/3), which inhibit presynaptic glutamate release, improves HD symptoms and pathology in heterozygous zQ175 knockin mice. Treatment of both male and female zQ175 mice with the potent and selective mGluR2/3 agonist LY379268 for either 4 or 8 weeks improves both limb coordination and locomotor function in all mice. LY379268 also reduces mutant huntingtin aggregate formation, neuronal cell death, and microglial activation in the striatum of both male and female zQ175 mice. The reduction in mutant huntingtin aggregates correlates with the activation of a glycogen synthase kinase 3β–dependent autophagy pathway in male, but not female, zQ175 mice. Furthermore, LY379268 reduces both Akt and ERK1/2 phosphorylation in male zQ175 mice but increases both Akt and ERK1/2 phosphorylation in female zQ175 mice. Taken together, our results indicate that mGluR2/3 activation mitigates HD neuropathology in both male and female mice but is associated with the differential activation and inactivation of cell signaling pathways in heterozygous male and female zQ175 mice. This further highlights the need to take sex into consideration when developing future HD therapeutics.

SIGNIFICANCE STATEMENT

The mGluR2/3 agonist LY379268 improves motor impairments and reduces pathology in male and female zQ175 Huntington's disease mice. The beneficial outcomes of LY379268 treatment in Huntington's disease mice were mediated by divergent cell signaling pathways in both sexes. We provide evidence that mGluR2/3 agonists can be repurposed for the treatment of Huntington's disease, and we emphasize the importance of investigating sex as a biological variable in preclinical disease-modifying studies.

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by premature loss of medium spiny striatal neurons that leads to progressive motor disturbances, cognitive impairments, behavior difficulties, and ultimately death (Martin and Gusella, 1986; Roos, 2010). The disease typically manifests at middle age and is caused by the expansion of a polyglutamine (CAG) repeat in the N-terminal region of the huntingtin protein (MacDonald et al., 1993). The proteolytic cleavage of polyglutamine-expanded huntingtin proteins at their N-terminus results in the formation of cytoplasmic and intranuclear aggregates that strongly correlates with HD symptoms and severity (Andrew et al., 1993; Furtado et al., 1996; DiFiglia et al., 1997). Despite this well characterized cause and the feasibility of early genetic diagnosis, the molecular mechanism(s) underlying HD pathogenesis remain poorly understood, and disease-modifying treatments for HD are lacking.

Glutamate is the major mediator of excitatory transmission in the brain, and considerable evidence suggests that impaired glutamate uptake and glutamate-induced toxicity contribute to the selective loss of striatal neurons in HD (Hassel et al., 2008; Fan et al., 2009; Ribeiro et al., 2011, 2017). Previous reports indicate that the function of glutamate transporter-1,

This work was supported by grants from the Huntington's Society of Canada, the Krembil Foundation, and Canadian Institutes for Health Research [PJT-148656, PJT-153317, and PJT-165967].

No author has an actual or perceived conflict of interest with the contents of this article.

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https://doi.org/10.1124/jpet.121.000735.

ABBREVIATIONS: AD, Alzheimer's disease; Akt, protein kinase B; ALS, amyotrophic lateral sclerosis; ATG14, autophagy related protein 14; ERK1/2, extracellular signal-regulated kinase 1/2; GSK3β, glycogen synthase kinase 3β; HD, Huntington disease; HRP, horseradish peroxidase; Iba1, ionized calcium-binding adaptor molecule 1; mGluR, metabotropic glutamate receptor; mHtt, mutant huntingtin; NeuN, neuronal nuclear protein; NMDAR, *N*-methyl-D-aspartate receptor; TBST, Tris buffered saline with Tween 20; Wt, wild type; ZBTB16, zinc finger and BTB domain-containing protein 16.

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the primary glial glutamate transporter responsible for the uptake of about 90% of the extracellular glutamate, is impaired in HD mice (Behrens et al., 2002; Huang et al., 2010). Moreover, intrastriatal injection of glutamate and its analog kainic acid induces a similar pattern of enzymatic changes, including large losses of glutamic acid decarboxylase and choline acetyltransferase in the striatum, as those reported in HD. Similarly, the *N*-methyl-D-aspartate receptor (NMDAR) agonist quinolinic acid causes striatal lesions that mimic the selective depletion of medium spiny striatal neurons seen in HD (Beal et al., 1991). Furthermore, genetic deletion of metabotropic glutamate receptor 5 (mGluR5), a heterotrimeric G protein–coupled receptor highly expressed in the striatum, in a Q111 knockin HD mouse model improves Rotarod performance and reduces the size of mutant huntingtin (mHTT) aggregates (Ribeiro et al., 2014). Pharmacological blockade of mGluR5 with the negative allosteric modulator CTEP (2-chloro-4-[2-[2,5-dimethyl-1-[4-(trifluoromethoxy)phenyl]imidazol-4-yl]ethynyl]-pyridine) in male zQ175 knockin (zQ175) HD mice also improves motor function, prevents neuronal cell death, and promotes autophagic removal of mHTT aggregates (Abd-Elrahman et al., 2017; De Souza et al., 2020). Therefore, it is evident that reducing postsynaptic glutamatergic signaling can ameliorate HD neuropathology and should be investigated as a treatment strategy.

mGluR2/3 are mainly located on corticostriatal presynaptic terminals, and their activation reduces excessive glutamate release via negative feedback mechanisms (Conn and Pin, 1997; Ribeiro et al., 2017). Previous studies have shown that selective mGluR2/3 agonists can protect against *N*-methyl-D-aspartate–induced neuronal death in vitro (Buisson and Choi, 1996; Battaglia et al., 1998; Kingston et al., 1999). Interestingly, a reduction in the expression of mGluR2/3 is reported in symptomatic R6/2 HD transgenic mice in the absence of detectable striatal neuron loss (Cha et al., 1998) . Furthermore, chronic administration of the maximum tolerated dose of the mGluR2/3 agonist LY379268 in R6/2 transgenic mice improves survival time and some motor deficits, but the effects of LY379268 on HD neuropathology have not been investigated in HD mice that better reproduce HD phenotype (Schiefer et al., 2004; Reiner et al., 2012). There is also growing evidence that sex may influence HD phenotype and neuropathology in HD rodent models and patients (Dorner et al., 2007; Bode et al., 2008; Zielonka and Stawinska-Witoszynska, 2020; Hentosh et al., 2021). This is particularly important given that we and others have previously reported sex-specific differences in glutamate signaling in Alzheimer's disease and HD (Padovan-Neto et al., 2019; Abd-Elrahman, et al., 2020a). Therefore, it is of interest to study the disease-modifying properties of mGluR2/3 agonists in the heterozygous zQ175 mouse model of HD that better reflects the slow and progressive nature of HD pathology in both male and female mice.

LY379268 is a potent and selective agonist of mGluR2/3 that was first developed in 1999 and showed a potent agonist activity toward both receptors with an EC_{50} in the low nanomolar range (Monn et al., 1999). LY379268 did not produce any measurable effects on mGluR1, mGluR5, mGluR4, mGluR7, and mGluR8 at concentrations up to 10 μM, but showed weak agonist activity toward mGluR6 at high nanomolar concentrations (Monn et al., 1999). LY379268 has also shown effectiveness in animal models of HD (Schiefer et al., 2004; Reiner et al., 2012), amyotrophic lateral sclerosis (ALS) (Battaglia et al., 2015), Parkinson disease (Battaglia et al., 2003), seizure (Moldrich et al., 2001), and drug abuse (Bossert et al., 2005). Importantly, analogs of LY379268 were found to be safe and well tolerated in a phase 2 clinical trial for patients with schizophrenia (Imre, 2007; Patil et al., 2007). Based on its safety and efficacy in other neurologic and neurodegenerative diseases, we investigate whether targeted activation of mGluR2/3 using LY379268 improves HD symptoms and neuropathology in both male and female zQ175 HD mice. We find that chronic treatment of zQ175 mice with LY379268 improves motor impairment and reduces mHTT aggregate pathology in both male and female heterozygous zQ175 mice. However, we find that LY379268 activates and inactivates divergent cell signaling pathways in male and female zQ175 mice. Our findings highlight the therapeutic potential of activating mGluR2/3 in HD and further implicate the importance of investigating sex as a biologic variable in preclinical disease-modifying studies (Shansky and Murphy, 2021).

Materials and Methods

Reagents. (1R,4R,5S,6R)-4-Amino-2-oxabicyclo [3.1.0] hexane-4,6-dicarboxylic acid (LY379268; 2453) was purchased from Cedarlane (Burlington, Canada). Horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G secondary antibody (G21234), HRP-conjugated anti-mouse secondary (G21040), and rabbit anti-ERK1/2 (61-7400) were from Thermo Fisher Scientific (Waltham, MA). Rabbit anti–phospho-p44/42 ERK1/2 (T202/Y2204, 9101S), anti–phospho-GSK3β (S9, 9323s), anti–phospho-Akt (S473, 9271S), and mouse anti-GSK3β (9832S) and anti-Akt (2920S) were from Cell Signaling Technology (Danvers, MA). Mouse anti-p62 (ab56416), anti-vinculin (ab29002), and rabbit anti-ZBTB16 (ab39354), and anti-Iba1 (ab178847) were from Abcam (Cambridge, MA). Rabbit anti-ATG14 (PD026) was from Cedarlane (Burlington, Canada). Mouse anti–neuronal nuclei (NeuN) (ABN78) and anti-Huntingtin (clone mEM48; MAB5374) were from Sigma-Aldrich (St. Louis, USA). Reagents used for Western blotting were purchased from Bio-Rad (Mississauga, Canada). All other biochemical reagents were from Sigma-Aldrich (St. Louis, MO).

Animals. All animal experimental protocols were approved by the University of Ottawa Institutional Animal Care Committee and were in accordance with the Canadian Council of Animal Care guidelines. Animals were caged in groups and housed under a constant 12-hour light/dark cycle, and food and water were given ad libitum. Wild-type and heterozygous zQ175 HD mice carrying ~188 CAG repeat expansions were obtained from the Jackson Laboratory and bred to establish littermate-controlled male and female wild-type (Wt) and heterozygous zQ175 (zQ175) mice. Groups of 24 male and female Wt and zQ175 mice were aged to 12 months, and 12 mice from each group were treated with either saline or LY379268 (3 mg/kg per day dissolved in saline). Both saline and LY379268 were delivered via subcutaneously implanted Alzet Osmotic Pumps (2002), and pumps were replaced once 4 weeks after initial implantation. The drug dose was calculated at the time of pump implantation according to body weight and was based on a dose range that was proven to be tolerable and effective in ALS mice (Battaglia et al., 2015). All groups of mice were tested in a series of behavior experiments after 4 weeks and 8 weeks of drug treatment. At the end of 8 weeks of treatment, mice were sacrificed, and the brains were collected and randomized for biochemical experiments and immunostaining.

Behavioral Analysis. All animals were habituated in the testing room for a minimum of 30 minutes before testing. All behavioral tests were performed blindly and during the animal's dark cycle.

Forelimb Grip Strength. The grip strength of each mouse was measured using the Chatillon DEF II Grip Strength Meter (Columbus Instruments). Mice were held over the grid of the instrument by their

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tails and allowed to firmly grip the bar. The mice were then pulled horizontally away from the bar using constant force and at a speed of ~2.5 cm/s until they released the bar. Each mouse was tested eight times with a break of 5 seconds in between each trial, and the values of maximal peak force were recorded (Abd-Elrahman et al., 2017).

Open Field Test. Mice were individually placed in the bottom-left corner of an opaque and illuminated (~300 lux) open field arena (45 × 45 × 45 cm) and allowed to explore for 10 minutes. Activities of the mice were recorded by an overhead camera connected to a computer in another room. Total distance traveled and velocity were calculated using the Noldus Ethovision software (Abd-Elrahman et al., 2017).

Rotarod Test. Mice were introduced to the Rotarod apparatus (IITC, Life Science Woodlands Hills, CA) by placing them on the Rotarod at rest for 3 minutes on the first day. Four 5-minute-long trials were then performed daily for 2 consecutive days using an accelerating protocol (from 5 to 45 RPM in 300 seconds) with 10 minutes of rest between each trial. Any mice remaining on the Rotarod after 300 seconds were removed, and the time was scored as 300 seconds. Average of the latency to fall obtained from the four trials of the second day was used for analysis (Abd-Elrahman et al., 2017).

Horizontal Ladder Test. The forelimb and hindlimb coordination and placement of the mice were tested using a horizontal ladder. The mice were required to traverse a horizontal ladder with a total of 121 regularly (1 cm apart) and irregularly (0.5–2.5 cm apart) spaced metal rungs (0.15 cm in diameter and 2 cm from the bottom of the wall). The mice were first trained (one trial) and then filmed crossing the ladder for four to five trials using a high-definition camera. The time to finish the task and the number of successful and missed steps during the two best consecutive trials were quantified, and percentage error was calculated (Abd-Elrahman et al., 2017).

Immunoblotting. Each mouse brain was dissected, and striatum was lysed in ice-cold lysis buffer (25 mM HEPES, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% Triton-X) containing protease inhibitor cocktail (100 μM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 2 μM leupeptin, 80 nM aprotinin, 5 μM bestatin, 1.5 μM E-64, and 1 μM pepstatin A) and phosphatase inhibitors (10 mM NaF and 500 μM Na_3VO_4). Tissue debris was pelleted and removed by centrifugation twice at 20,000*g* at 4°C for 10 minutes. Supernatants were collected, and their protein concentrations were measured using Bradford Protein Assay (Bio-Rad). Homogenates were diluted to a protein concentration of 1 μg/μl in a mix of lysis buffer and β-mercaptoethanol–containing 3× loading buffer and then boiled for 10 minutes at 90°C. Aliquots containing 50 μg of total protein were resolved by electrophoresis on 7.5% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). Blots were blocked for 1 hour at room temperature in Tris-buffered saline (pH 7.6) containing 0.05% Tween 20 (TBST) containing 5% nonfat dry milk. Blots were incubated overnight at 4°C with primary antibodies diluted (1:1000) in TBST containing 1% nonfat dry milk. Blots were washed three times (5 minutes per wash) with TBST the next day and incubated with anti-rabbit/mouse secondary antibodies (1:5000) diluted in TBST containing 1% nonfat dry milk for 1 hour at room temperature. Blots were washed again in TBST, and then bands representing our proteins of interest were detected using SuperSignal West Pico PLUS Chemiluminescent Substrate using Bio-Rad chemiluminescence (Abd-Elrahman et al., 2018; Gupta et al., 2019).

Immunohistochemistry. One hemisphere of each brain sample was fixed in 4% paraformaldehyde and then transferred to 70% ethanol for storage at 4°C. The samples were embedded in paraffin and then coronally sectioned through the striatum at a thickness of 5 μm. Sections were then incubated with the mouse monoclonal EM48 antibody at 1:100, NeuN antibody at 1:1500, or Iba1 antibody at 1:8000 dilution for 30 minutes at room temperature and detected using an HRP-conjugated compact polymer system. Slides were then stained using 3,3′-diaminobenzidine as the chromogen, counterstained with hematoxylin, mounted, and cover slipped. Slides were scanned using a Leica Aperio Slide scanner at 20×, and the number of EM-positive aggregates and NeuN- or Iba1-positive cells were counted in representative 900-μm^2 areas of the striatum. Six sections per mouse were analyzed, and for each section, two regions of interest in the striatum were quantified (Abd-Elrahman et al., 2017, 2020b).

Statistical Analysis. Means ± S.E.M. for each independent experiment are shown in the various figure legends. GraphPad Prism 9 software was used to analyze the data for statistical significance. Statistical significance was determined by Student's *t* test or a series of 2 (strain) × 2 (drug treatment) ANOVAs, followed by Fisher's least significant difference comparisons to determine the source of significant interactions. Statistical details of individual experiments are indicated in figure legends.

Results

LY379268 Treatment Rescued Motor Deficits in Both Male and Female zQ175 Mice. To investigate the potential role of presynaptic regulated glutamate release in HD progression and pathology, we first tested whether mGluR2/3 activation would rescue motor deficits in symptomatic heterozygous male and female zQ175 mice. The 12-month-old male and female wild-type and heterozygous zQ175 mice were treated with either saline or the mGluR2/3 agonist LY379268 using implanted osmotic pumps (releasing drug at a rate of 3 mg/kg per day), and their motor and locomotor performance was assessed after 4 weeks (13-month-old) and 8 weeks (14-month-old) of drug treatment. Both saline-treated 13- and 14-month-old male and female zQ175 mice showed significant impairment in forelimb grip strength compared with age- and sex-matched, saline-treated wild-type mice (Fig. 1, A and B). LY379268 treatment of either 4 or 8 weeks resulted in a significant improvement in grip strength in both male and female zQ175 mice when compared with saline-treated counterparts (Fig. 1, A and B). When tested on an accelerating Rotarod, saline-treated male and female zQ175 mice spent less time on the Rotarod when compared with age- and sex-matched wild-type controls (Fig. 1, C and D). LY379268 treatment of both 4 and 8 weeks improved the performance of both male and female zQ175 mice to a level that was comparable to age- and sex-matched, saline-treated wild-types (Fig. 1, C and D). However, LY379268 treatment of either 4 or 8 weeks did not result in a statistically significant improvement in Rotarod behavior in male zQ175 mice when compared with saline-treated male zQ175 mice, although there was a trend toward behavior phenotype improvement that could not be addressed by prolonging drug treatment due to ethical concerns (Fig. 1, C and D). In contrast, LY379268 treatment of female zQ175 mice for either 4 or 8 weeks resulted in a significant improvement in Rotarod performance when compared with saline-treated female zQ175 mice (Fig. 1, C and D).

To further examine the potential effects of LY379268 treatment on motor deficits in zQ175 mice, we examined the performance of our mice in the horizontal ladder task and their locomotor activity in open field. We found that both male and female saline-treated zQ175 mice made significantly more errors in the horizontal ladder rung test than age- and sex-matched wild types (Fig. 2, A and B). However, we found that the treatment of both male and female zQ175 with LY379268 for either 4 or 8 weeks significantly improved limb coordination and error scores in the horizontal ladder rung test when compared with age- and sex-matched, saline-treated wild-type mice (Fig. 2, A and B). We also found that male and female zQ175 mice treated with saline for either 4 or 8 weeks

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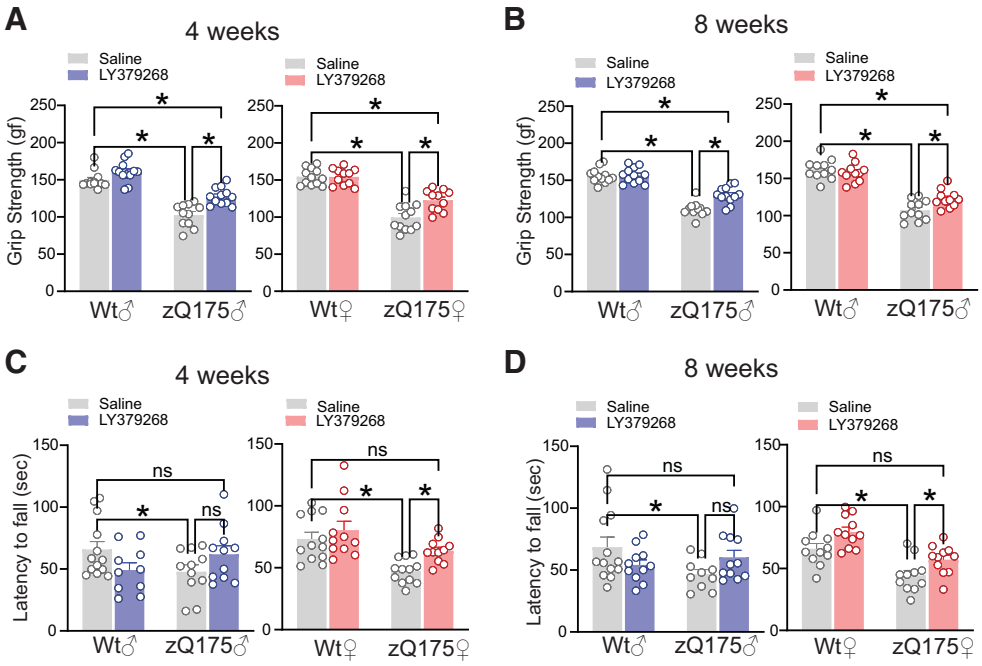

Fig. 1. LY379268 improves grip strength and Rotarod performance in male and female zQ175 mice. Mean ± S.E.M. of grip strength [gram-force (gf)] after 4 weeks (A) and 8 weeks (B) of treatment with saline or LY379268 (3 mg/kg per day subcutaneously via osmotic pump) of 12-month-old zQ175 and Wt male and female mice (n = 11 to 12 for each group). Mean ± S.E.M. of latency to fall (seconds) from accelerating Rotarod after 4 weeks (C) and 8 weeks (D) of treatment with saline or LY379268 of 12-month-old zQ175 and Wt male and female mice (n = 11 to 12 for each group). *$P < 0.05$ by two-way ANOVA and Fisher's least significant difference comparisons. ns, not significant.

exhibited reduced locomotor activity in an open field (reduced velocity) compared with saline-treated age- and sex-matched wild types (Fig. 2, C and D). LY379268 treatment of either 4 or 8 weeks significantly improved locomotor activity of both male and female zQ175 mice when compared with saline-treated, age- and sex-matched zQ175 mice (Fig. 2, C and D). However, locomotor activity of LY379268-treated zQ175 mice remained reduced overall when compared with age- and sex-matched, saline-treated wild types (Fig. 2, C and D). Together, these results indicated that LY379268 activation of mGluR2/3 reversed motor deficits in the majority of motor and locomotor behavior tests examined in both male and female heterozygous zQ175 mice.

LY379268 Treatment Reduced Huntingtin Aggregate Number and Neuronal Loss in Both Male and Female zQ175 Mice. mHTT aggregates represent the key pathological hallmark of HD, and we previously demonstrated that both genetic and pharmacological silencing of mGluR5 reduced the number of mHTT aggregates in Q111 and zQ175 HD mice, respectively (Ribeiro et al., 2014; Abd-Elrahman et al., 2017). This suggested that pathological glutamate signaling contributes to the accelerated mHTT deposition in HD brain. Therefore, we examined whether the improvement in motor deficits in LY379268-treated zQ175 mice was also accompanied by a reduction in the number of mHTT aggregates in the striatum. We focused on the striatum, as it harbors dense glutamatergic corticostriatal inputs with high expression of many glutamate receptors and exhibits the most profound neuropathological deficits in HD (Wüllner et al., 1994). We found that after 8 weeks of LY379268 treatment, the number of mHTT aggregates in the striatum of both male and female zQ175 mice was significantly reduced when compared with sex-matched, saline-treated mice (Fig. 3A). We then tested whether the improvement in motor function and pathology was associated with a rescue in neuronal survival by staining for NeuN. The number of NeuN-positive cells was significantly lower in the striatum of saline-treated male and female zQ175 mice compared with sex-matched, saline-treated wild-type mice (Fig. 3, B and C). We found that the number of NeuN-positive striatal neurons was increased in both LY379268-treated male and female zQ175 mice when compared with sex-matched, saline-treated zQ175 mice, but this difference was more distinguishable in male LY379268-treated zQ175 mice (Fig. 3, B and C). Together, these findings indicated that the improvement in motor function after mGluR2/3 activation in heterozygous zQ175 mice was correlated with a reduction in mHTT deposition and rescue of neuronal loss.

LY379268 Reduced Microglial Activation in Both Male and Female zQ175 Mice. Microglial activation has been shown to contribute to the pathogenesis of several neurodegenerative diseases and was observed in presymptomatic gene carriers and symptomatic patients with HD (Tai et al., 2007; Björkqvist et al., 2008; Perry et al., 2010). Thus, we quantified the number of activated microglia in the striatum of wild-type and zQ175 mice treated with either saline or LY379268 by staining for ionized calcium-binding adapter molecule 1 (Iba1), a protein that specifically identifies activated microglia (Ito et al., 1998). We detected a significant increase in the number of Iba1-positive cells in the striatum of both saline-treated male and female zQ175 mice when compared with sex-matched, saline-treated wild-types (Fig. 4, A and B). LY379268 treatment reduced the number of Iba1-positive cells in both male and female zQ175 striatal tissue when compared with sex-matched, saline-treated zQ175 mice

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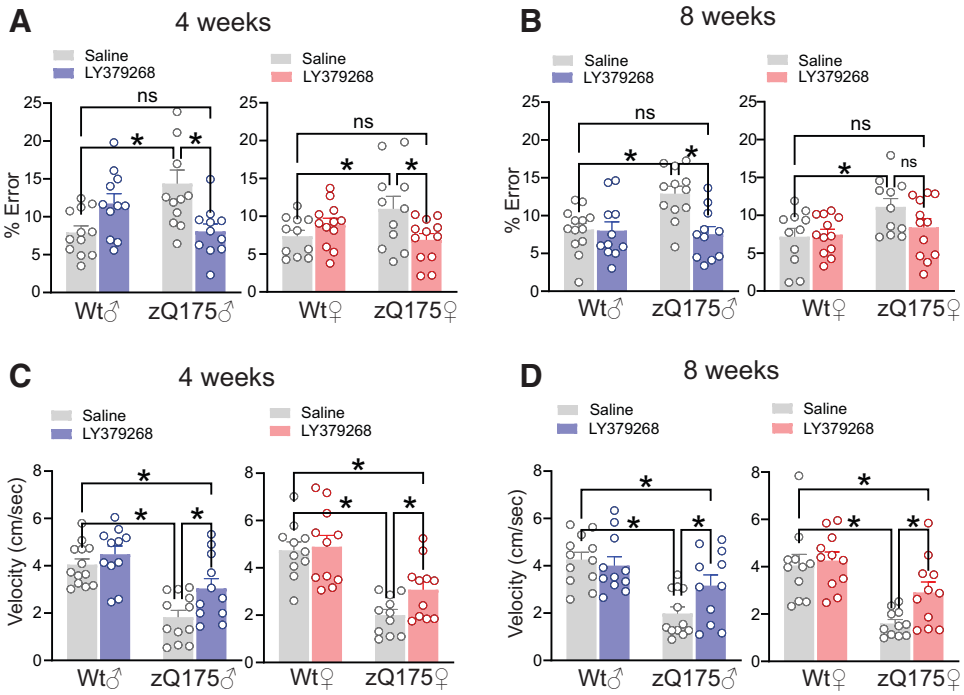

Fig. 2. LY379268 improves locomotor activity and performance on the ladder rung test in male and female zQ175 mice. Mean ± S.E.M. of percent error (% error) in limb placement while completing the horizontal ladder task after 4 weeks (A) and 8 weeks (B) of treatment with saline or LY379268 (3 mg/kg per day subcutaneously via osmotic pump) of 12-month-old zQ175 and Wt male and female mice (n = 11 to 12 for each group). Mean ± S.E.M. of velocity (centimeters per second) in open field arena after 4 weeks (C) and 8 weeks (D) of treatment with saline or LY379268 of 12-month-old zQ175 and Wt male and female mice (n = 11 to 12 for each group). *$P < 0.05$ by two-way ANOVA and Fisher's least significant difference comparisons. ns, not significant.

(Fig. 4, A and B). Thus, mGluR2/3 activation appeared to reduce microglial activation and associated neuroinflammation in both male and female heterozygous zQ175 HD mice, which potentially contributes to improved pathology and symptoms in HD mice of both sexes.

LY379268 Promoted GSK3β/ZBTB16/ATG14 Autophagy in Male but Not Female zQ175 Mice. We previously demonstrated that glutamate-mediated activation of mGluR5 downregulated a novel ZBTB16-dependent autophagic pathway, which may inhibit the removal of proteotoxic aggregates in the brain of HD and Alzheimer's disease mice (Abd-Elrahman et al., 2018, 2017; Abd-Elrahman and Ferguson, 2019; Ibrahim et al., 2021). Specifically, mGluR5 induced the inhibitory phosphorylation of GSK3β at S9 and the expression of ZBTB16, a member of ZBTB16-Cullin3-Roc1 E3-ubiquitin ligase complex. This resulted in the ubiquitination and proteasomal degradation of ATG14 protein and inhibition of neuronal autophagy (Zhang et al., 2015; Abd-Elrahman et al., 2017, 2018; Abd-Elrahman and Ferguson, 2019; Ibrahim et al., 2021). Therefore, we tested whether activating mGluR2/3 which can modulate glutamate release, using LY379268 enhanced the autophagic clearance of mutant huntingtin aggregates via the GSK3β/ZBTB16/ATG14 pathway in both male and female zQ175 mice. In saline-treated male zQ175 mice, we detected a significant increase in GSK3β-pS9 phosphorylation that was not detected in female mice (Fig. 5, A and B). LY379268 treatment of 8 weeks reduced GSK3β-pS9 phosphorylation in male zQ175 mice when compared with saline-treated male zQ175 mice but had no effect on GSK3β-pS9 phosphorylation levels in female zQ175 mice (Fig. 5, A and B). LY379268 treatment for 8 weeks also reduced ZBTB16 protein expression levels in male but not female zQ175 mice compared with sex-matched, saline-treated zQ175 mice (Fig. 5, C and D). In contrast, 8-week LY379268 treatment of male but not female zQ175 mice increased ATG14 expression compared with sex-matched, saline-treated zQ175 mice (Fig. 5, E and F). Consistent with these observation, 8-week LY379268 treatment reduced p62 expression levels in male but not female zQ175 mice when compared with sex-matched, saline-treated zQ175 mice (Fig. 5, G and H). Thus, although LY379268 improved HD pathology in both male and female heterozygous zQ175 mice, it only contributed to the activation of the GSK3β/ZBTB16/ATG14-regulated autophagy in the striatum of male heterozygous zQ175 mice. This suggested that the mechanism(s) by which LY379268 contributed to mitigating HD neuropathology in zQ175 mice was sex-specific and mediated by yet-to-be-defined cell signaling mediators and pathways.

Akt and ERK1/2 Phosphorylation in zQ175 Mice are Altered by LY379268 in a Sex-Dependent Manner. Akt and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) were shown previously to be activated after glutamate-dependent activation of both postsynaptic group I mGluRs and NMDARs (Gines et al., 2003; Rong et al., 2003; Zhou et al., 2009; Ibrahim et al., 2021). Importantly, both Akt and ERK1/2 could phosphorylate GSK3β at S9 to potentially inhibit autophagy via ZBTB16-dependent mechanisms (Stambolic and Woodgett, 1994; Hetman et al., 2002; Beaulieu et al., 2009; Abd-Elrahman and Ferguson, 2019). Thus, we investigated whether sex-specific alterations in GSK3β/ZBTB16/ATG14 autophagy are correlated with alteration in Akt and ERK1/2 phosphorylation in both male and female zQ175 mice.

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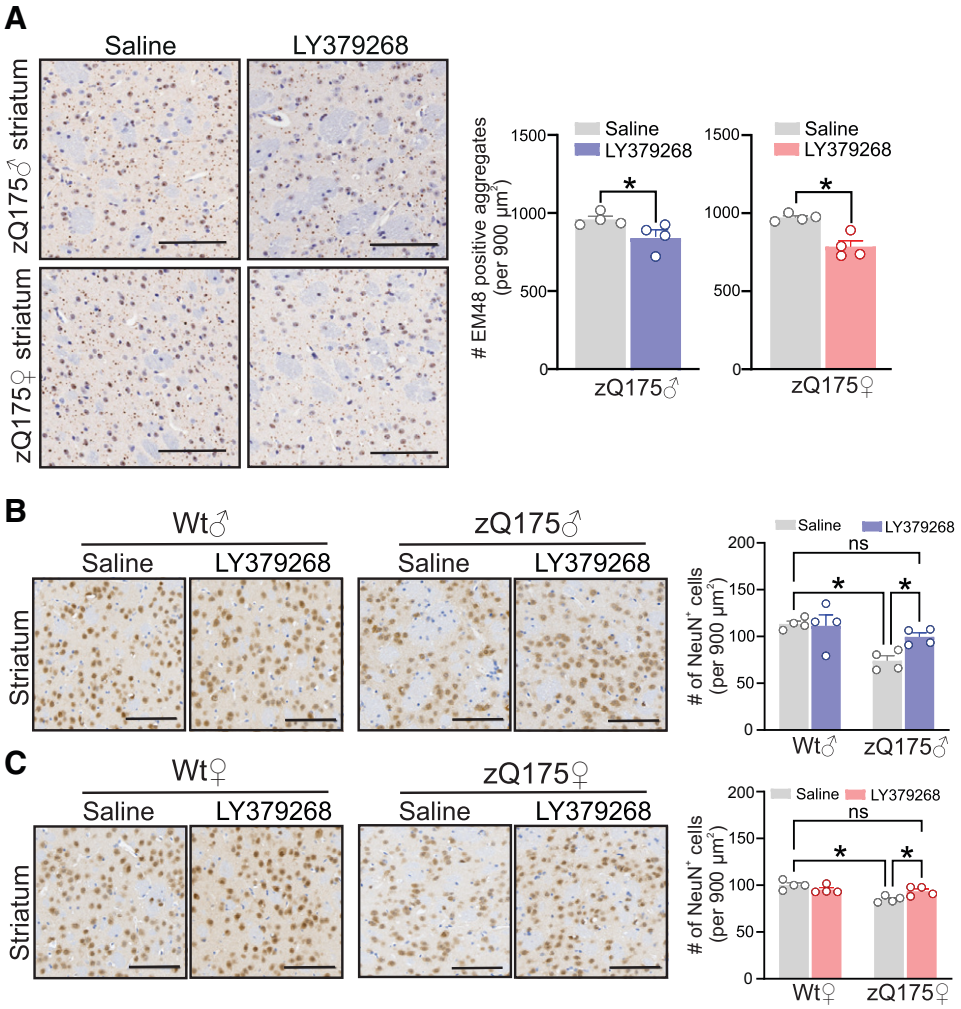

Fig. 3. LY379268 reduces mHTT aggregates and neuronal loss in male and female zQ175 mice. (A) Representative images of staining for mHTT using the EM48 antibody and quantification of the number of mHTT in striatal brain slices from 14-month-old male and female zQ175 mice after 8 weeks of treatment with saline or LY379268 (3 mg/kg per day subcutaneously via osmotic pump). Representative images of staining for NeuN-positive ($NeuN^+$) neurons and quantification of the number of $NeuN^+$ neurons in striatal brain slices from 14-month-old male (B) and female (C) zQ175 and Wt mice after 8 weeks of treatment with saline or LY379268. Scale bars, 100 μm. Data are quantified from two different 900-μm^2 regions of six sections per mouse, and four independent mouse brains from each group were used for analysis and presented as means ± S.E.M. *$P < 0.05$ by Student's t test for EM48 and two-way ANOVA and Fisher's least significant difference comparisons for NeuN. ns, not significant.

We found that Akt-pS473 and ERK1/2-PT202/Y204 phosphorylation were significantly increased in male, but not female, saline-treated zQ175 mice (Fig. 6, A–D). LY379268 treatment of male zQ175 mice restored Akt-pS473 and ERK1/2-pT202/Y204 phosphorylation to saline-treated male wild-type levels (Fig. 6, A and C). In contrast, Akt phosphorylation was not different and ERK1/2 phosphorylation was significantly lower in saline-treated female zQ175 striatum when compared with saline-treated female wild-type mice (Fig. 6, B and D). Unlike what we observed in male mice, LY379268 treatment enhanced Akt and ERK1/2 phosphorylation in the striatum of female zQ175 mice when compared with saline-treated female zQ175 striatum (Fig. 6, B and D). Thus, it was evident mGluR2/3 activation triggered sex-specific differences in the activation of both the ERK1/2 and Akt signaling pathways in heterozygous zQ175 mice, despite resulting in similar behavioral and pathologic outcomes in both sexes.

Discussion

Glutamate plays a key role in the pathophysiology of HD, and both the genetic and pharmacological silencing of one postsynaptic glutamate receptor, mGluR5, are able to halt disease progression and mitigate mHTT pathology in two HD mouse models (Ribeiro et al., 2014; Abd-Elrahman et al., 2017). However, glutamate triggers excitotoxicity in HD brain via other glutamate receptors, such as NMDARs and, potentially, mGluR1 (Heng et al., 2009; Ribeiro et al., 2011). Therefore, we investigated whether activating presynaptic mGluR2/3 might represent an effective disease-modifying approach to slow HD progression. Our results indicate that the activation of mGluR2/3 using the highly selective agonist LY379268 to reduce glutamate release improves overall motor deficits in both male and female heterozygous zQ175 HD mice. We also show that mGluR2/3 agonist reduces mHTT aggregates, microglial activation, and neuronal loss in the striatum of

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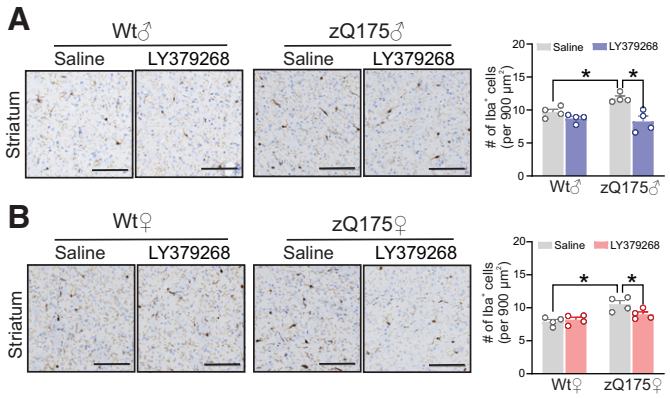

Fig. 4. LY379268 reduces microglial activation in heterozygous male and female zQ175 mice. Representative images of staining for microglia using Iba1 antibody and quantification of the number of Iba1-positive ($Iba1^+$) cells in striatal brain slices from 14-month-old male (A) and female (B) zQ175 and Wt mice after 8 weeks of treatment with either saline or LY379268 (3 mg/kg per day subcutaneously via osmotic pump). Scale bars, 100 μm. Data are quantified from two different 900-μm^2 regions of six sections per mouse, and four independent mouse brains from each group were used for analysis and presented as means ± S.E.M. *$P < 0.05$ by two-way ANOVA and Fisher's least significant difference comparisons.

heterozygous zQ175 HD mice of both sexes. Interestingly, mGluR2/3 agonist-induced improvement in HD neuropathology was likely mediated by distinct cell signaling/receptor-dependent mechanisms in male and female heterozygous zQ175 HD mice.

Previous studies have demonstrated that LY379268 at the maximum tolerated dose (20 mg/kg per day) improves the survival and motor deficits in the R6/2 model of HD and that a lower dose of LY379268 (1.2 mg/kg per day) improves survival and motor function in R6/2 mice without reducing the formation of intranuclear mHTT aggregates (Schiefer et al., 2004; Reiner et al., 2012). For our studies, we chose the heterozygous zQ175 model, as it presents with less aggressive phenotype compared with the R6/2 model but recapitulates the slow and progressive manifestations of HD in humans, such as accumulation of mHTT aggregates in striatal and cortical neurons, neuronal loss, and motor impairments (Heikkinen et al., 2012; Menalled et al., 2012; Smith et al., 2014). We delivered LY379268 at a dose of 3 mg/kg per day via osmotic pumps since a similar treatment paradigm (1–5 mg/kg per day for 4 weeks) has proven to improve motor deficits, survival, and more importantly pathology in SOD1G93A mouse model of ALS (Battaglia et al., 2015). We find that prolonged administration of LY379268 for either 4 or 8 weeks results in a significant reduction in motor/locomotor impairment, striatal neuron death, and mHTT aggregate formation in both male and female zQ175 mice at 12 months of age. This correlates with previously published work from our group and others reporting similar motor deficits at this age in this mouse model (Menalled et al., 2012; Smith et al., 2014; Abd-Elrahman et al., 2017). We also find a significant impairment in forelimb grip force in both male and female zQ175 mice, a motor deficit that we have previously detected for male zQ175 mice and was reported in patients with HD (Reilmann et al., 2001; Menalled et al., 2012; Abd-Elrahman et al., 2017).

Evidence indicates that activated microglia in the brains of presymptomatic, symptomatic, and post-mortem patients with HD along with elevated proinflammatory cytokines contributes to HD pathology (Tai et al., 2007; Björkqvist et al., 2008; Silvestroni et al., 2009). Similarly, we also detect an increase in the number of microglia in the striatum of both male and female zQ175 mice. LY379268 -mediated activation of mGluR2/3 reduces the number of activated microglia in male and female zQ175 mice to levels that are observed in wild-type mice. It is worth noting that microglia express mGluR2/3, and when microglia are activated, they release glutamate, which may contribute to the exacerbation of neuronal excitotoxicity (Barger et al., 2007; Garaschuk and Verkhratsky, 2019). Moreover, impaired glutamate uptake by the glial glutamate transporter-1 was reported in HD mice (Behrens et al., 2002; Huang et al., 2010). Therefore, mGluR2/3 agonist treatment may represent an effective approach to reduce glutamate overspill from microglia to prevent the activation of excitotoxic glutamate receptor signaling in both microglia and neurons, thereby interrupting key cellular mechanisms involved in HD pathophysiology.

Defects in autophagy, a catabolic process responsible for clearing toxic cellular cargos and protein aggregates, have been implicated in the pathophysiology of HD, and it is thought that this potentially exacerbates the deposition of mHTT in the brain (Li and Li, 2004; Cortes and La Spada, 2014; Abd-Elrahman et al., 2017; Croce and Yamamoto, 2019). We previously demonstrated that the improvement in motor and cognitive function in zQ175 mice after chronic mGluR5 blockade was dependent on the activation of ZBTB16-dependent autophagy to enhance clearance of mHTT aggregates (Abd-Elrahman et al., 2017; Abd-Elrahman and Ferguson, 2019). In fact, pathologic glutamate signaling via mGluR5 induced an inhibitory phosphorylation of GSK3β that leads to ubiquitin-mediated degradation of the autophagy adaptor ATG14 via the ZBTB16-Cullin3-Roc1 E3-ubiquitin ligase complex (Zhang et al., 2015; Abd-Elrahman et al., 2017; Ibrahim et al., 2021). Here, we show that LY379268-mediated activation of mGluR2/3 in male Q175 mice also reduces the inhibitory Ser-9 phosphorylation of GSK3β and ZBTB16 expression in the striatum, which is accompanied by a rescue in ATG14 expression and induction of autophagy, as reflected by a reduction in p62 protein expression. Thus, it is likely that mGluR2/3 activation reduces synaptic glutamate, leading to a reduction in pathologic postsynaptic glutamate signaling, resulting in the activation of autophagy and improved HD neuropathology in male zQ175 mice. However, we do not detect any changes in the GSK3β/ZBTB16/ATG14 pathway in either saline- or LY379268-treated female zQ175 striatum, which is consistent with our previous studies in AD mice, in which we did not detect engagement of these cell signaling pathways in female mice (Abd-Elrahman, et al., 2020a). Indeed, we showed that, unlike males, GSK3β/ZBTB16/ATG14 pathway is not altered in female AD mice, and hence, mGluR5 inhibition can only reactivate autophagy in male AD brain (Abd-Elrahman, et al., 2020a). Here, we report a very similar sex-specific change in GSK3β/ZBTB16/ATG14 pathway in HD mice after treatment with LY379268, suggesting that the favorable outcomes of mGluR2/3 activation in HD mice are dependent at least in part on reducing postsynaptic mGluR5 signaling. It also shows that the reduction in mHTT aggregates in female zQ175 mice after mGluR2/3 activation is mediated via yet-to-be-identified cell signaling mechanism(s) that do not involve the GSK3β/ZBTB16/ATG14 autophagy pathway. This finding further supports a sex-specific contribution of pathologic glutamate

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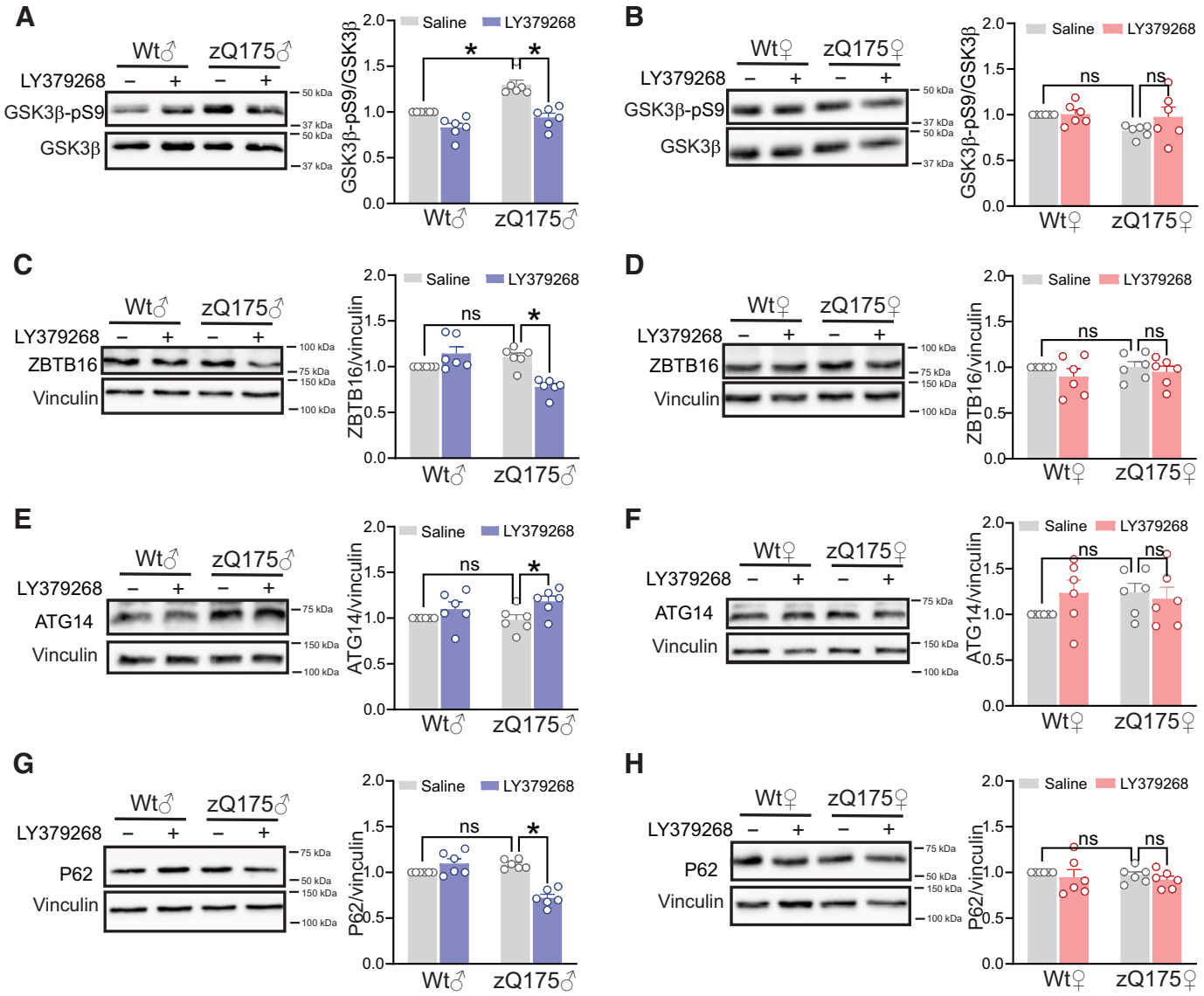

Fig. 5. LY379268 activates the GSK3β/ZBTB16/ATG14 autophagy pathway in heterozygous male but not female zQ175 mice. Representative immunoblots and quantification of GSK3β-pS9 with total GSK3β as the loading control in striatal lysates from 14-month-old male (A) and female (B) zQ175 and Wt mice after 8 weeks of treatment with either saline or LY379268 (3 mg/kg per day subcutaneously via osmotic pump). Representative immunoblots and quantification of ZBTB16 [(C) male and (D) female], ATG14 [(E) male and (F) female], and p62 [(G) male and (H) female] with vinculin as the loading control in striatal lysates from 14-month-old zQ175 and Wt mice after 8 weeks of treatment with either saline or LY379268. Quantification is presented as means ± S.E.M. of fold change in GSK3β-pS9, ZBTB16, ATG14, and p62 band intensity relative to corresponding saline-treated Wt values (n = 6). *$P < 0.05$ by two-way ANOVA and Fisher's least significant difference comparisons. ns, not significant.

signaling to the neuropathology of many neurodegenerative diseases.

Although it remains less clear how mGluR5 directly regulates GSK3β phosphorylation, the most plausible candidates are Akt and ERK1/2 pathways. NMDAR activation is known to trigger both Akt and ERK1/2-dependent signaling in neurons (Gines et al., 2003; Zhou et al., 2009). Additionally, agonist-dependent activation of group I mGluRs enhances Akt signaling in response to phosphoinositide 3-kinase activation (Rong et al., 2003; Guhan and Lu, 2004) and also triggers phosphorylation of ERK1/2 (Eng et al., 2016; Stoppel et al., 2017; Ibrahim et al., 2021). Both Akt and ERK1/2 are known to phosphorylate GSK3β at S9 and inhibit its catalytic activity (Stambolic and Woodgett, 1994; Hetman et al., 2002; Beaulieu et al., 2009). We detect a significant increase in Akt and ERK1/2 phosphorylation in the striatum of male zQ175 mice that may be attributable to exacerbated glutamate signaling. This is consistent with the enhancement of ERK1/2 and Akt phosphorylation we have previously observed in male homozygous zQ175 brain samples (Abd-Elrahman et al., 2017; Abd-Elrahman and Ferguson, 2019). Similar to what we observe after mGluR5 antagonism, LY379268-mediated activation of mGluR2/3 restores Akt and ERK1/2 phosphorylation in male zQ175 mice.

In female zQ175 mice, ERK1/2 and Akt signaling are not altered, providing an explanation for the lack of change in GSK3β phosphorylation or ZBTB16-dependent autophagy. Rather, ERK1/2 signaling is reduced in saline-treated female zQ175 mice, and LY379268 enhances the phosphorylation of Akt and ERK1/2 in the striatum of female zQ175 mice. Interestingly, mGluR2/3 is known to activate ERK1/2 and

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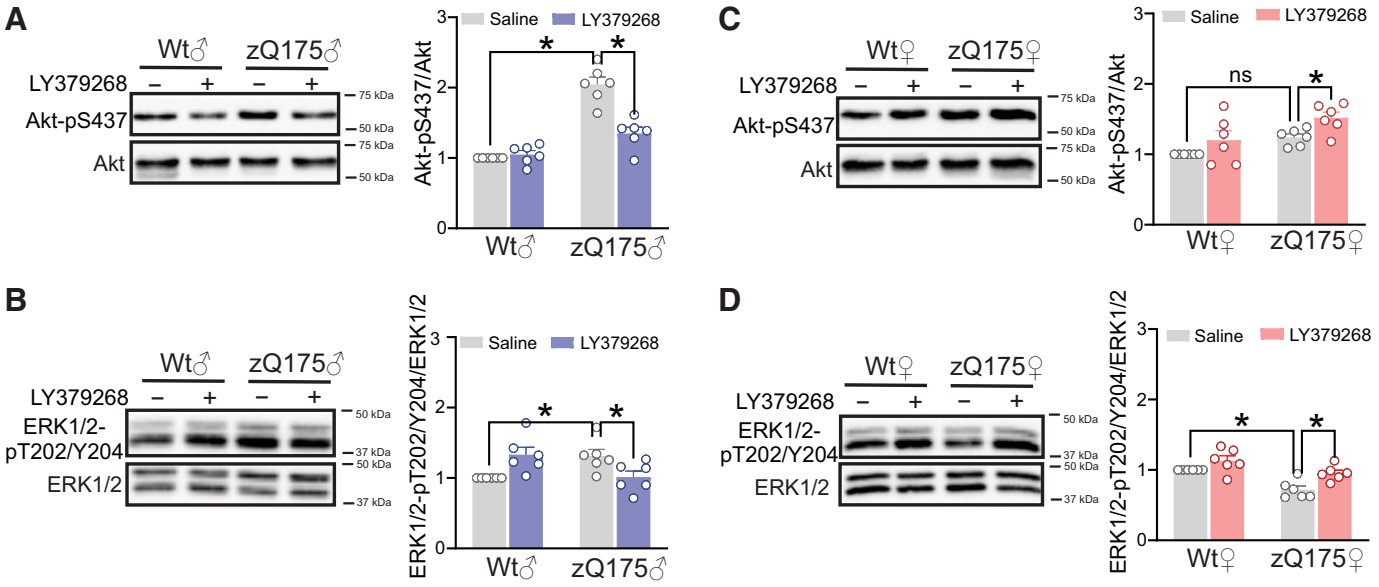

Fig. 6. LY379268 alters Akt and ERK1/2 phosphorylation in heterozygous zQ175 mice in a sex-selective manner. Representative immunoblots and quantification of Akt-pS437 with corresponding Akt as the loading control in striatal lysates from 14-month-old male (A) and female (C) zQ175 and Wt mice after 8 weeks of treatment with saline or LY379268 (3 mg/kg per day subcutaneously via osmotic pump). Representative immunoblots and quantification of ERK1/2-pT202/Y204 with corresponding ERK1/2 as the loading control in striatal lysates from 14-month-old male (B) and female (D) zQ175 and Wt mice after 8 weeks of treatment with either saline or LY379268. Quantification is presented as means ± S.E.M. of fold change in Akt-pS437 and ERK1/2-pT202/Y204 band intensity relative to corresponding saline-treated Wt values ($n = 6$). *$P < 0.05$ by two-way ANOVA and Fisher's least significant difference comparisons. ns, not significant.

phosphoinositide 3-kinase, and it is possible that these signaling mechanisms are only activated in female zQ175 mice after treatment with LY379268 since ERK1/2 and Akt are already activated in male zQ175 mice (Lin et al., 2014). Thus, it is possible that LY379268 supports neuronal survival and differentiation in female zQ175 mice by triggering the cell survival mechanisms regulated by Akt and ERK1/2 downstream of mGluR2/3 (Rai et al., 2019). It is also possible that mGluR2/3 activates ERK1/2 and Akt signaling via transactivation of receptor tyrosine kinase, as has been reported for other members of the mGluR family (Wang et al., 2007). Although the underlying molecular basis of sex-specific mGluR2/3 signaling remains unclear, we cannot rule out that mGluR2/3 may heterodimerize with other G protein–coupled receptors in a sex-selective manner to trigger differential cell signaling between sexes (De Bartolomeis et al., 2013).

In conclusion, we demonstrate that mGluR2/3 can be an effective pharmacological target to mitigate motor deficits, reduce mHTT aggregates accumulation, and rescue neuronal cell death in both male and female zQ175 HD mice. We also provide evidence that there are sex-specific differences in cell signaling mechanisms contributing to the pathophysiology of male and female zQ175 HD mice. mGluR2/3 agonists have proven to be safe and effective in clinical trials for schizophrenia, and this study suggests they can be repurposed for the treatment of HD. We also further emphasize the importance of delineating sex-specific differences in the pathophysiology of all neurodegenerative disease when designing novel approaches for treatment.

Acknowledgments

S.S.G.F holds a Tier I Canada Research Chair in Brain and Mind. S.H.L is the recipient of Ontario Graduate Scholarship. K.S.A.-E. is a Lecturer at the Department of Pharmacology & Toxicology, Faculty of Pharmacy, University of Alexandria, Egypt, and is supported by clinician postdoctoral fellowship from the Alberta Innovates Health Solutions and Canadian Institutes for Health Research. The authors thank Shaunessy Hutchinson for breeding the animals and Behavior and Physiology Core at the University of Ottawa for their assistance. Graphical abstract was created using Biorender.com.

Authorship Contributions

Participated in research design: Li, Abd-Elrahman, Ferguson.
Conducted experiments: Li, Colson, Abd-Elrahman.
Performed data analysis: Li, Abd-Elrahman.
Wrote or contributed to the writing of the manuscript: Li, Abd-Elrahman, Ferguson.

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