

mGluR2/3 Activation Improves Motor Performance and Reduces Pathology in heterozygous zQ175 Huntington's Disease Mice

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List of abbreviations: Akt, protein kinase B; ATG14, autophagy related protein 14; ERK1/2, extracellular signal-regulated kinase 1/2; GSK3 β , glycogen synthase kinase 3 β ; HD, Huntington's disease; Iba1, Ionized calcium binding adaptor molecule 1; mGluR, metabotropic glutamate receptor; mHtt, mutant huntingtin; NMDAR, N-methyl-D-aspartate receptor; NeuN, neuronal nuclear protein; ZBTB16, zinc finger and BTB domain-containing protein 16

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Abstract

Huntington's Disease (HD) is an autosomal dominant neurodegenerative disease that leads to progressive motor impairment with no available disease-modifying treatments. 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 knock-in 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 microglia activation in the striatum of both male and female zQ175 mice. The reduction in mutant huntingtin protein correlates with the activation of a GSK3 β -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.

Keywords: Glutamate, mGluR, Sex, Autophagy, Huntingtin, Neurodegeneration, GPCR

Significance statement: The mGluR2/3 agonist LY379268 improves motor impairments and reduces pathology in male and female zQ175 Huntington's mice. The beneficial outcomes of LY379268 treatment in Huntington's mice were mediated by divergent cell signalling 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 (MSNs) that leads to progressive motor disturbance, cognitive impairment, behaviour 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 correlate 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, 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). More so, intra-striatal injection of glutamate and its analogue 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 MSN seen in HD (Beal *et al.*, 1991). Furthermore, genetic deletion of metabotropic glutamate receptor 5 (mGluR5), a heterotrimeric G protein-coupled receptor (GPCR) highly expressed in the striatum, in a Q111 knock-in 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 in male zQ175 knock-in (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.

Metabotropic glutamate receptors 2 and 3 (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 NMDA-induced neuronal death *in vitro* (Buisson, 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 the 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 and Huntington's diseases (Padovan-Neto *et al.*, 2019; Abd-Elrahman, Albaker, *et al.*, 2020). 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 towards 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 μ M, but showed weak agonist activity towards mGluR6 at high nanomolar concentrations (Monn *et al.*, 1999). LY379268 has also shown effectiveness in animal models of Huntington's disease (Schiefer *et al.*, 2004; Reiner *et al.*, 2012), amyotrophic lateral sclerosis (ALS) (Battaglia *et al.*, 2015), Parkinson's disease (Battaglia *et al.*, 2003), seizure (Moldrich *et al.*, 2001) and drug abuse (Bossert *et al.*, 2005). Importantly, analogues of LY379268 were found to be safe and well-tolerated in a phase-2 clinical trial for schizophrenia patients (Imre, 2007; Patil *et al.*, 2007). Based on the safety and efficacy in other neurological 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 biological 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 Thermofisher Scientific (Waltham, USA). Rabbit anti-phospho-p44/42 ERK1/2 (T202/Y2204, 9101S), anti-phospho-GSK3 β (S9, 9323s), anti-phospho-Akt (S473, 9271S) and mouse anti-GSK3 β (9832S), anti-Akt (2920S) were from Cell Signalling Technology (Danvers, USA). Mouse anti-p62 (ab56416), anti-vinculin (ab29002) and rabbit anti-ZBTB16 (ab39354), anti-mGluR2/3 (ab6438) and anti-Iba1 (ab178847) were from Abcam (Cambridge, USA). Rabbit anti-ATG14 (PD026) was from Cedarlane (Burlington, Canada). Mouse anti-NeuN (ABN78) and anti-Huntingtin (clone mEM48; MAB5374) were from Sigma-Aldrich (St. Louis, USA). Rabbit anti- β -Tubulin (T2200) was from Sigma-Aldrich. Reagents used for Western blotting were purchased from Bio-Rad (Mississauga, Canada). All other biochemical reagents were from Sigma-Aldrich (St. Louis, USA).

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 group caged 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 repeats 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 of age and 12 mice from each group were treated with either saline or LY379268 (3mg/kg/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 is based on a dose range that was proven to be tolerable and effective in amyotrophic lateral sclerosis (ALS) mice (Battaglia *et al.*, 2015). All groups of mice were tested in a series of behaviour experiments after 4 weeks and 8 weeks of drug treatments. At the end of 8 weeks of treatment, mice were sacrificed, and the brains were collected and randomized for biochemical experiments and immunostaining.

Behavioural analysis

All animals were habituated in the testing room for a minimum of 30 minutes before testing. All behavioural 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 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 ~2.5cm/s until they released the bar. Each mouse was tested 8 times with a break of 5s 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 (45cm X 45cm X 45cm) and allowed to explore for 10 min. Activity of the mice were recorded by an overhead camera connected to a computer in another room. Total distance travelled and velocity were calculated using the Noldus Ethovision software (Abd-Elrahman *et al.*, 2017).

Rotarod test

Mice were introduced to the rotarod apparatus (IITC, Woodlands Hills, CA, USA) by placing them on the rotarod at rest for 3 minutes on the first day. Four 5-min-long trails were

then performed daily for two 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 scored as 300s. 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 (1 trial) and then filmed crossing the ladder for 4-5 trials using 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

Mouse brain was dissected, and striatum was lysed in ice-cold lysis buffer [25 mM HEPES, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% Triton-X] containing protease inhibitors cocktail (100 μM AEBSF, 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 Na₃VO₄). Tissue debris was pelleted and removed by centrifugation twice at 20,000 xg 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 3X 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-buffer saline (pH 7.6) containing 0.05% Tween 20 (TBST) containing 5% non-fat dry milk. Blots were incubated overnight at 4°C with

primary antibodies diluted (1:1000) in TBST containing 1% non-fat dry milk. Blots were washed 3 times (5min/wash) with TBST the next day and incubated with anti-rabbit/mouse secondary antibodies (1:5000) diluted in TBST containing 1% non-fat 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, Neuronal Nuclei (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 (DAB) as the chromogen, counterstained with Hematoxylin, mounted and cover slipped. Slide were scanned using a Leica Aperio Slide scanner at 20x and the number of EM positive aggregates, NeuN or Iba1 positive cells were counted in representative 900 µm² areas of the striatum. Six sections per mouse were analyzed and for each section 2 ROIs in the striatum were quantified (Abd-Elrahman *et al.*, 2017; Abd-Elrahman, Hamilton, *et al.*, 2020) .

Statistical Analysis

Means ± SEM for each independent experiment is 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) Analysis of Variance (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 pre-synaptic 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. Twelve-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 3mg/kg/day) and their motor and locomotor performance were 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 fore limb grip strength compared to age- and sex-matched, saline-treated wild-type mice (Fig. 1A and 1B). LY379268 treatment for 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. 1A and 1B). When tested on an accelerating rotarod, saline-treated male and female zQ175 mice spent less time on the rotarod when compared to age- and sex-matched wild-type controls (Fig.1C and 1D). LY379268 treatment for 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. 1C and 1D). However, LY379268 treatment for either 4 or 8 weeks did not result in a statistically significant improvement in rotarod behavior in male zQ175 mice when compared to saline-treated male zQ175 mice, although there was a trend towards behavior phenotype improvement that could not be addressed by prolonging drug treatment due to ethical concerns (Fig.1C and 1D). In contrast, LY379268 treatment of female zQ175 mice for either 4 or 8 weeks resulted in a significant improvement in rotarod performance when compared to saline-treated female zQ175 mice (Fig.1C and 1D).

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. 2A and 2B). 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. 2A and 2B). We also found that male and female zQ175 mice treated with saline for either 4 or 8 weeks exhibited reduced locomotor activity in an open field (reduced velocity) compared to saline-treated age- and sex-matched wild-types (Fig. 2C and 2D). LY379268 treatment for 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. 2C and 2D). However, locomotor activity of LY379268-treated zQ175 mice remained reduced overall when compared with age- and sex-matched, saline-treated wild-types (Fig. 2C and 2D). 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 contributed 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 harbours 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 following 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 neuronal nuclei (NeuN). The number of NeuN-positive cells was significantly lower in the striatum of saline-treated male and female zQ175 mice compared to sex-matched, saline-treated wild-type mice (Fig. 3B and 3C). 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. 3B and 3C). Together, these findings indicated that the improvement in motor function following mGluR2/3 activation in heterozygous zQ175 mice was correlated with a reduction in mHTT deposition and rescue in neuronal loss.

LY379268 reduced microglial activation in both male and female zQ175 mice

Microglia activation has been shown to contribute to the pathogenesis of several neurodegenerative diseases and was observed in pre-symptomatic gene carriers and symptomatic HD patients (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. 4A and 4B). 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 (Fig. 4A and 4B). Thus, mGluR2/3 activation appeared to reduce microglia activation and associated neuroinflammation in both male and female heterozygous zQ175 HD mice which potentially contribute to improved pathology and symptoms in HD mice of both sexes.

LY379268 promoted GSK3 β /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 AD mice (Abd-Elrahman et al., 2018, 2017; Abd-Elrahman and Ferguson, 2019; Ibrahim et al., 2021). Specifically, mGluR5 induced the inhibitory phosphorylation of GSK3 β at S9 and the expression of Zinc finger and BTB domain-containing protein 16 (ZBTB16), a member of ZBTB16-Cullin3-Roc1 E3-ubiquitin ligase complex. This resulted in the ubiquitination and proteasomal degradation of the autophagy related 14 (ATG14) protein and inhibition of neuronal autophagy (Abd-Elrahman et al., 2018, 2017; Abd-Elrahman and Ferguson, 2019; Ibrahim et al., 2021; Zhang et al., 2015). Therefore, we tested whether activating mGluR2/3 that can modulate glutamate release using LY379268 enhanced the autophagic clearance of mutant huntingtin aggregates via the GSK3 β /ZBTB16/ATG14 pathway in both male and female zQ175 mice. In saline-treated male zQ175 mice, we detected a significant increase in GSK3 β -pS9 phosphorylation that was not detected in female mice (Fig. 5A and 5B). LY379268 treatment for 8 weeks reduced GSK3 β -pS9 phosphorylation in male zQ175 mice, when compared with saline-treated male zQ175 mice, but had no effect on GSK3 β -pS9 phosphorylation levels in female zQ175 mice (Fig. 5A and 5B). Eight-week LY379268 treatment also reduced ZBTB16 protein expression levels in male but not female zQ175 mice compared to sex-matched saline-treated zQ175 mice (Fig. 5C and 5D). In contrast, 8-week LY379268 treatment of male but not female zQ175 mice increased ATG14 expression

compared to sex-matched saline-treated zQ175 mice (Fig. 5E and 5F). 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. 5G and 5H). Thus, although LY379268 improved HD pathology in both male and female heterozygous zQ175 mice, it only contributed to the activation of the GSK3 β /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 is altered by LY379268 in a sex-dependent manner

Protein kinase B (Akt) and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) were shown previously to be activated following glutamate-dependent activation of both postsynaptic group I mGluRs and NMDARs (Gines et al., 2003; Ibrahim et al., 2021; Rong et al., 2003; Zhou et al., 2009). Importantly, both Akt and ERK1/2 could phosphorylate GSK3 β 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 GSK3 β /ZBTB16/ATG14 autophagy are correlated with alteration in Akt and ERK1/2 phosphorylation in both male and female zQ175 mice. We found that Akt-pS473 and ERK1/2-PT202/Y204 phosphorylation were significantly increased in male, but not female, saline-treated zQ175 mice (Fig. 6A-D). LY379268 treatment of male zQ175 mice restored Akt-pS473 and ERK1/2-pT202/Y204 phosphorylation to saline-treated male wild-type levels (Fig. 6A and 6C). In contrast, Akt phosphorylation was not different and ERK1/2 phosphorylation was significantly lower in saline-treated female zQ175 striatum when compared to saline-treated female wild-type mice (Fig. 6B and 6D). Unlike what we observed in male mice, LY379268 treatment enhanced Akt and ERK1/2 phosphorylation in the

striatum of female zQ175 mice when compared to saline-treated female zQ175 striatum (Fig. 6B and 6D). 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 pathological 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, is 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, microglia activation and neuronal loss in the striatum of 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/day) improves the survival and motor deficits in the R6/2 model of HD and a lower dose of LY379268 (1.2 mg/kg/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 to 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/day via osmotic pumps since a similar treatment paradigm (1-5 mg/kg/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 fore limb 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 HD patients (Reilmann *et al.*, 2001; Menalled *et al.*, 2012; Abd-Elrahman *et al.*, 2017).

Evidence indicates that activated microglia in the brains of pre-symptomatic, symptomatic and post-mortem HD patients 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 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 releases glutamate that may contribute to the exacerbation of neuronal excitotoxicity (Barger *et al.*, 2007; Garaschuk and Verkhratsky, 2019). More so, impaired glutamate uptake by of 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 following 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, pathological glutamate signaling via mGluR5 induced an inhibitory phosphorylation of GSK3 β that lead 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 showed that LY379268-mediated activation of mGluR2/3 in male Q175 mice also reduced inhibitory Ser-9 phosphorylation of GSK3 β 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 was likely that mGluR2/3 activation reduces synaptic glutamate leading to a reduction in pathological post-synaptic glutamate signaling resulting in the activation of autophagy and improved HD neuropathology in male zQ175 mice. However, we did not detect any changes in the GSK3 β /ZBTB16/ATG14 pathway in either saline or LY379268-treated female zQ175 striatum, which was consistent with our previous studies in AD mice where we did not detect engagement of these cell signaling pathways in female mice (Abd-Elrahman, Albaker, *et al.*, 2020). Indeed, we showed that unlike males, GSK3 β /ZBTB16/ATG14 pathway is not altered in female AD mice and hence, mGluR5 inhibition can only reactivate autophagy in male AD brain (Abd-Elrahman, Albaker, *et al.*, 2020). Here we report a very similar sex-specific change in GSK3 β /ZBTB16/ATG14 pathway in HD mice following 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 following mGluR2/3 activation was mediated via, yet to be identified, cell signaling mechanism(s) that do not involve the GSK3 β /ZBTB16/ATG14 autophagy pathway.

This finding further supports a sex-specific contribution of pathological glutamate signaling to the neuropathology of many neurodegenerative diseases.

While it remains less clear how mGluR5 directly regulates GSK3 β 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 (PI3K) activation (Rong *et al.*, 2003; Guhan and Lu, 2004) and also triggers phosphorylation of ERK1/2 (Eng *et al.*, 2016; Ibrahim *et al.*, 2021; Stoppel *et al.*, 2017). Both Akt and ERK1/2 are known to phosphorylate GSK3 β 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 following 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 GSK3 β phosphorylation or ZBTB16-dependent autophagy. Rather, ERK1/2 signaling was reduced in saline-treated female zQ175 mice and LY379268 enhanced the phosphorylation of Akt and ERK1/2 in the striatum of female zQ175 mice. Interestingly, mGluR2/3 is known to activate ERK1/2 and PI3K and it is possible that these signaling mechanisms are only activated in female zQ175 mice after treatment with LY379268 since ERK1/2 and Akt were 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). While the underlying molecular basis of sex-specific mGluR2/3 signaling remains unclear, we can not rule out that mGluR2/3 may heterodimerize with other GPCRs 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 difference in the pathophysiology of all neurodegenerative disease when designing novel approaches for treatment.

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Figure 1. LY379268 improves grip strength and rotarod performance in male and female zQ175 mice.

Mean \pm SEM of grip strength [gram-force (gf)] after 4 weeks **(A)** and 8 weeks **(B)** of treatment with saline or LY379268 (3mg/kg/day subcutaneously via osmotic pump) of 12-month-old zQ175 and wild-type (Wt) male and female mice (n= 11-12 for each group). Mean \pm SEM of latency to fall (sec) 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= 11-12 for each group). * $P < 0.05$ by two-way ANOVA and Fisher's least significant difference (LSD) comparisons.

Figure 2. LY379268 improves locomotor activity and performance on the ladder rung test in male and female zQ175 mice.

Mean \pm SEM 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 (3mg/kg/day subcutaneously via osmotic pump) of 12-month-old zQ175 and wild-type (Wt) male and female mice (n= 11-12 for each group). Mean \pm SEM of velocity (cm/sec) 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= 11-12 for each group). * $P < 0.05$ by two-way ANOVA and Fisher's least significant difference (LSD) comparisons.

Figure 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 following 8 weeks of treatment with saline or LY379268 (3mg/kg/day subcutaneously via osmotic pump). Representative images of staining for neuronal nuclei (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 following 8 weeks of treatment with saline or LY379268. Scale bars is 100µm. Data are quantified from two different 900µm² regions of 6 sections per mouse and four independent mouse brains from each group were used for analysis and presented as mean ± SEM. **P* < 0.05 by Student's t-test for EM48 and two-way ANOVA and Fisher's least significant difference (LSD) comparisons for NeuN.

Figure 4. LY379268 reduces microglia 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 wild-type (Wt) mice following 8 weeks of treatment with either saline or LY379268 (3mg/kg/day subcutaneously via osmotic pump). Scale bars, 100µm. Data are quantified from two different 900µm² regions of 6 sections per mouse and four independent mouse brains from each group were used for analysis and presented as mean ± SEM. **P* < 0.05 by two-way ANOVA and Fisher's least significant difference (LSD) comparisons.

Figure 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 wild-type (Wt)

mice following 8 weeks of treatment with either saline or LY379268 (3mg/kg/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 following 8 weeks of treatment with either saline or LY379268. Quantification is presented as mean \pm SEM of fold change in GSK3 β -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 (LSD) comparisons.

Figure 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 wild-type (Wt) mice following 8 weeks of treatment with saline or LY379268 (3mg/kg/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 following 8 weeks of treatment with either saline or LY379268. Quantification is presented as mean \pm SEM of fold change in Akt-pS437 and ERK1/2-pT202/Y204 band intensity relative to corresponding saline-treated Wt values (n = 6). Data. **P* < 0.05 by two-way ANOVA and Fisher's least significant difference (LSD) comparisons.

Figure 1

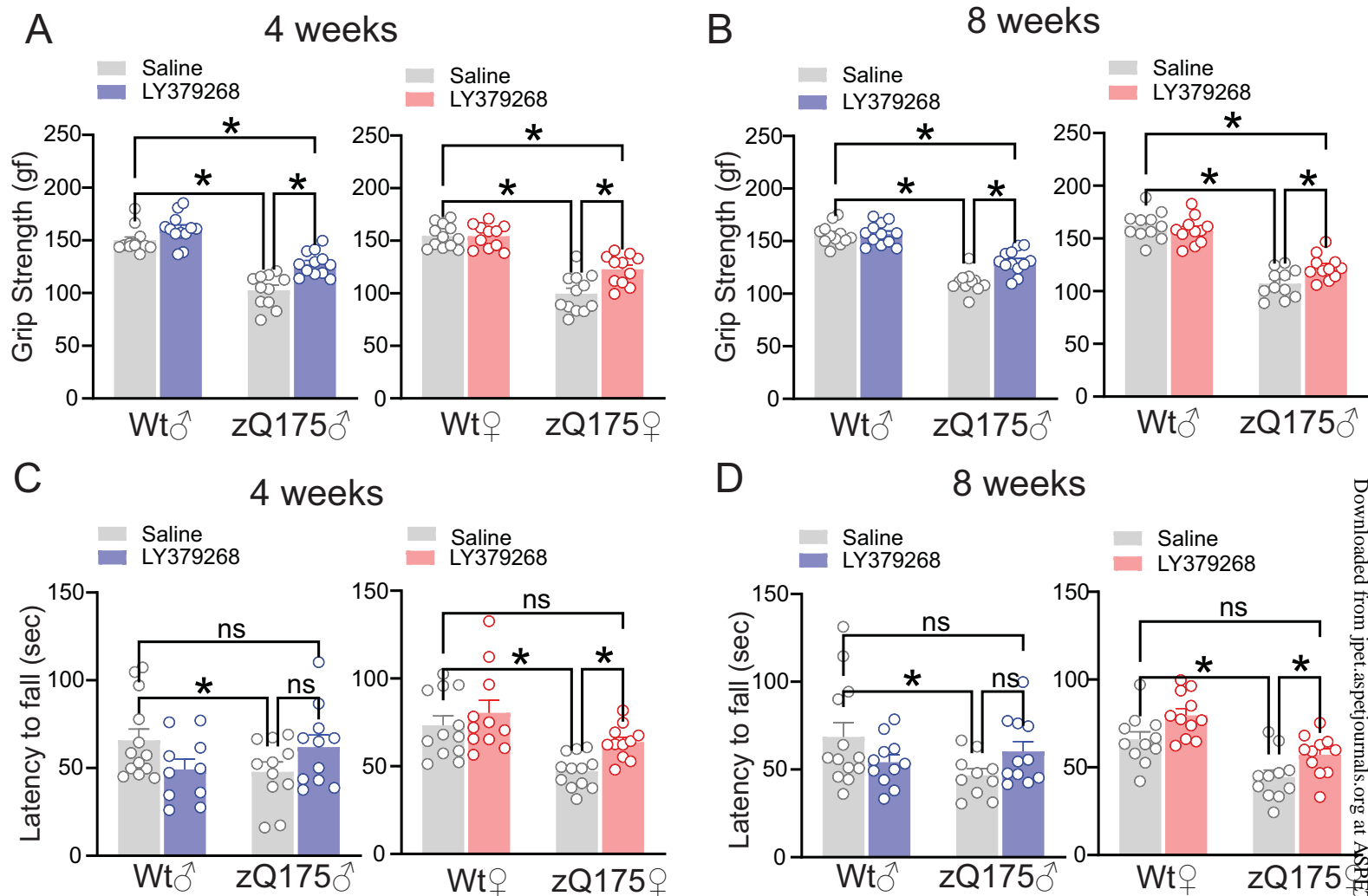


Figure 2

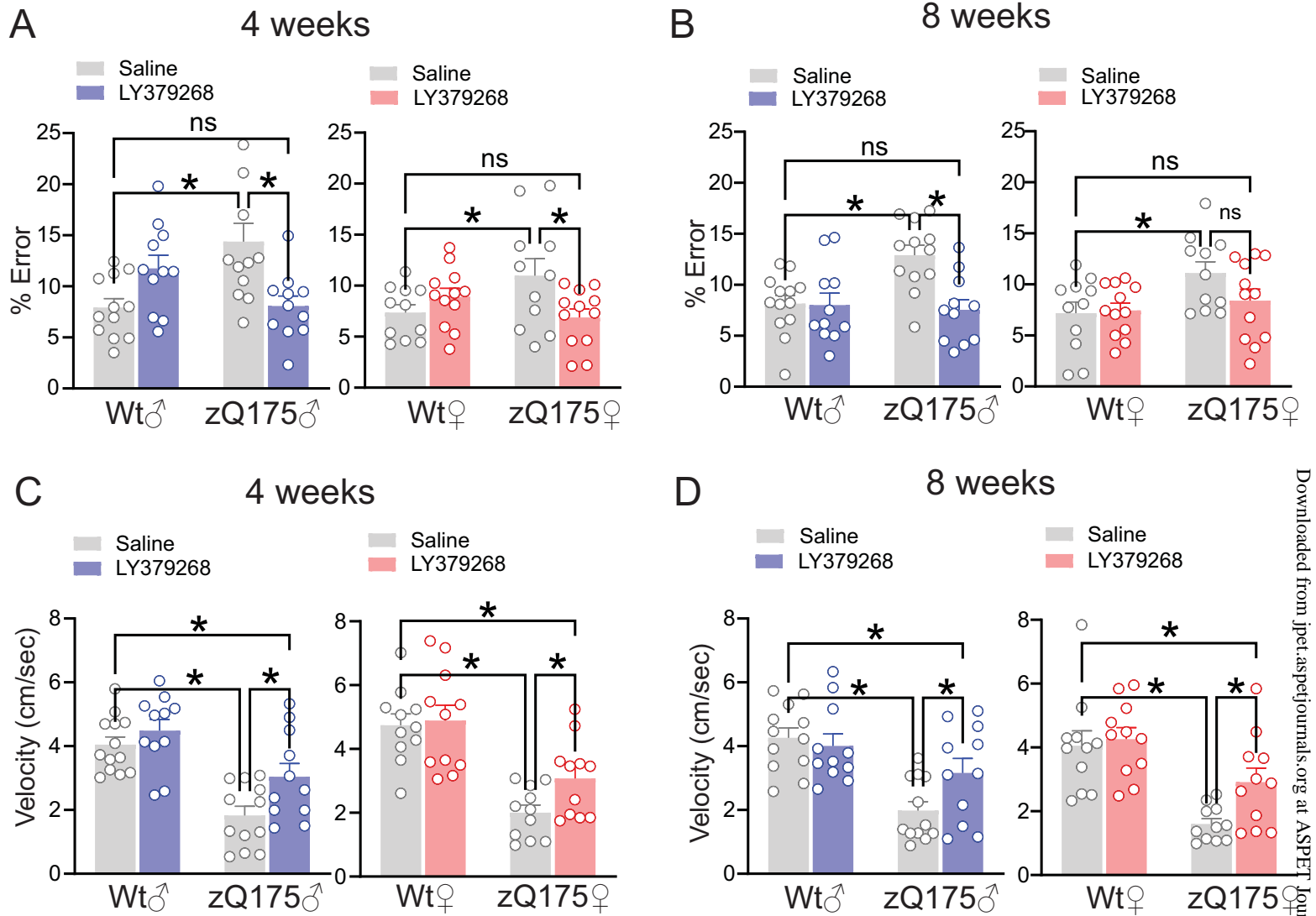
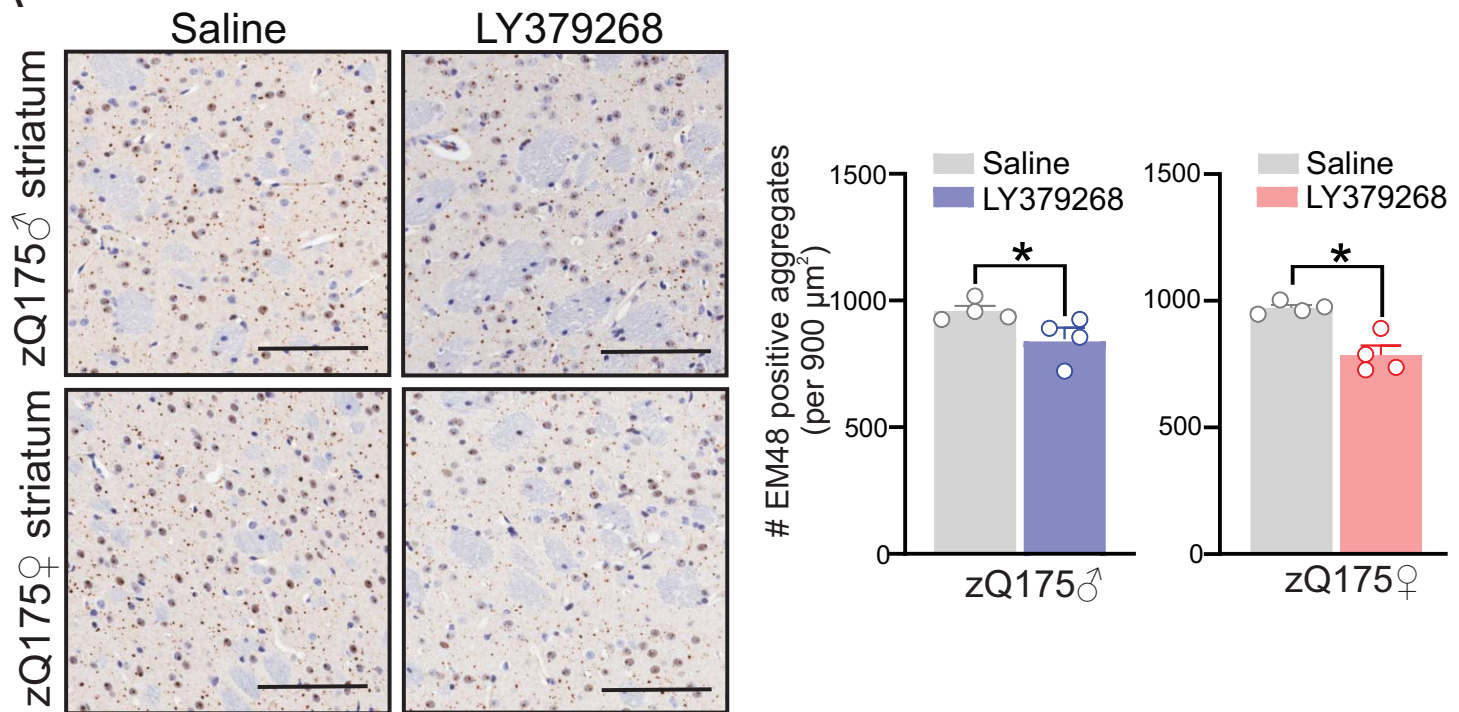
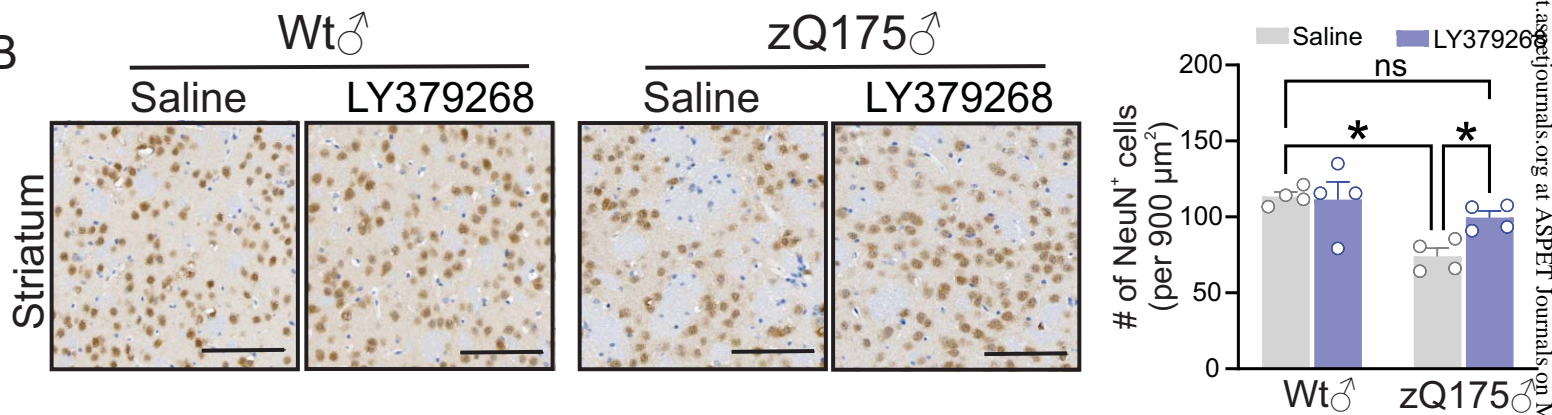


Figure 3

A



B



C

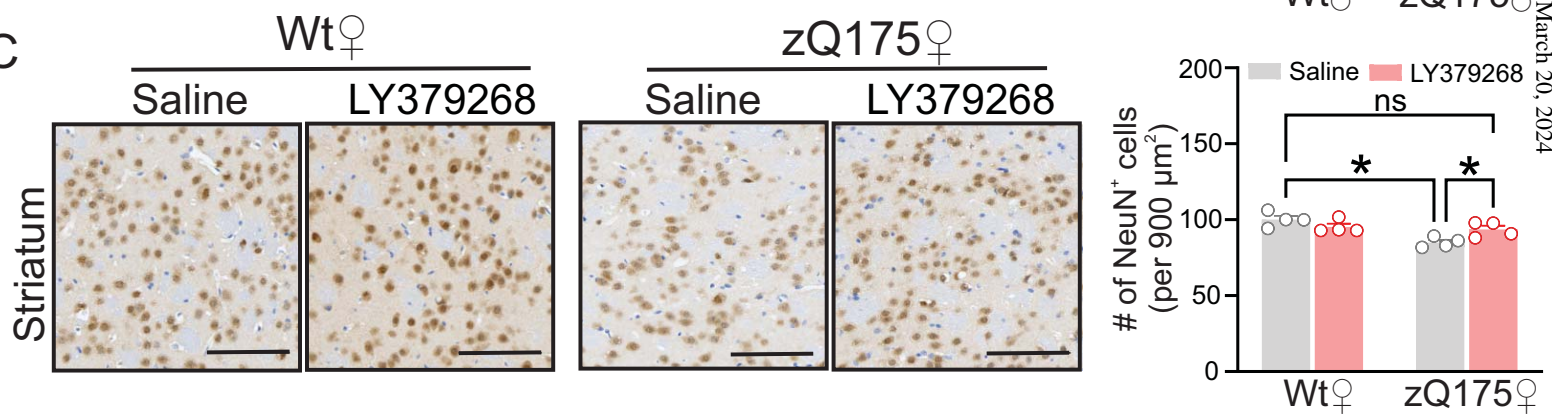


Figure 4

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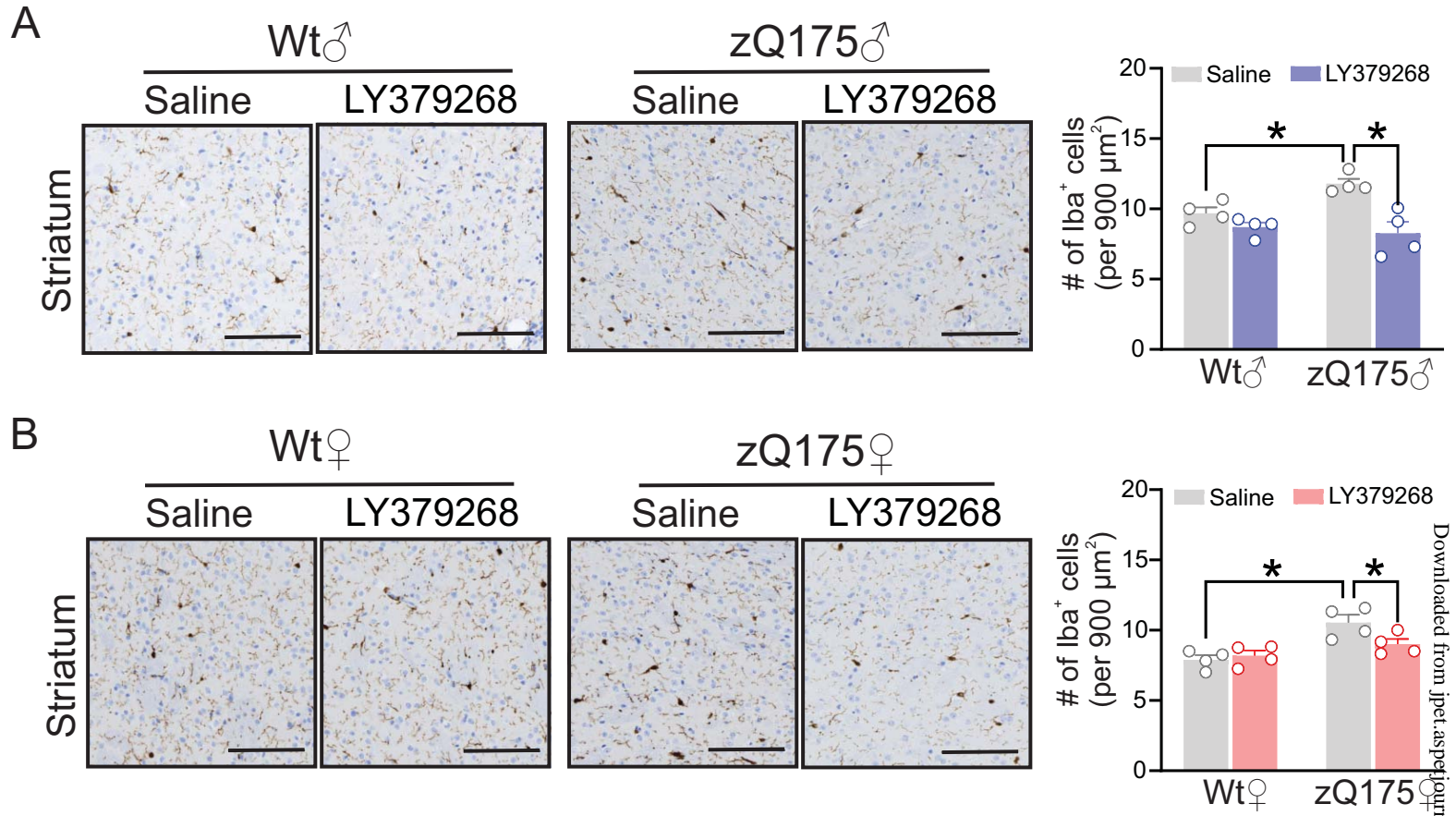


Figure 5

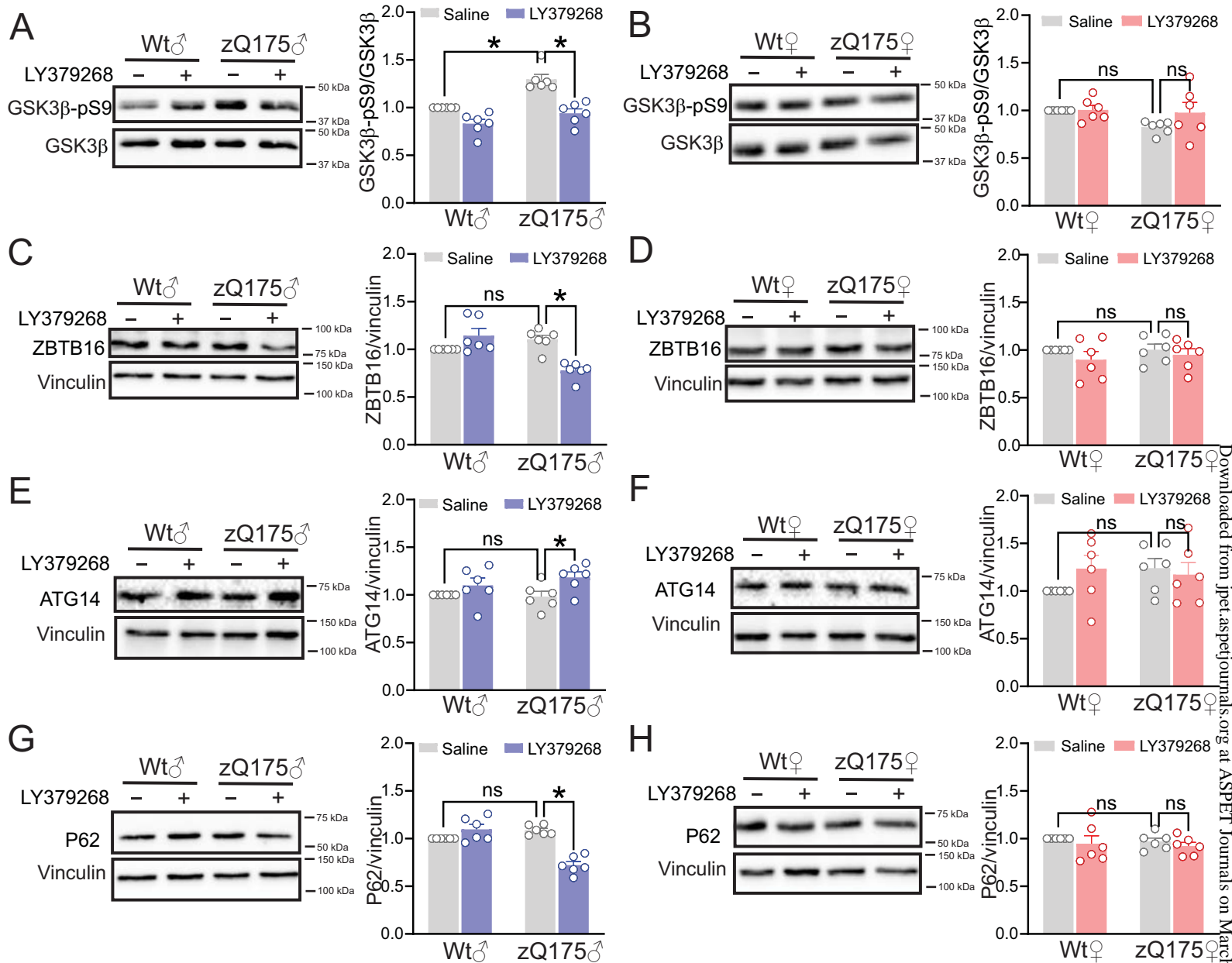
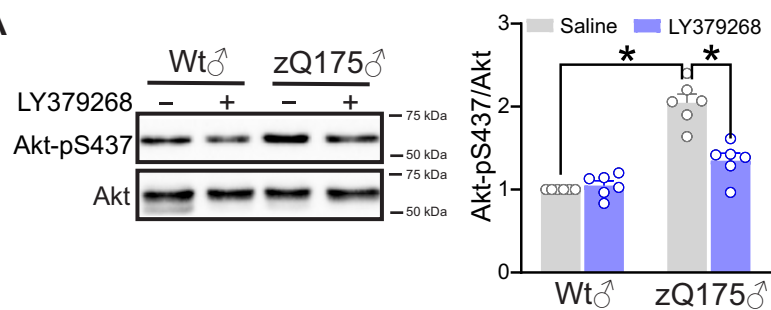
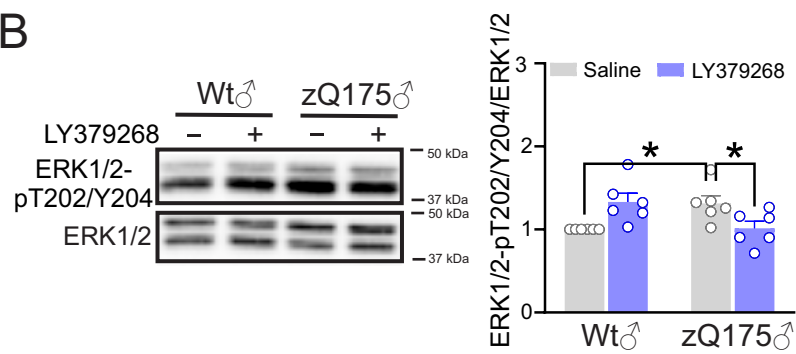


Figure 6

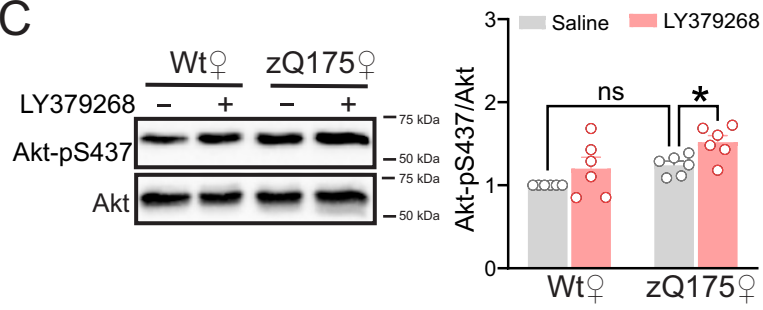
A



B



C



D

