Metabotropic glutamate receptor signalling and phenotype progression in Huntington's disease mice

Si Han Li

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Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

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Abstract

Huntington's disease (HD) is an inherited autosomal-dominant neurodegenerative disease caused by the abnormal expansion of CAG repeats in exon 1 of the huntingtin gene located on chromosome 4. This disease is characterized by the premature loss of medium spiny neurons in the striatum and behavioural deficits that typically manifest at middle-age. Despite the identification of its cause decades ago, there is still no disease modifying treatment available for HD patients. Current evidence indicates that exacerbated glutamate signalling in the striatum plays a key role in the pathophysiology of HD. Within the striatum, metabotropic glutamate receptor (mGluR) 2/3 are predominantly expressed on presynaptic terminals, whereas mGluR5 is predominantly localized to postsynaptic terminals. Here, we show that both the activation of mGluR2/3 and the inhibition of mGluR5 can improve HD symptoms in the zQ175 HD mouse model. Specifically, treating zQ175 HD mice with either the mGluR2/3 agonist LY379268 or the mGluR5 negative allosteric modulator (NAM) CTEP rescues motor deficits, reduces mutant huntingtin aggregate formation, improves neuronal survival and alleviates microglia activation. We also provide evidence that shows sex can influence the progression of HD symptoms and the efficacy of therapeutic agents. We found that chronic administration of LY379268 differentially activated and inactivated cell signalling pathways in male and female zQ175 mice. Furthermore, female zQ175 mice required a longer treatment duration with CTEP than male mice to show improvement in their rotarod performance. Using FDNQ175 mice, a newer HD mouse model derived from the zQ175 line, we demonstrated that female FDNQ175 mice were less susceptible to decline in limb function than male mice but showed higher levels of insoluble mutant huntingtin aggregates at a younger age.

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List of abbreviations

Aβ	Amyloid β
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4- isoxazole propionate
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
ATG	Autophagy related protein
BAC	Bacteria artificial chromosome
BAD	BCL2-associated agonist of cell death
Bcl-$_{XL}$	B-cell lymphoma-extra large
BDNF	Brain-derived neurotrophic factor
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
CBP	CREB-binding protein
CNS	Central nervous system
CREB	cAMP response element-binding protein
DAG	Diacylglycerol
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
GDNF	Glial derived neurotrophic factor
GIRK	G-protein-coupled inward rectifying potassium channels
GKAP	Guanylate kinase-associated protein
GLAST	Glial-glutamate-aspartate transporter
GLT-1	Glutamate transporter-1
GPCR	G-protein-coupled receptor
GSK3β	Glycogen synthase kinase 3β
HAP1	Huntingtin-associated protein-1
HD	Huntington's disease

Hip1	Huntingtin-interacting protein 1
Iba1	Ionized calcium binding adaptor molecule 1
iGluR	Ionotropic glutamate receptor
IL	Interleukin
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
mGluR	Metabotropic glutamate receptor
mHtt	Mutant huntingtin
MSN	Medium spiny neurons
mTOR	Mammalian target of rapamycin
NAM	Negative allosteric modulator
NeuN	Neuronal nuclear protein
NFκB	Nuclear factor Kappa B
NKCC1	Na-K-Cl cotransporter isoform 1
NMDA	N-methyl-D-aspartate
NRSE	Neuron-restrictive silencer elements
PAM	Positive allosteric modulator
PD	Parkinson's disease
PDK1/2	Phosphoinositide-dependent kinase 1/2
PI3K	Phosphoinositide 3-kinases
PIKE	Phosphoinositide 3 kinase enhancers
PIP2	Phosphatidylinositol-4,5-biphosphate
IP3	Inositol-1,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C

PLC	Phospholipase C
PSD95	Postsynaptic density protein 95
REST/NRSF	Repressor Element-1 Transcription Factor/Neuron Restrictive Factor
ROS	Reactive oxygen species
SHANK	SH3 and multiple ankyrin repeat domains protein
SNAP-25	Synaptosome-associated protein 25
SP1	Specificity protein 1
Stx4	Syntaxin 4
TAFII130	TATA binding protein-associated factor II130
TBP	TATA-binding protein
TGFβ	Transforming growth factor β
TrkB	Tropomyosin receptor kinase B
ULK1	Unc-51-like kinases 1
VPS	Vacuolar protein sorting 34
YAC	Yeast artificial chromosome
ZBTB16	Zinc finger and BTB domain-containing protein 16

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Chapter 1

General Introduction

1.1 Glutamate in the Central Nervous System

Glutamate is ubiquitously distributed throughout the mammalian central nervous system (CNS) and is present at a higher level than any other free amino acids. Most importantly, glutamate is the primary excitatory neurotransmitter in the brain, which plays a role in long-term potentiation, synaptic plasticity, learning and memory (Fonnum, 1984). Similar to other signalling molecules, glutamate exerts its functions through interacting with glutamate receptors expressed on the cell surface. Moreover, the concentration of glutamate in the extracellular space is regulated by release from presynaptic neurons and reuptake through a family of transporter proteins to ensure the appropriate amount of glutamate is available at the correct location and time (Zhou & Danbolt, 2014). In fact, both the over-abundance and lack of glutamate has been shown to contribute to the pathogenesis of a wide range of neurological disorders (Li et al., 2022a). Thus, glutamate signalling has garnered enormous scientific interest.

Over the years, two families of glutamate receptors have been identified: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) (**Figure 1.1**). The iGluRs are all ligand gated cation channels and they are divided into three major types based on the original agonists that were identified to activate them: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) (Dingledine et al., 1999). iGluRs are tetrameric non-selective cation channels consist of four large subunits: GluA subunits in AMPA receptors, GluN subunits for NMDA receptors and GluK subunits for kainate receptors (Traynelis et al., 2010). There are 16 genes encoding different subtypes of iGluR subunits, including four GluA subunits (GluA1-4), five GluK subunits (GluK1-5), one GluN1 subunit, four GluN2 subunits (GluN2A-D) and two GluN3 subunits (GluN3A and GluN3B) (**Figure 1.1**) (Traynelis et al., 2010). Each subunits contains an extracellular N-terminal domain, a ligand-

binding domain and an intracellular C-terminal domain. Kainate and AMPA receptors can be either homo- or hetero-tetramers, whereas NMDA receptors are obligatory hetero-tetramers with two essential GluN1 subunits and two GluN2 or GluN3 subunits (Paoletti et al., 2013). iGluRs with different subunit compositions have distinct biophysical, pharmacological and signalling properties. Furthermore, their activity can be further regulated by protein interaction, post-translational modification, alternative splicing and gene expression. For example, while GluN1 is encoded by a single gene, it has eight isoforms due to alternative splicing around exon 5 or exon 21 and exon 22 (Paoletti et al., 2013). The mGluRs are family C G-protein-coupled receptors (GPCRs) with seven transmembrane domains and transmit external signals into the cell through the α, β and γ subunits of heterotrimeric G proteins (Dhami & Ferguson, 2006). Eight mGluRs, numbered from 1 to 8, have been reported and they are further classified into three groups based on sequence homology and signal transduction mechanisms (**Figure 1.1**). Group I consists of mGluR1 and mGluR5, which are receptors coupled to $G\alpha_{q/11}$ and their activation stimulates the cleavage of phosphoinositide by phospholipase C and the release of Ca^{2+} from intracellular stores (Abdul-Ghani et al., 1996; Conn & Pin, 1997). Group II is made up of mGluR2 and mGluR3 and their activation inhibits adenylyl cyclase as they are predominantly linked to $G\alpha_{i/o}$ (Niswender & Conn, 2010). Group III includes all four remaining mGluRs (mGluR4, mGluR6, mGluR7 and mGluR8) and they are also coupled predominantly coupled to $G\alpha_{i/o}$ and inhibit adenylyl cyclase (Niswender & Conn, 2010).

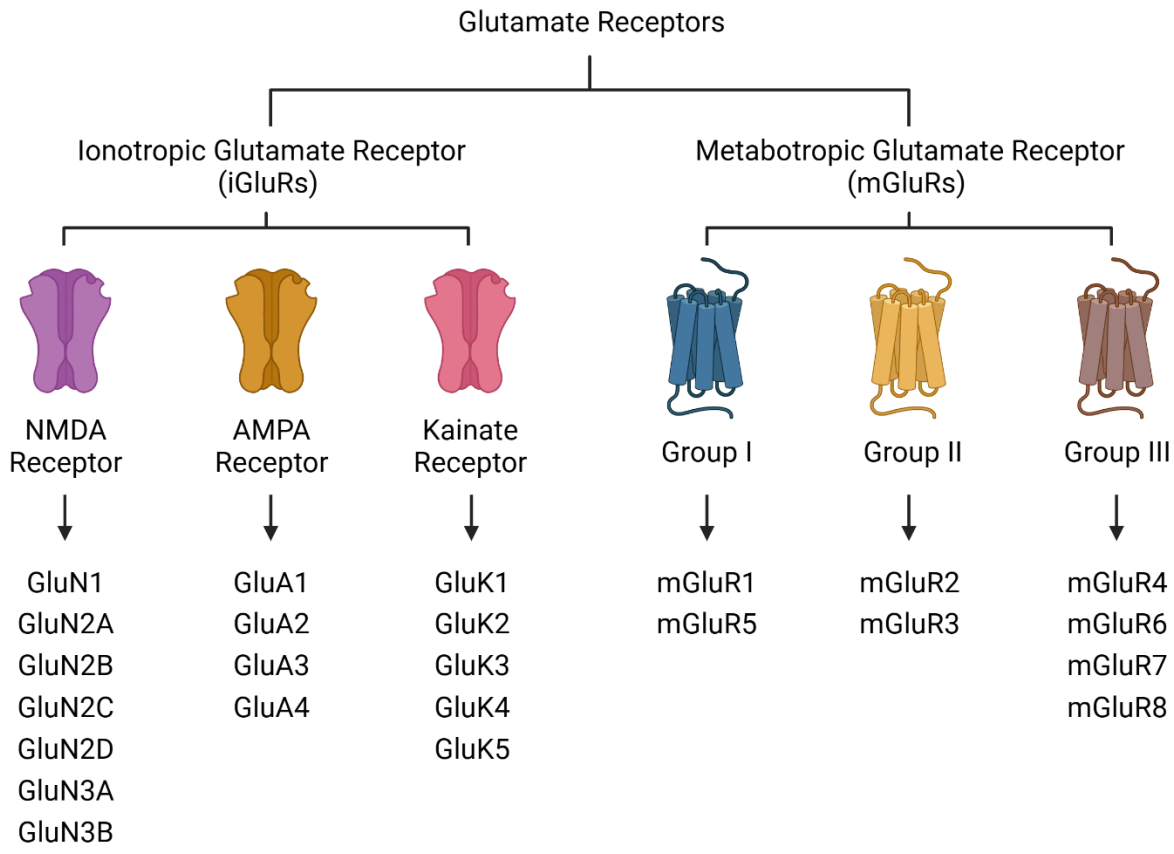

Figure 1.1 Types of Glutamate Receptors in the Brain. Glutamate receptors in the brain are separated into ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). iGluRs are tetrameric ligand-gated ion channels and they are further categorized based on their ligands: NMDA, AMPA and kainate. There are 7 different NMDA receptor subunits, 4 subtypes of AMPA receptor subunits and 5 subtypes of kainate receptor subunits. Subunit composition dictates the biophysical, pharmacological and signalling properties of each iGluR. mGluRs are G-protein-coupled receptors with 7 transmembrane domains. They are divided into three groups based on sequence homology and signal transduction mechanisms. $G\alpha_{q/11}$-coupled mGluR1 and mGluR5 are place in group I. $G\alpha_{i/o}$-coupled mGluR2 and mGluR3 are classified as group II. $G\alpha_{i/o}$-coupled mGluR4, mGluR6, mGluR7 and mGluR8 belongs to Group III. Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4- isoxazole propionate; NMDA, N-methyl-D-aspartate. The figure is created using BioRender.

1.2 Glutamatergic Signalling

Despite their distinct functions and properties, both mGluRs and iGluRs detect glutamate binding via their extracellular clamshell-shaped ligand-binding domain. Upon glutamate binding, the ligand-binding domain closes, which initiates a series of conformational change to trigger channel opening or G-protein activation. In addition to glutamate, both types of glutamate receptors also respond to other signals, including cations, anions, other endogenous ligands and change in membrane voltage. For example, membrane depolarization removes blockage of NMDA receptor pore by external magnesium ions and facilitates glutamate-induced current (Reiner & Levitz, 2018). Overall, iGluRs respond very fast to glutamate, with AMPA and kainate receptors opening and closing within a few milliseconds, and NMDA receptors reacting on a time scale of 10 to hundreds of milliseconds (Reiner & Levitz, 2018). In contrast, responses downstream of mGluR activation are often measured in the range of seconds to even minutes (Reiner & Levitz, 2018).

Glutamate receptors are critical for the regulation of synaptic plasticity. They greatly contribute to long-lasting changes in synaptic strength, which are critical for memory and learning, through a variety of mechanisms. In the hippocampus, it was demonstrated that long-term potentiation (LTP) is dependent on NMDA receptor activation and calcium ion (Ca^{2+}) influx. Specifically, Ca^{2+} activates Ca^{2+}/calmodulin-dependent kinase II (CaMKII), which enhances the AMPA receptor function at the postsynaptic terminals (Herring & Nicoll, 2016). Following LTP induction, CaMKII phosphorylates the C-terminus of AMPA receptor GluA1 subunit at serine 831, which appears to promote AMPA receptor trafficking to the synaptic site and increase their channel conductance (Kristensen et al., 2011). Group I mGluRs also play a role in LTP, as knocking out mGluR5 or mGluR1 reduces the amplitude of LTP in CA1 or CA3, respectively (Conquet et al.,

1994; Lu et al., 1997). Furthermore, co-activation of NMDA and mGluR5 can induce LTP in cultured hippocampal neurons, suggesting that both mGluRs and iGluRs work in concert to promote synaptic plasticity (Kotecha et al., 2003).

Long-term depression (LTD) is another form of synaptic plasticity, and it can be triggered at glutamatergic synapses via activation of both group I mGluRs and NMDA receptors. Furthermore, unlike LTP in which mGluRs and NMDA receptors work synergistically, group I mGluRs and NMDA receptors induce LTD through mechanistically distinct pathways (Oliet et al., 1997). NMDA receptors induce LTD by facilitating dephosphorylation of AMPA receptors at serine 845 and promoting their endocytosis (Lee et al., 2000). It remains unclear whether Ca^{2+} influx is required for NMDA receptor-induced LTD, as there is evidence showing that NMDA receptor can induce LTD even in the presence of the pore blocker MK-801 (Reiner & Levitz, 2018; Weilinger et al., 2016). Moreover, several studies have shown that ligand binding can trigger conformational changes in the c-terminal domain of NMDA receptor and alter its interaction with kinases and phosphatases (Vissel et al., 2001; Weilinger et al., 2016). Group I mGluR-induced LTD are dependent on the activation of several kinase signalling pathways downstream of G-protein activation and requires rapid dendritic protein synthesis (Huber et al., 2000; Reiner & Levitz, 2018). Due to the different roles of group I mGluRs in LTD versus LTP induction, it has been proposed that activation of group I mGluRs can regulate neural plasticity based on the extent of NMDA receptor activation, inducing LTP if NMDA receptor is co-activated and LTD if not (Reiner & Levitz, 2018). Some studies have also suggested that mGluR-induced LTD are more prominent in mature synapses, whereas NMDA receptors play a more important role in LTD induced at newer synapses (Holbro et al., 2009).

$G\alpha_{i/o}$-coupled mGluRs and iGluRs can also induce LTD at presynaptic terminals

throughout the brain. For example, group II mGluRs have been shown to induce presynaptic LTD at hippocampal mossy fiber-CA3 synapses in a NMDA receptor-independent manner (Kobayashi et al., 1996). This appears to be facilitated by inhibition of adenyl cyclase to downregulate cAMP production and cAMP-dependent signalling pathways (Tzounopoulos et al., 1998). Furthermore, it is also dependent on a rise in presynaptic Ca^{2+} concentration and activation of CamIIKα (Kobayashi et al., 1999; Tzounopoulos et al., 1998). Among the iGluRs, kainate receptors contribute to both short-term and long-term presynaptic plasticity. Pharmacological blockade and genetic knockout of kainate receptors partially blocks the induction of presynaptic LTP (Contractor et al., 2001; Wallis et al., 2015). Therefore, it appears that group II mGluRs and kainate receptor play opposed roles in mediating presynaptic neural plasticity. However, kainate receptors have also been found to facilitate presynaptic depression, potentially via a metabotropic pathway as G-protein inhibitors block kainate receptor-dependent depression (Frerking et al., 2001). Finally, presynaptic NMDA receptors may also contribute to presynaptic LTP at cortico-striatal synapses in a brain derived neurotrophic factor (BDNF)-dependent manner (Park et al., 2014).

It is clear that glutamatergic signalling plays a vital role in a wide range of neural processes such as learning and memory formation. However, dysregulation of glutamate signalling has been implicated in psychiatric disorders such as schizophrenia and neurodegenerative diseases such as Alzheimer's disease (AD), Parkingson's disease (PD) and Huntington's disease (HD). Therefore, delineating the events downstream of iGluRs and mGluRs activation at different glutamatergic synapses could help identify new therapeutic targets for treating these diseases.

1.3 Glutamate and Excitotoxicity

It is now well established that the over-abundance of glutamate can trigger neuronal degeneration through a process termed excitotoxicity. The neurotoxic effect of glutamate was first

demonstrated when injection of glutamate triggered lesions in several tissues, including the retina and nuclei of the hypothalamus (Lucas & Newhouse, 1957; Olney, 1969). Later studies have linked the neurotoxic potential of glutamate to prolonged depolarization of neurons, disruption of calcium homeostasis and activation of programmed cell death (Doble, 1999). Furthermore, substantial evidence indicate that excitotoxicity is a fundamental mechanism involved in neurodegenerative diseases such as Alzheimer's disease, Huntington's disease and Parkinson's disease (Li et al., 2022a).

Excessive depolarization of postsynaptic membrane is responsible for the initial stage of excitotoxic damage (**Figure 1.2**). The influx of sodium (Na^+) and chloride (Cl^-) ions into the neuron disrupts the osmotic balance, leading to water entering the cell and rupturing the cell membrane (Rothman, 1985). Removal of either Na^+ or Cl^- from extracellular solution of cultured hippocampal neurons blocked the toxicity effect of glutamate, NMDA and kainate (Rothman, 1985). In contrast, removing Ca^{2+} from the culture media did not have an effect on the toxic action of glutamate, NMDA and kainate (Rothman, 1985). Similar results were also observed in another study using isolated chicken retina tissues, in which the absence of either Na^+ or Cl^- but not Ca^{2+} in the extracellular environment abolished the toxic effect of kainate and NMDA (Chen et al., 1998). Subsequent studies show cation-dependent Cl^- transport protein Na-K-Cl cotransporter isoform 1 (NKCC1) contribute to NMDA-mediated excitotoxicity by facilitating the entry of Na^+ and Cl^- (Beck et al., 2003). Furthermore, blocking the activity of NKCC1 with the bumetanide prevented glutamate-induced neurotoxicity and cell swelling in cultured cortical neurons (Beck et al., 2003).

Another mechanisms underlying glutamate-induced excitotoxicity involves elevated levels of intracellular Ca^{2+} (**Figure 1.2**). An early study showed that while the removal of Na^+ prevented

immediate cell damage following glutamate exposure, the presence of Ca^{2+} in the environment can still lead to delayed cell death 24 hours after glutamate exposure (Choi, 1985). Therefore, excitotoxicity can be roughly separated into two stages: an initial stage caused by Na^{+} and Cl^{-} influx that leads to cell membrane rupture, and a delayed wave of neurodegeneration caused by elevated levels of intracellular Ca^{2+}. The exact mechanism underlying the toxic action of Ca^{2+} overload remains unclear, primarily due to the vast and complicated Ca^{2+}-dependent signalling pathways that have been implicated in neuronal cell death. However, it has been proposed that the entry of Ca^{2+} through Ca^{2+}-permeable glutamate receptors, especially NMDA receptors, can activate such signalling pathways more efficiently (Arundine & Tymianski, 2003). Multiple studies have shown that Ca^{2+} can trigger apoptotic pathways by activating the serine-threonine phosphatase calcineurin. Activated calcineurin dephosphorylates the pro-apoptotic protein BCL2-associated agonist of cell death (BAD), leading to its translocation to the mitochondria where it associates with B-cell lymphoma-extra large (Bcl-$_{XL}$) and promotes cell death (Wang et al., 1999). Ca^{2+} can also activate the Ca^{2+}-dependent protease calpain, whose substrates have been suggested to play critical roles in promoting neuronal degeneration (Wu et al., 2004). For example, calpain can cleave and convert calcineurin into constitutively active forms during both glutamate- and kainate-induced neuronal excitotoxicity (Wu et al., 2004). Furthermore, calpain has been shown to cleave off the calcineurin-binding domain of CAINcabin1, an endogenous inhibitor of calcineurin, leading to activation of calcineurin and cell death (Kim et al., 2002).

Abnormal increase in intracellular Ca^{2+} concentration can also lead to oxidative stress. Increased activity of Ca^{2+}-dependent enzymes phospholipase-A_2, nitric oxide synthase and xanthine oxidase causes higher production of free radicals, which can damage proteins, lipids, carbohydrates and nucleic acids. Most notably, nitric oxide has been shown to upregulate the

expression of metalloproteinase, triggering proteolytic cascades that degrade extracellular environment for neurons (Arundine & Tymianski, 2003). Furthermore, the downstream metabolite of nitric oxide, peroxynitrite ($ONOO^-$), can promote DNA fragmentation and prevent protein phosphorylation required for proper signal transduction (Parathath et al., 2006). Both increased intracellular Ca^{2+} load and excessive production of free radicals can also disrupt mitochondrial functions. For example, excessive mitochondrial Ca^{2+} intake can result in over-production of reactive oxygen species (ROS), which in turn inhibits mitochondrial enzymes and electron transport chain complexes that facilitates cellular energy production (Mehta et al., 2013). In addition, high levels of Ca^{2+} and ROS directly induces opening of the mitochondrial permeability transition pore, releasing proapoptotic factors and stored Ca^{2+} into the cytosol (Plotegher et al., 2021).

To date, numerous mechanisms are identified as contributing factors in excitotoxic neuronal death triggered by excessive glutamate exposure. However, it remains to be determined how excitotoxicity is involved in the pathogenesis of different neurodegenerative disease. Therefore, examining signalling events downstream of both ionotropic and metabotropic glutamate receptors in the context of specific neurodegenerative diseases may lead to the discovery of therapeutic interventions for treating each disease.

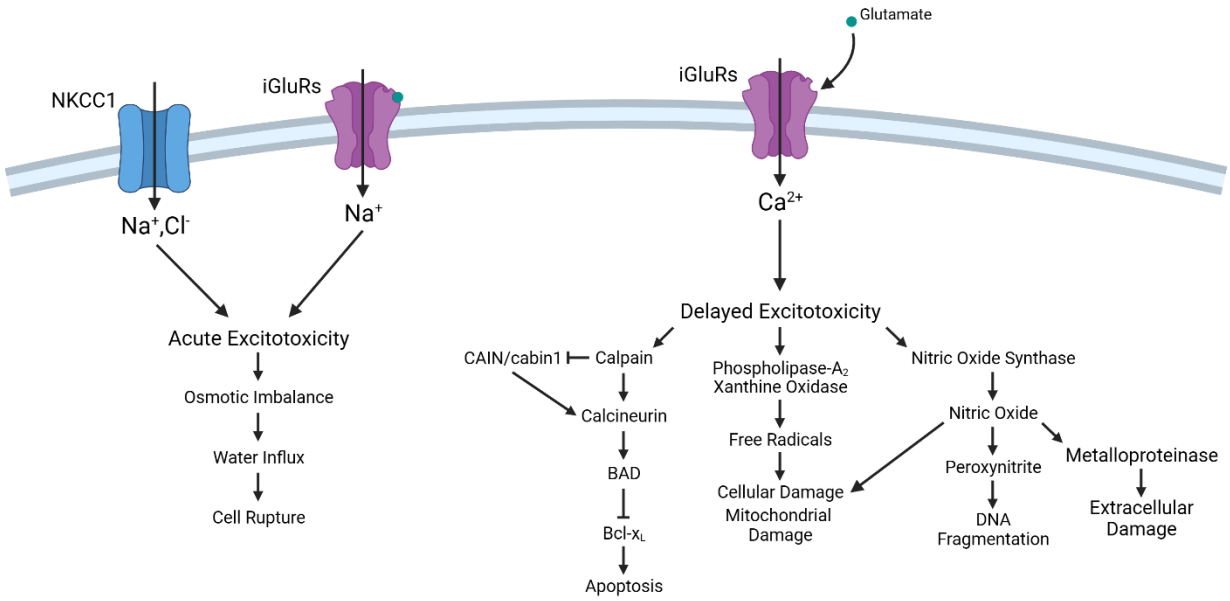

Figure 1.2 Mechanisms of Excitotoxicity. In acute excitotoxicity, the influx of sodium (Na^+) and chloride (Cl^-) ions through ionotropic glutamate receptors (iGluRs) and NKCC1 disrupts osmotic balance inside neurons. This leads to excessive water entering the cell and rupturing the cell membrane. Delayed excitotoxicity is triggered by elevated levels of intracellular calcium (Ca^{2+}) following excessive glutamate exposure. Ca^{2+} activates calpain to convert calcineurin into constitutively active forms and prevent CAIN/cabin1-mediated inhibition of calcineurin. Calcineurin dephosphorylates BAD, triggering its translocation to the mitochondria where it associates with Bcl-x_L and promotes cell death. High Ca^{2+} concentration cause increased production of free radicals, which can damage proteins, lipids, carbohydrates and nucleic acids. The free radical nitric oxide can upregulate metalloproteinase to degrade extracellular environment. Nitric oxide can promote DNA fragmentation through its downstream metabolite peroxynitrite. Abbreviations: BAD, BCL2 associated agonist of cell death; Bcl-x_L, B-cell lymphoma-extra large; NKCC1, Na-K-Cl cotransporter isoform 1. The figure is created using BioRender.

1.4 Huntington's Disease

Huntington's disease (HD) is an inherited, progressive and autosomal-dominant neurodegenerative disorder first described in vivid detail by Dr. George Huntington in the late 1800's. In the vast majority of cases, the symptoms of HD appear between the ages of 30 to 50, but they can occur at anytime between the ages of 1 to 80 (Roos, 2010). HD patients can live completely healthy lives until symptoms gradually begin to develop, starting with subtle signs such as irritability, disinhibition, unreliable at work, difficulty in multitasking, forgetfulness and anxiety (Walker, 2007). Eventually, distinct symptoms including chorea, motor impairments and behaviour difficulties manifests, leading to diagnosis of the disease (Walker, 2007). The genetic cause of HD is the abnormal expansion of CAG repeats in exon 1 of the human huntingtin gene and the resulting production of mutant huntingtin protein with an expanded polyglutamine tract in its N-terminus (MacDonald et al., 1993). Proteolytic cleavage of mutant huntingtin proteins results in the deposition of insoluble cytoplasmic and intranuclear aggregates, which are the pathological hallmarks of HD (DiFiglia et al., 1997). Moreover, the length of the CAG repeats directly correlates with HD progression, with longer repeats leading to earlier age of disease onset and more severe disease progression (Andrew et al., 1993; Furtado et al., 1996).

Despite the identification of its cause, the exact mechanism(s) underlying HD pathogenesis remains unclear and disease-modifying treatments for HD patients is still not available. Huntingtin is ubiquitous throughout the body and interacts with a wide variety of cellular structures, including the endoplasmic reticulum (ER), microtubules mitochondrial and synaptic vesicles (Young, 2003). Huntingtin also appears to be important for development, as full knockout of huntingtin in mice is embryonically lethal (Duyao et al., 1995). Furthermore, heterozygous huntingtin knockout mice showed significant loss of neurons in the globus pallidus and subthalamic nucleus (O'Kusky et al.,

1999). Similarly, conditional knockout of huntingtin in mice results in progressive motor phenotype and neurodegeneration (Dragatsis et al., 2000). Interestingly, the wide distribution of huntingtin protein is not reflected in the toxic activity of mutant huntingtin, as the neurodegeneration in HD is highly selective and specifically targets medium sized spiny neurons (MSNs) within the striatum (Imarisio et al., 2008).

Mutation in the huntingtin gene has been demonstrated to trigger HD-associated neurodegeneration through a wide variety of mechanisms (**Figure 1.3**). Some evidence suggest that the loss of wild-type huntingtin leads to disruption of critical cellular pathways that require it. For example, huntingtin is involved in the movement of mitochondria in neurons. In a conditional knockout mouse model that expresses significantly lower levels of wild-type huntingtin, movement of mitochondria is impaired (Trushina et al., 2004). Besides mitochondria, huntingtin promotes the vesicular transport of BDNF along the microtubules (Gauthier et al., 2004). Specifically, wild-type huntingtin interacts with huntingtin-associated protein-1 (HAP1) and the $p150^{Glued}$ subunit of dynactin. The inclusion of huntingtin in this complex enhances the transport efficiency of vesicles containing BDNF, as reducing the levels of huntingtin by RNA interference attenuates BDNF transport and results in neuronal death due to the lack of neurotrophic support (Gauthier et al., 2004). In addition to its transport, wild-type huntingtin increases the production of BDNF by sequestering repressor element-1 transcription factor/neuron restrictive factor (REST/NRSF) in the cytoplasm (Zuccato et al., 2003). This interaction prevents REST/NRSF from relocating to the nucleus and binding the neuron-restrictive silencer elements (NRSE) located in the promoter region of the BDNF gene (Zuccato et al., 2003). Mutant huntingtin is less effective at interacting with REST/NRSF, and thus the loss of wild-type huntingtin results in the suppression of BDNF gene transcription due to more REST/NRSF binding to NRSE (Zuccato et al., 2003).

Wild-type huntingtin has also been shown to sequester huntingtin-interacting protein 1 (Hip1), a protein that in its free form can interact with Hip1 protein interactor (Hippi) to activate the pro-apoptotic caspase-8 (Gervais et al., 2002; Kalchman et al., 1997). As the polyglutamine tract in huntingtin expands, it losses affinity towards Hip1, leading to increased formation of the Hippi-Hip1 complex and triggering apoptotic pathways (Gauthier et al., 2004). There is also evidence that suggests huntingtin binds and regulates the activity of caspase-3 (Zhang et al., 2006). Mutant huntingtin has significantly weaker interaction with caspase-3 and consequently increases cell death in response to stress stimuli (Zhang et al., 2006).

In addition to the loss of wild-type huntingtin's beneficial effects, it is also proposed that the expansion of the polyglutamine tract may be associated with a gain of toxic function (**Figure 1.3)**. The insoluble aggregates formed from the N-terminal fragments of mutant huntingtin have been shown to be capable of recruiting transcription factors such as TATA-binding protein (TBP) and specificity protein 1 (SP1) into their matrix, thus potentially interfering with their normal activities and causing transcriptional dysregulation (Landles & Bates, 2004). Similarly, mutant huntingtin complexes also trap wild-type huntingtin and key components of the vesicular trafficking machinery, including kinesin and the $p150^{Glued}$ subunit of dynactin, into insoluble aggregates (Trushina et al., 2004). However, despite the observation that mutant huntingtin aggregates remove important cellular proteins from the soluble pool, the overall impact of the aggregates is still under debate as they also reduce the level of soluble mutant huntingtin fragments. One study has shown that neuronal cell death in tissue cultures can occur in the absence of aggregate deposition (Arrasate et al., 2004). In fact, the study demonstrated that neurons without aggregates are at higher risk of death (Arrasate et al., 2004). Therefore, it is possible that the formation of aggregates protects the cell by sequestering the more toxic protein species, most

notably the N-terminal fragments of mutant huntingtin. Consistent with this hypothesis, multiple studies indicate that interaction between mutant huntingtin proteins and other proteins can significantly disrupt normal cellular functions. For example, several studies have now demonstrated that the soluble form of mutant huntingtin can interact with transcription factors to trigger transcriptional dysregulation, and nuclear targeting of mutant huntingtin *in vitro* enhances their toxicity (Peters et al., 1999). More specifically, the expanded polyglutamine tract of mutant huntingtin enhances its affinity for the transcription factor SP1 and TATA binding protein (TBP)-associated factor II130 (TAFII130) (Li et al., 2002; Shimohata et al., 2000). Association of mutant huntingtin to SP1 and TAFII130 inhibits their binding to DNA, leading to transcriptional dysregulation that occurs early in HD patients (Dunah et al., 2002). Soluble mutant huntingtin also disrupts gene transcription through interacting with cAMP response element-binding protein (CREB)-binding protein (CBP), a histone acetyltransferase that functions as an important transcriptional co-activator, at a much higher affinity than wild-type huntingtin and promoting its degradation via the proteasomal pathway (Cong et al., 2005). Suppression of CBP activity leads to histone hypoacetylation and heterochromatin formation. Indeed, administration of histone deacetylase inhibitors attenuates neurodegeneration caused by the polyglutamine-containing domain of mutant huntingtin in drosophila (Steffan et al., 2001). Other transcription factors whose function is disrupted by the binding of soluble mutant huntingtin include TATA box binding protein (TBP), RNA polymerase II-associating protein 30 (RAP30) and CAAT box transcription factor NF-Y (Labbadia & Morimoto, 2013).

The N-terminal fragment of mutant huntingtin also directly associates with mitochondria and impairs its association with microtubule-based transport proteins *in vitro* (Orr et al., 2008). In fact, the presence of mutant huntingtin fragments can directly reduce the velocity of mitochondrial

transport despite physiological levels of wild-type huntingtin (Orr et al., 2008). Moreover, the association of mutant huntingtin fragments with mitochondria increases with age and correlates with disease progression in HD mice (Orr et al., 2008). There is also evidence that suggest mutant huntingtin fragments interfere with the function of electron transport chain complexes II and III, which correlates with the defects in energy metabolism and exacerbated oxidative damage in HD brains (Solans et al., 2006). Another aspect that is impaired by mutant huntingtin expression is the proteosome degradation pathway, as there is an accumulation of lysine 48-linked polyubiquitin chain in the brains of HD patients and mouse models of HD (Bennett et al., 2007). However, mutant huntingtin oligomers do not appear to inhibit the activity of proteasome directly, but instead simply overwhelm the proteosome and causes an inadequate removal of misfolded proteins (Labbadia & Morimoto, 2013). Another study suggests that autophagic degradation of bulk proteins is impaired in HD as well. This appears to be caused by a defect in the ability of autophagic vacuoles to trap cytosolic cargo in their lumen despite normal rate or even enhanced rate of formation and elimination (Martinez-Vicente et al., 2010).

Collectively, mutant huntingtin can trigger cell death through disrupting a wide variety of critical cellular functions, from gene transcription to protein degradation. However, it is unclear the exact mechanism(s) by which mutant huntingtin selectively targets MSNs of the striatum in HD. The identification of such mechanism represents a critical step in the development of therapeutic strategy for treating HD patients.

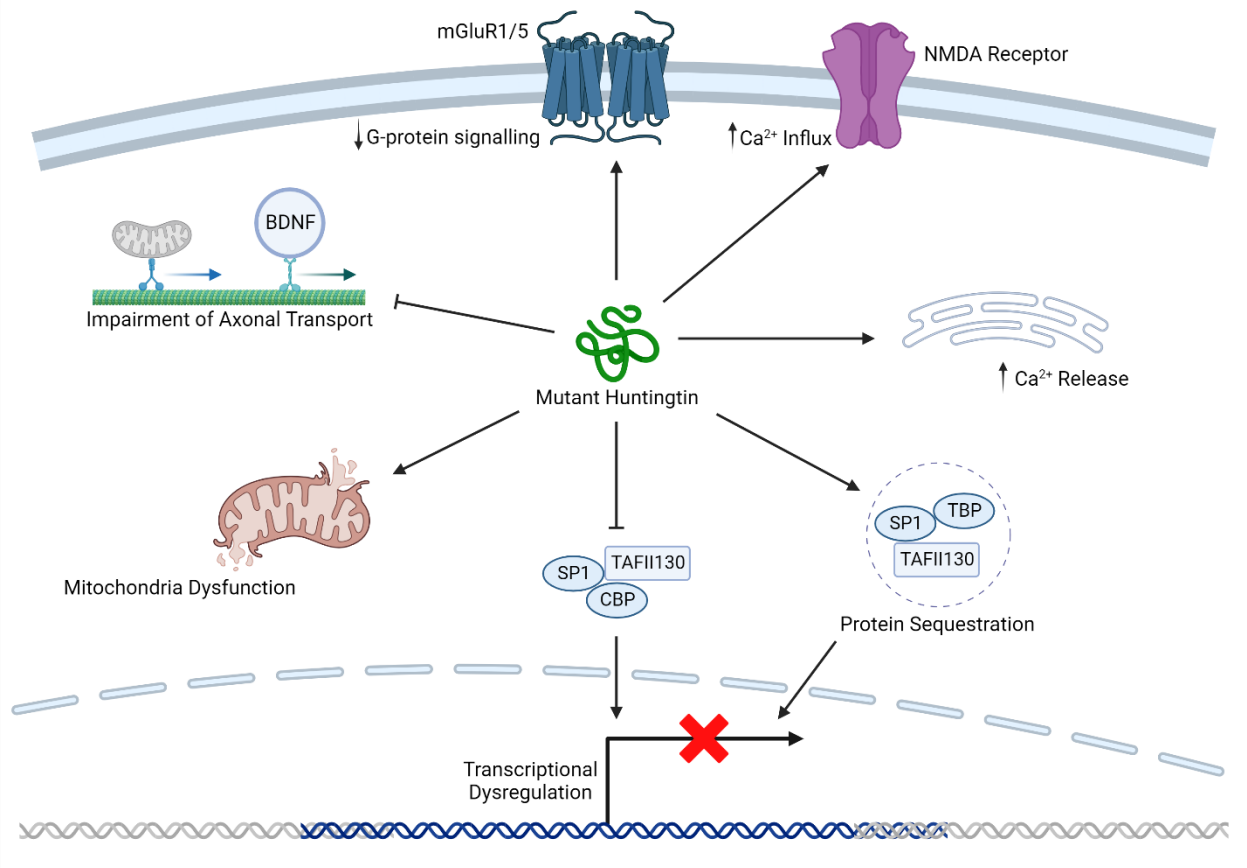

Figure 1.3 Neurotoxic Effects of Mutant Huntingtin. Mutant huntingtin interacts with mGluR1/5 and decouples mGluR1/5 signalling from IP3 formation through PKC-mediated mechanisms. Mutant huntingtin sensitizes NMDA receptor, leading to increased influx of Ca^{2+} into the neurons. Mutant huntingtin also interacts and sensitizes IP3 receptors on the endoplasmic reticulum (ER), leading to increased release of Ca^{2+} from intracellular storages. Insoluble mutant huntingtin aggregates traps transcription factor into their matrix, decreasing their availability for proper transcriptional regulation. Soluble mutant huntingtin interacts with transcription factors to directly trigger transcriptional dysregulation. Mutant huntingtin can also disrupt mitochondrial function and transport. Furthermore, axonal transport of BDNF is impaired due to mutant huntingtin interfering with vesicular trafficking machinery. Abbreviations: CBP, cAMP response element-binding protein-binding protein; mGluR1/5, metabotropic glutamate receptor 1/5; NMDA, N-methyl-D-aspartate; SP1, specificity protein 1; TAFII130, TATA binding protein-associated factor II130; TBP, TATA binding protein. The figure is created using BioRender.

1.5 Mouse Models of HD

The creation of mouse models that replicate the clinical, pathological and biochemical changes observed in HD patients is one of the most critical steps in the research of treatment for HD. The earliest rodent models of HD are generated by direct injection of chemicals such as monosodium glutamate, quinolinic acid and kainic into the striatum, which led to neuronal degenerations that are similar to those observed in HD (Beal et al., 1986; Coyle & Schwarcz, 1976). With the discovery of the CAG repeat mutation in the huntingtin gene as the cause for HD, various genetic mouse models of HD have now been created. Based on the way in which the huntingtin mutation is incorporated into the mouse genome, mouse models of HD can be separated into three categories: i) transgenic mice that express a toxic N-terminal fragment of human mutant huntingtin in addition to mouse wild-type huntingtin, ii) transgenic mice that express full length human mutant huntingtin alongside mouse wild-type huntingtin, and iii) knock-in mice with expanded CAG repeats in their endogenous huntingtin gene (Ferrante, 2009). Transgenic HD mouse lines that express the N-terminal fragments of mutant huntingtin include the R6, N171-82Q and Tg100, with the R6/2 line being the most extensively used one (Farshim & Bates, 2018). Both R6/2 and R6/1 express one copy of a human mutant huntingtin N-terminal fragment that contains the gene's promotor sequence, exon 1 and part of intron 1 (Mangiarini et al., 1996). The R6/2 line has a longer CAG repeats (~150 vs 115) and higher expression (75% of endogenous level vs 31%) than the R6/1 line (Menalled & Chesselet, 2002). Due to the unstable nature of the CAG repeat length, R6/2 mice used by different laboratories have varying length of polyglutamine tract. In general, the R6/2 mice have been reported to develop motor and cognitive impairments as early as 5 weeks of age followed by death at 10 weeks of age (Menalled & Chesselet, 2002). This early age of onset and aggressive phenotype progression make R6/2 mice the most utilized model for HD research.

However, since the R6/2 mice only expresses an N-terminal fragment of huntingtin, the role of proteolytic cleavage on mutant huntingtin toxicity is not taken into consideration. Moreover, HD in humans predominantly shows progressive decline starting at middle-age and thus, the R6/2 mouse model seems to be better suited for modelling the rarer juvenile HD.

More recently, transgenic HD mice expressing full-length human mutant huntingtin have been generated using yeast artificial chromosome (YAC) and bacteria artificial chromosome (BAC). In comparison to the R6 mouse lines, YAC and BAC HD mice show normal survival time and disease phenotypes that progress steadily over several months. Both YACHD and BACHD mice express multiple copies of human mutant huntingtin due to random integration, with YACHD mice having 4 copies and BACHD mice having 5 copies (Farshim & Bates, 2018). There are multiple lines of YACHD mice with roughly 46, 72 and 120 CAG repeats, named YAC46, YAC72 and YAC128 respectively. Just like HD in humans, the severity of the disease in YAC mice directly correlates with the length of the CAG repeats. Both YAC46 and YAC72 mice only show mild behaviour abnormalities whereas YAC128 mice can show robust motor and cognitive deficits as early as 4 weeks of age. Furthermore, YAC128 mice are available on both C56BL/6 and FVB/N background strains, with the FVB/N strain showing more vulnerability to HD-associated neurodegeneration (Van Raamsdonk et al., 2007). Cytoplasmic and intranuclear aggregates are also observed throughout the brains of YAC128 mice, which is accompanied by widespread neurodegeneration (Bayram-Weston et al., 2012). BACHD mice express full length human mutant huntingtin with 97 CAG repeats under the control of huntingtin gene regulatory machinery on the BAC (Gray et al., 2008). BACHD mice develop behaviour deficits progressively between the age of 2 to 6 months. The decline in motor function precedes the neuropathology but occurs at the same time as electrophysiological dysfunction in striatal MSNs (Spampanato et al., 2008).

Interestingly, the deposition of mutant huntingtin aggregates in BACHD mice is predominantly cytoplasmic since EM48-positive nuclear inclusions are complete absent in their striatum and cortex (Gray et al., 2008). Both YAC128 and BACHD mice are resistant to somatic instability, likely due to the presence of CAA codons interspersed within the CAG repeats (Farshim & Bates, 2018). More importantly, both mouse models are poor choices for studying the effects of mutant huntingtin on energy metabolism and weight loss, because both YAC128 and BACHD mice gain weight whereas HD patients lose weight as the disease progresses (Farshim & Bates, 2018).

Knock-in mouse models of HD, in which the disease-causing mutation is directly inserted into the mouse endogenous huntingtin gene, represent the most genetically accurate models of HD, as they express the mutant protein in the correct genomic context. Knock-in HD mice are generated in two ways: i) the normal mouse polyQ sequence is replaced with an expanded CAG repeats, and ii) entire exon 1 of the mouse huntingtin gene is replaced by exon 1 of mutant human huntingtin, resulting in the creation of a chimeric protein. The HdhQ150 mice are generated using the first approach and their huntingtin gene contains roughly 150 CAG repeats and is unstable in gametic transmission, leading to the generation of various other mouse lines with CAG repeat lengths ranging from 50 to 365. Compared to transgenic HD mouse models, the HdhQ150 mice show cognitive deficits at 24 weeks of age and motor deficits at around 40 weeks of age (Brooks et al., 2006). HdhQ150 mice also display widespread aggregate formation throughout the brain, transcriptional dysregulation in the striatum and reduced striatal volume (Bayram-Weston et al., 2012; Woodman et al., 2007). Several knock-in mouse lines have also been generated using the second method. For example, the huntingtin gene of HdhQ111 mice contains human exon 1 engineered with 111 CAG repeats (Wheeler et al., 2000). HdhQ111 mice show nuclear aggregate deposition and neuronal atrophy in the striatum, but motor deficits do not appear until 24 months

of age and only subtle gait abnormalities can be observed at this age (Wheeler et al., 2000). zQ175 HD mice is another knock-in mouse model with a chimeric huntingtin gene and its human exon 1 contains 188 CAG repeats (Menalled et al., 2012). The zQ175 knock-in mouse model is particularly important for HD research as it is the first model to show robust phenotypes on a heterozygous background. The heterozygous background is of particular interest since homozygosity is very rare in HD patients. Heterozygous and homozygous zQ175 mice show gene dosage-dependent progression in certain disease phenotypes, with heterozygous mice exhibiting decreased body weight and hypoactivity at a much later age than heterozygous mice (Menalled et al., 2012). Removing the 5' neo cassette from the huntingtin gene of zQ175 mice and changing the background strain to FVB/N led to the creation of the Q175FDN mouse line (Southwell et al., 2016). Q175FDN mice express higher levels of mutant huntingtin, have earlier age of onset, display more severe and wider variety of HD-like symptoms than other knock-in HD models, making them a valuable addition to HD research (Southwell et al., 2016).

1.6 NMDA Receptor-Mediated Excitotoxicity in HD

Excitotoxicity has received considerable attention in the effort to determine why striatal MSNs are selectively targeted in HD despite the ubiquitous expression of huntingtin. Now, several lines of evidence support the excitotoxicity hypothesis, which stipulates that excessive activation of glutamate receptors, likely due to a combination of increased release of glutamate from cortico-striatal afferents, reduced uptake by glial cells, hypersensitivity of glutamate receptors and pathological signalling downstream of glutamate receptor activation, lead to death of MSNs in the striatum. Early radioligand-binding studies show that there is disproportionate loss of NMDA receptor in the striatum of symptomatic and pre-symptomatic HD patients (Albin et al., 1990; Young et al., 1988). Around the same time, it was also demonstrated that direction injection of

NMDA receptor agonist, such as quinolinic acid, into rodent striatum leads to HD-like lesions in which MSNs are selectively lost but interneurons remain largely unaffected (Beal et al., 1991; Beal et al., 1986). Analysis of NMDA receptor subunit expression reveals decreased mRNA and protein levels of NR2A and NR2B subunits in the striatum of HD mouse models (Cepeda et al., 2001). Furthermore, striatal neurons from transgenic mouse model of HD show increased sensitivity to NMDA receptor-mediated cell death that directly correlates with the length of polyglutamine repeats (Shehadeh et al., 2006; Zeron et al., 2002). Based on these observations, it appears that striatal MSNs are more vulnerable to HD-associated neurodegeneration and lost preferentially in HD patients due to their high expression of NMDA receptors.

More recent evidence indicates that mutant huntingtin directly alters the activity of NMDA receptors through multiple mechanisms. The post-synaptic density protein 95 (PSD95) interacts with both wild-type huntingtin and the C-terminus of NMDA receptor to regulate the clustering of receptor on the plasma membrane (Sun et al., 2001). Mutant huntingtin interferes with the interaction between PSD95 and wild-type huntingtin, which enhances the binding of NMDA receptor to PSD95 and increase their activity to eventually trigger excitotoxic neuronal death (Sun et al., 2001). Mutant huntingtin also changes the phosphorylation of NMDA receptors by tyrosine kinases. In cells expressing mutant huntingtin, the level of phosphorylated NR2B subunits is increased due to higher activity of the Src family kinases (Song et al., 2003). Phosphorylation of Y837 in NR1, Y842 in NR2A and Y1472 in NR2B stabilizes cell surface NMDA receptors by blocking the binding of clathrin adapter protein AP-2 and inhibiting NMDA receptor endocytosis (Roche et al., 2001). Consistent with these results, NMDA receptor responsiveness is found to be significantly increased in striatal neurons of multiple HD mouse models. In fact, the amplitude of currents generated by NMDA exposure is higher in both pre-symtomatic and symptomatic HD

mice (Raymond, 2003). Enhanced NMDA receptor activity results in higher basal intracellular Ca^{2+} levels and stronger calcium response upon NMDA exposure (Raymond, 2003).

Some studies have suggested that the specific vulnerability of medium spiny neurons in HD is caused by the subunit composition of their NMDA receptors. Specifically, MSNs expresses the NR2B units at a much higher level than other NR2 subunits (Rigby et al., 1996). In fact, the ratio of NR2B to NR2A is significantly higher in the striatum than in the hippocampus and cortex, two brain regions that are much less vulnerable in HD (Raymond, 2003). In cell cultures, mutant huntingtin expression increases the current through NMDA receptors composed of NR1/NR2B subunits, whereas NMDA receptors with NR1/NR2A subunits show similar levels of current when co-expressed with either mutant or wild-type huntingtin (Chen et al., 1999). Furthermore, cells co-transfected with mutant huntingtin and NR1/NR2B NMDA receptors are significantly more vulnerable to NMDA-induced cell death than cells co-transfected with mutant huntingtin and NR1/NR2A NMDA receptors (Zeron et al., 2001). NMDA receptor antagonist that are selective for the NR1/NR2B receptor subtype are just as effective as MK801, an inhibitor that targets both NR1/NR2A and NR1/NR2B receptor subtypes, at protecting striatal neurons from NMDA-induced cell death (Zeron et al., 2002). Finally, in cerebellar granule neurons from wild-type and HD mice, NMDA exposure induced similar levels of cell death as these neurons only express NMDA receptors containing NR2A and NR2C subunits (Vallano et al., 1996).

Recent studies have begun to distinguish the role of synaptic NMDA receptors from extrasynaptic NMDA receptors. The earliest piece of evidence supporting this line of thought comes involves the demonstration that Ca^{2+} entry though synaptic and extrasynaptic NMDA receptors have the opposite effect on CREB activity and BDNF expression (Hardingham et al., 2002). In hippocampal neurons, stimulation of synaptic NMDA receptors triggered

phosphorylation of CREB at serine-133, increased CREB activity in the nucleus and induced BDNF gene expression (Hardingham et al., 2002). In contrast, activation of extrasynaptic NDMA receptors via glutamate bath initiated a dominant shut off signal for CREB activity, as phosphorylation of CREB at serine 133 rapidly decayed (Hardingham et al., 2002). More importantly, extrasynaptic NMDA receptors in hippocampal neurons are predominantly composed of NR1 and NR2B subunits, whereas synaptic NDMA receptor contains a mixture of both NR2A and NR2B (Hardingham et al., 2002). Therefore, the selective inhibitor of NR2B-containing NMDA receptor ifenprodil prevented initiation of the CREB shut-off pathway by extrasynaptic NMDA receptors (Hardingham et al., 2002). Similar results were also obtained in primary cortical neurons in which selective activation of extrasynaptic NMDA receptors did not activate beneficial extracellular signal-regulated kinase (ERK) pathways like synaptic NMDA receptors but instead triggered mitochondrial dysfunction and cellular damage (Leveille et al., 2008). Furthermore, synaptic NMDA receptors boost antioxidant defense in rat neurons, but activation of both synaptic and extrasynaptic NMDA receptors simultaneously blocks this process. In transgenic HD mice expressing full-length human mutant huntingtin, there is increased expression of NR2B-containing extrasynaptic NMDA receptors in the striatum (Milnerwood et al., 2010). This is accompanied by augmented extrasynaptic NMDA receptor signalling at ages before and after phenotype onset (Milnerwood et al., 2010). Treatment of the HD mice with low doses of the weak NMDA receptor antagonist memantine, which preferentially targets extrasynaptic NMDA receptor signalling, rescued their motor learning deficit (Milnerwood et al., 2010). A later study expanded upon this discovery and showed that low doses of memantine reduces extrasynaptic NMDA receptor expression, rescues CREB shut-off and blocks excessive activation of p38 mitogen-activated protein kinases (MAPK) in transgenic HD mice (Dau et al., 2014).

Collectively, ample amount of evidence has shown that dysregulation of NMDA receptor signalling in HD contributes to the selective loss of MSNs and plays a key role in the pathogenesis of the disease. Therefore, multiple therapeutic agents targeting NMDA receptors have been tested for treatment of HD. NMDA receptor antagonists including amantadine, memantine, ketamine, milacemide, remaceide and riluzole have been gone through clinical trials (Fan & Raymond, 2007). Amongst them, amantadine and memantine showed promising results in preliminary studies (Beister et al., 2004; Heckmann et al., 2004). Both amantadine and memantine are weak, non-competitive but highly selective NMDA receptor antagonists (Lipton, 2006). These properties greatly enhance the clinical tolerability of the two drugs by allowing them to limit the pathological hyperactivity of NMDA receptors, especially at the extrasynaptic sites, while sparing normal synaptic activities (Lipton, 2006).

1.7 AMPA and Kainate Receptors in HD

While NMDA receptors have received the most amount of attention in HD research, some evidence also show that deficits in AMPA receptor is involved in HD. In postmortem HD brains, the expression of AMPA receptor GluA2 subunit is significantly reduced in the putamen compared to control levels (Fourie et al., 2014). In contrast, the levels of GluA2 expression in hippocampal dentate gyrus, CA1 area and CA3 area remained similar between HD and healthy brains (Fourie et al., 2014). Furthermore, expression of mutant huntingtin in neuronal cultures reduced the amplitude and frequency of AMPA receptor-mediated excitatory postsynaptic current, whereas expression of wild-type huntingtin increased both (Mandal et al., 2011). Similarly, AMPA receptor-mediated synaptic transmission is impaired in a transgenic mouse model of HD. It appears that mutant huntingtin disrupts the trafficking of AMPA receptors by interfering with HAP1 and kinesin-related protein 5 (Kif5), as the complex of GluA2/Kif5/HAP1 is found to be dissociated

from microtubules in transgenic mouse model of HD (Mandal et al., 2011). A recent study in 2009 showed that upregulating the activity of AMPA receptors through the use of positive allosteric modulators (PAM) can normalize BDNF levels, rescue synaptic plasticity and improve long-term memory in knock-in mouse model of HD (Simmons et al., 2009). More long-term treatment with AMPA receptor PAM in R6/2 HD mice slows the progression of HD phenotypes, blocks the decrease in total striatal area, prevents the loss of striatal neurons and reduces the size of huntingtin aggregates (Simmons et al., 2011). It was also revealed that decreased BDNF levels in HD impairs BDNF-Tropomyosin receptor kinase B (TrkB) signalling, which subsequently contributes to the dysregulation of AMPA receptor surface diffusion (Zhang et al., 2018). Impaired BDNF-TrkB signalling reduces interaction between transmembrane AMPA receptor regulatory proteins and PSD95 (Zhang et al., 2018). This prevents the accumulation of AMPA receptors at synapses following synaptic potentiation stimuli, leading to poor activity-dependent synaptic plasticity in HD (Zhang et al., 2018). Restoring BDNF levels with the antidepressant tianeptine rescues hippocampal plasticity and hippocampus-dependent memory function in transgenic HD mice (Zhang et al., 2018). Therefore, disruption of AMPA receptor function can contribute to cognitive impairments and psychiatric disturbances in HD patients.

Compared to both NMDA and AMPA receptors, the role of kainate receptors in HD is much less clear. The injection of kainic acid into the striatum of rats caused biochemical changes and neurodegeneration that mimic those observed in HD patients (Coyle & Schwarcz, 1976). Early study showed that kainate receptor binding is found to be significantly reduced in layer VI of frontal cortex in HD brains (Wagster et al., 1994). Furthermore, a study linked genotype variations in kainate receptor GluK2 subunit to age of onset in HD patients (Rubinsztein et al., 1997). However, a later study using a much larger sample found no evidence that polymorphism in the

gene encoding GluK2 has any significant effect on the onset of motor, cognitive and psychiatric impairments in HD patients (Lee et al., 2012). GluK2 knockout mice does not display more striatal damage and motor impairments than wild-type mice following treatment with 3-nitropropionic acid, a chemical that causes striatal metabolic failure and excitotoxic damage similar to those observed in HD (Diguet et al., 2004). Nevertheless, GluK2 knockout mice show earlier age of death and motor symptoms after 3-nitopropionic acid injection, suggesting that GluK2-containing kainate receptors is still implicated in the neurodegeneration process (Diguet et al., 2004). So far, evidence indicate that kainate receptors, especially ones containing the GluK2 subunit, plays a complex role in the pathogenesis of HD. However, the mechanism(s) underlying their contribution to HD remains unclear. Therefore, better understanding of kainate receptor signalling in HD brains may help the discovery of treatment for the disease.

1.8 Group I Metabotropic Glutamate Receptors

In the 1990s, functional cloning studies led to the discovery of eight subtypes of mGluRs, which are subsequently divided into three groups based on the sequence homology and signal transduction mechanisms (**Figure 1.1)**. The 4 splice variants of mGluR1 and 2 splice variants of mGluR5 make up the group I mGluRs. Activation of mGluR1 and mGluR5 is positively coupled to the activity of phospholipase C (PLC), which cleaves phosphatidylinositol-4,5-biphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) (Aramori & Nakanishi, 1992). Both DAG and IP3 can activate a variety of signalling pathways, including release of Ca^{2+} from the ER due to the binding of IP3 to IP3 receptors on the ER surface and activation of protein kinase C (PKC) by DAG (**Figure 1.4**) (Ribeiro et al., 2017).

In-situ hybridization studies reveal high levels of mGluR1 mRNA in the neurons of the cerebellum, olfactory bulb, hippocampus, lateral septum, thalamus, globus pallidus,

entopeduncular nucleus, ventral pallidum, magnocellular preoptic nucleus, substantia nigra, and dorsal cochlear nucleus (Shigemoto et al., 1992). In comparison, mGluR5 is found in the olfactory bulb, anterior olfactory nuclei, olfactory tubercle, cerebral cortex, hippocampus, lateral septum, striatum, nucleus accumbens, inferior colliculus, and spinal trigeminal nuclei (Shigemoto et al., 1993). Interestingly, mGluR5 but not mGluR1 mRNA is detected in astrocytes and microglia (Biber et al., 1999). Furthermore, while group I mGluRs are found in presynaptic terminals, they are predominantly located in postsynaptic terminals at the periphery of the synaptic sites. The effects of group I mGluRs activation is primarily involved in postsynaptic excitatory responses. In fact, mGluR5 physically links with NMDA receptors through Homer, SH3 and multiple ankyrin repeat domains protein (SHANK), guanylate kinase-associated protein (GKAP) and PSD95 (**Figure 1.4**) (Abd-Elrahman & Ferguson, 2022). The production of DAG and the release of intracellular Ca^{2+} downstream of group I mGluR signalling lead to the activation of PKC, which has been shown to increase NMDA receptor currents by sequentially activating the focal adhesion tyrosine kinase Pyk2 and Src protein tyrosine kinases (**Figure 1.4**) (Lu et al., 1999; Nicodemo et al., 2010). Pyk2 is located within the postsynaptic density and can recruit Src to the NMDA receptor complex to potentiate NMDA receptor current (Abd-Elrahman & Ferguson, 2022). Another mechanism by which mGluR5 activation can lead to increased NMDA receptor current involves CamKII (**Figure 1.4**). It has been reported that CamKIIα binds directly to the C-terminal tail and intracellular loop 2 of mGluR5, and upon mGluR5-stimulated Ca^{2+} release, CamKIIα dissociates from mGluR5 to phosphorylate NR2B (Jin et al., 2013). mGluR5 activation is also linked to the activation of Fyn, another Src family protein that phosphorylates NR2B following mGluR5 activation (Abd-Elrahman & Ferguson, 2022). Interestingly, the activation of PKC by mGluR5 signalling also lead to rapid desensitization of mGluR5 due to PKC-mediated

phosphorylation of mGluR5 at multiple sites (Gereau & Heinemann, 1998). Activation of NMDA receptor reverses PKC-mediated desensitization of mGluR5 by activating protein phosphatase 2B (PP2B)/Calcineurin (CaN) to dephosphorylate mGluR5 (Alagarsamy et al., 1999).

In addition to its effects on NMDA receptors, mGluR5 signalling is also involved in a wide variety of other critical cellular pathways. For example, mGluR5 has been shown to promote the activity of extracellular signal-regulated kinases 1/2 (ERK1/2), an important regulator of gene transcription and protein synthesis through multiple independent pathways (**Figure 1.4**). First, PKC has been proposed to activate mitogen-activated protein kinase kinases (MEK) to upregulate the activity of ERK1/2 (Exton, 1999; Naor et al., 2000; van Rossum & Patterson, 2009). However, mGluR5-mediated activation of ERK1/2 has also been shown to be resistant to PKC and PLC inhibitors (Abd-Elrahman & Ferguson, 2022). This is likely because mGluR5 signalling also facilitate ERK1/2 activation through separate mechanisms involving β-arrestin 2 and Homer (Eng et al., 2016; Mao et al., 2005; Stoppel et al., 2017). In fact, it is reported that ERK1/2 needs to be co-activated through both the PKC- and Homer-mediated pathways to phosphorylate two transcription factors, Elk-1 and CREB, that are important for c-Fos expression (Mao et al., 2005). Currently, the relative contribution of these pathways to mGluR5-mediated ERK1/2 activation under normal physiological conditions or in neurodegenerative diseases is unclear.

Homer protein also plays an important role in linking mGluR5 signalling to the phosphoinositide 3 kinases (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway (**Figure 1.4**). Specifically, upon agonist activation, there is enhanced complex formation between mGluR5, Homer and the GTPase phosphoinositide 3 kinase enhancers (PIKE) (Guhan & Lu, 2004; Rong et al., 2003). This leads to increased binding of PIKE to the p85 subunit of PI3K and activation of PI3K activity (Guhan & Lu, 2004). Subsequently, PI3K converts PIP2 into

phosphatidylinositol-3,4,5-triphosphate, which in turn activates phosphoinositide-dependent kinase 1/2 (PDK1/2) (Porta et al., 2014). Phosphatidylinositol-3,4,5-triphosphate also recruits Akt to the cell membrane where it is phosphorylated by PDK1/2 (Porta et al., 2014). Akt is a key regulator of many cellular pathways, with the mTOR signalling complex being one of its most important downstream target that plays a central role in cell metabolism, growth, proliferation and survival (Laplante & Sabatini, 2009). There is also evidence that suggests the activation of mGluR5 increases the DNA binding activity of nuclear factor κB (NF-κB), a dimeric transcription factor involved in synaptic plasticity and long-term memory formation (Albensi & Mattson, 2000; Mattson et al., 2000). Furthermore, mGluR5 appears to activate NF-κB through several signalling pathways involving PI3K, PKC, ERK1/2 and p38-MAPK (O'Riordan et al., 2006). Ultimately, the activation of mGluR5 likely leads to enhanced phosphorylation of the inhibitor κB (IκB) by the IκB kinase complex (IKK), promoting its degradation and allowing nuclear translocation of NF-κB. In cultured striatal neurons, mGluR5 activation caused a dynamic transactivation of epidermal growth factor (EGF) receptor (**Figure 1.4**) (Yang et al., 2006). This event triggers increased phosphorylation of c-Jun N-terminal kinase (JNK), which subsequently phosphorylates and activates c-Jun to enhance activator protein 1 (AP-1)-mediated transcription (Yang et al., 2006).

Overall, mGluR5 plays a significant role in synaptic plasticity, protein synthesis and gene transcription through multiple G-protein-dependent and independent pathways. The vast interacting partners and diverse signal transduction mechanisms of mGluR5 make it an attractive pharmacological target for the treatment of neurodegenerative conditions. Indeed, there is now substantial evidence that suggest aberrant mGluR5 signalling is involved in neurodegenerative diseases such HD and targeting the receptor using allosteric modulators can be highly beneficial while minimizing side effects.

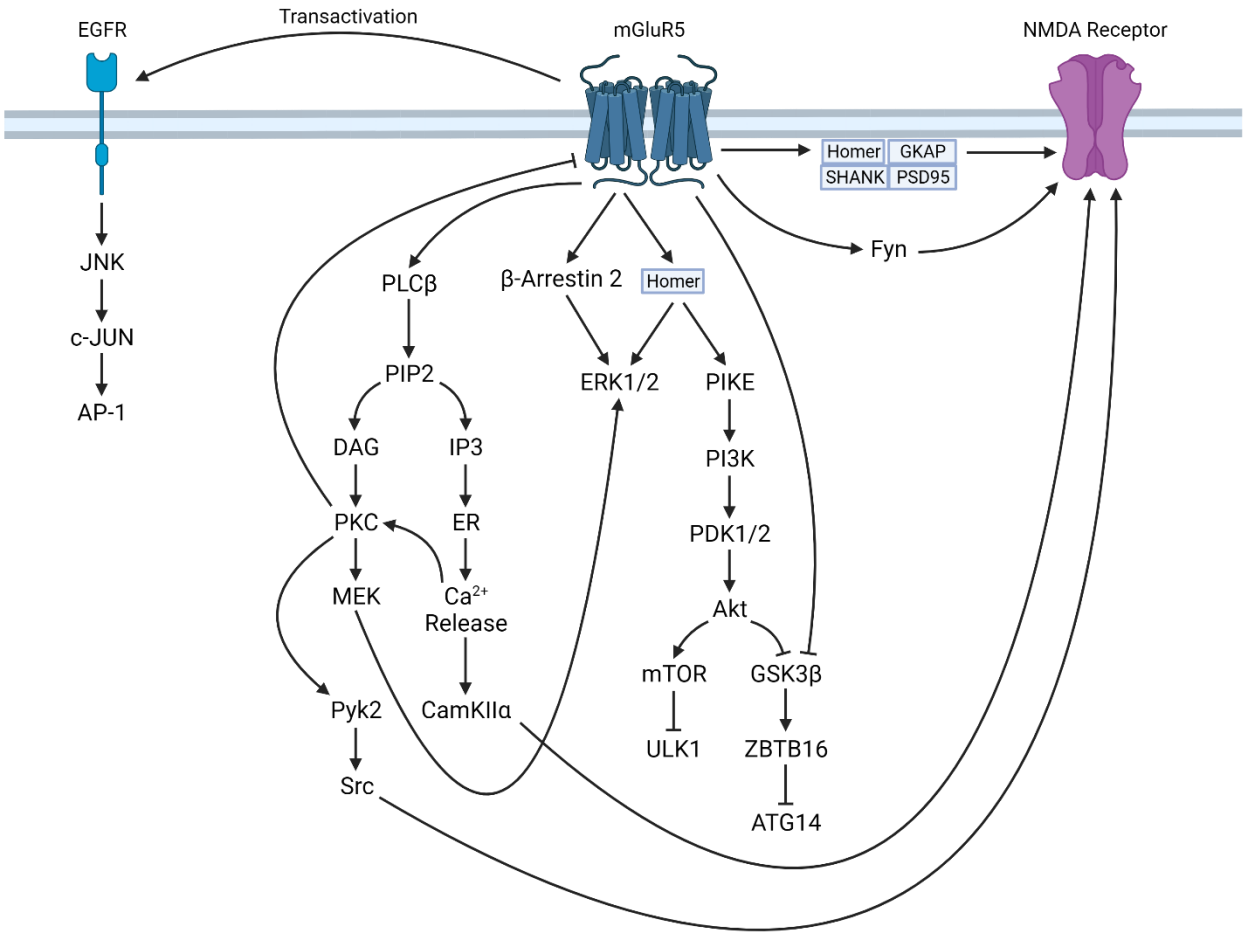

Figure 1.4 Overview of mGluR5 signalling. Activation of mGluR5 stimulates PLCβ to cleave PIP2 into DAG and IP3. IP3 triggers Ca^{2+} release from internal storages. Both Ca^{2+} and DAG activate PKC, which activates MEK to promote the activity of ERK1/2. PKC also facilitates mGluR5 desensitization. Furthermore, mGluR5 signalling activates ERK1/2 through β-arrestin 2- and Homer-mediated pathways, independent of PKC. mGluR5 is physically linked to NMDA receptors via Homer, SHANK, GKAP and PSD95. mGluR5 sequentially activates Pyk2 and Src via PKC to potentiate NMDA receptor current. In addition, mGluR5-stimulated Ca^{2+} release activates CaMKII, which can stimulate NMDA receptor activity. mGluR5 is also linked to the activation of Fyn, another Src family kinase that can promote NMDA receptor activity. mGluR5 signalling enhances the activity of the PIKE/PI3K/PDK1/2 pathway, leading to the activation of Akt. Akt can activate mTOR to regulate cell metabolism, growth, proliferation and survival. mTOR also phosphorylates ULK1 and inhibits its activity. Moreover, mGluR5 directly, or indirectly via Akt, inhibits GSK3β-mediated phosphorylation and degradation of ZBTB16. This consequently promotes the ubiquitination and proteasomal degradation of ATG14. mGluR5 activation leads to transactivation of EGF receptors, which subsequently phosphorylates and activates c-Jun to enhance AP-1 activity. Abbreviations: Akt, protein kinase B; AP-1, activator

protein 1; ATG14, autophagy-related protein 14; CaMKII, Ca2+/calmodulin-dependent protein kinase II; DAG, diacylglycerol; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinase 1/2; GKAP, guanylate kinase-associated protein; GSK3β, glycogen synthesis kinase 3β; MEK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NMDA, N-methyl-D-aspartate; PDK1/2, phosphoinositide-dependent kinase 1/2; PI3K, phosphoinositide 3 kinase; PIKE, phosphoinositide 3 kinase enhancer; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol-1,4,5-triphosphate; PKC, protein kinase C; PLCβ, phospholipase C-β; PSD95, postsynaptic density protein 95; SHANK, SH3 and multiple ankyrin repeat domains protein; ULK1, unc-51-like kinases 1, ZBTB16, zinc finger and BTB domain–containing protein 16. The figure is created using BioRender.

1.9 Metabotropic Glutamate Receptor 5 in HD

mGluR5 activation can greatly increase the intracellular Ca^{2+} concentration by triggering its release from intracellular storage and enhancing its influx via sensitizing NMDA receptors. Since the disruption of calcium homeostasis and the resulting excitotoxic damage is heavily implicated in the pathogenesis of HD, mGluR5 has received considerable attention as a potential pharmacological target for treating HD. mGluR5 is highly expressed in brain regions affected by HD, including the striatum, cortex and hippocampus (Shigemoto et al., 1993). More importantly, mGluR5 has been shown to interact with wild-type and mutant huntingtin as part of a complex that includes the autophagy adapter protein optineurin and the small GTPase Rab8 (Anborgh et al., 2005; Esseltine et al., 2012). This interaction appears to have functional consequences, as in striatal cells derived from HdhQ111 HD mice, IP3 formation following mGluR5-stimulation by quisqualate is significantly hindered compared to striatal cells from control mice (**Figure 1.3)** (Anborgh et al., 2005; Ribeiro et al., 2010). Furthermore, the decoupling of mGluR5 signalling from IP3 formation is mediated by PKC and inhibition of PKC activity restored IP3 formation to normal levels (Ribeiro et al., 2010). Interestingly, PKC-mediated desensitization of mGluR5 is actually protective against glutamate-induced neuronal cell death and it is only present in presymptomatic mice, suggesting that this is actually a compensatory mechanism and it is gradually lost with age in HD (Ribeiro et al., 2010). A potential mechanism underlying the protective effect of mGluR5 desensitization may be the fact that mutant huntingtin, but not wild-type huntingtin, can sensitize type 1 IP3 receptors located on the ER membrane, leading to elevated Ca^{2+} release upon mGluR5 activation (Tang et al., 2003). In fact, treating R6/2 HD mice with the mGluR5 antagonist MPEP improves their survival time and rescues the decline in motor coordination (Schiefer et al., 2004). Consistent with these observations, treatment of HdhQ111

mice with the mGluR5 antagonist MTEP increases their locomotor activity (Ribeiro et al., 2010). Moreover, genetic deletion of mGluR5 in HdhQ111 mice improves their rotarod performance and reduces the number of mutant huntingtin aggregates in their brain (Ribeiro et al., 2010).

Similarly, administration of the mGluR5 negative allosteric modulator (NAM) CTEP in zQ175 HD mice rescues their motor and cognitive impairments (Abd-Elrahman et al., 2017). The improvements in disease symptoms are accompanied by decreases in neuronal apoptosis, caspase-3 activity and mutant huntingtin aggregate deposition (Abd-Elrahman et al., 2017). The reduction in huntingtin aggregate burden appears to be facilitated by the activation of two different autophagy pathways. First, mGluR5 antagonism leads to decreased inhibitory phosphorylation of Unc-51-like kinases 1 (ULK1), which is an important regulator of autophagosome formation (**Figure 1.4**) (Chan et al., 2007). Active ULK1 forms a complex with autophagy-related protein 13 (ATG13) and focal adhesion kinase family-interacting protein of 200 kDa (FIP200) at the autophagic isolation membrane to promote the formation of autophagosomes and initiate autophagy (Ganley et al., 2009). This reduction in ULK1 phosphorylation may be facilitated by mGluR5 antagonism correcting enhanced PI3K/Akt/mTOR signalling in zQ175 mice, as mTOR is well known to directly phosphorylate ULK1 (Abd-Elrahman & Ferguson, 2019; Kim et al., 2011). The second autophagy pathway activated by chronic CTEP administration involves glycogen synthesis kinase 3β, zinc finger and BTB domain–containing protein 16 (ZBTB16) and autophagy-related 14 (ATG14) (**Figure 1.4**) (Zhang et al., 2015). ZBTB16 is a part of multiple cullin-RING-based E3 ubiquitin–protein ligase complexes that can ubiquitinate ATG14, a key component of the vacuolar protein sorting 34 (VPS34) autophagic complex, to promote its proteasomal degradation (Zhang et al., 2015). However, GSK3β can phosphorylate ZBTB16 at S184/T282 to promote ZBTB16 autoubiquitination and lysosomal degradation (Zhang et al., 2015).

Furthermore, GSK3β-mediated phosphorylation of ZBTB16 disrupts its binding to ATG14, leading to increased availability of ATG14 to promote autophagy (Zhang et al., 2015). CTEP-mediated antagonism of mGluR5 can activate this autophagy pathway by reducing inhibitory phosphorylation of GSK3β, which resulted in reduced ZBTB16 protein level, stabilization of ATG14 and increased autophagic removal of huntingtin aggregates (Abd-Elrahman et al., 2017). mGluR5 signalling has also been linked to REST/NRSF, a key regulator of huntingtin-mediated gene transcription whose expression is found to be dysregulated in HD (Zuccato et al., 2003). REST/NRSF expression is regulated by the canonical Wnt/β-catenin pathway and mGluR5 modulates the phosphorylation of β-catenin by Src kinase (de Souza et al., 2020; Grigoryan et al., 2008). Both antagonism of mGluR5 signalling with CTEP in zQ175 HD mice and genetic ablation of mGluR5 in BACHD mice reduce REST/NRSF expression and increase expression of its target genes (de Souza et al., 2020). Chronic mGluR5 antagonism also appears to promote CREB activity to enhance the expression of cFos and BDNF (Abd-Elrahman & Ferguson, 2019).

Despite ample evidence suggesting that mGluR5 signalling contributes to the pathogenesis of HD and its antagonism may improve HD symptoms, it is important to consider that mGluR5 can also play a neuroprotective role. In fact, several pathways downstream of mGluR5 activation, including ERK1/2 and Akt/mTOR, are important for cell survival and genetic knockout of mGluR5 in mice leads to neurodegeneration similar to that observed in HD mice (Carvalho et al., 2019). Particularly, increased Akt activity is neuroprotective in HD since it has been shown that Akt can phosphorylate mutant huntingtin at serine 421 and reduces nuclear accumulation of mutant huntingtin fragments (Humbert et al., 2002; Warby et al., 2009). Indeed, activation of mGluR5 with its positive allosteric modulators (PAM) CDPPB and VU1545 protects neurons against glutamate-induced cell death through mechanism that is dependent on Akt activation (Doria et al.,

2013). Furthermore, treatment of BACHD mice with CDPPB increases both ERK1/2 and Akt activation, enhances the level of BDNF mRNA and decreases huntingtin aggregate formation (Doria et al., 2015). These improvements are accompanied by normalization of memory deficits and partial rescue of motor coordination (Doria et al., 2015). More recently, it was demonstrated that another mGluR5 PAM, VU0409551, can increase the level of mGluR5 at the plasma membrane. This consequently increases the expression of several genes important for synaptic plasticity, including BDNF, cytoskeletal-associated protein (Arc/Arg3.1), c-Fos, syntaxin 1A and PSD-95 (Doria et al., 2018). VU0409551 treatment for 8 days in BACHD mice also fully rescues their memory deficits and increases the density of mature dendritic spines and presynaptic terminals in their hippocampus (Doria et al., 2018). Interestingly, genetic ablation of mGluR5 in BACHD mice actually exacerbates some of the HD-related alterations (de Souza et al., 2022). One important characteristic of mGluR5 PAMs is their ability to bias mGluR5 signalling to favor the activation of pro-survival pathways over those that contribute to excitotoxicity. Indeed, both CDPPB and VU0409551 are shown to promote Akt phosphorylation without triggering an increase in intracellular Ca^{2+} concentrations (Doria et al., 2013).

Overall, considerable evidence indicates that mGluR5 plays an important role in HD. However, the exact mechanism(s) by which mGluR5 influences the neurotoxic actions of mutant huntingtin is still unclear. This is further complicated by the fact that the activation of mGluR5 appears to have a dual function and is capable of activating either pro-survival or pro-death pathways depending on the type of drug. So far, both mGluR5 NAMs and PAMs have proven to be beneficial in multiple mouse models of HD. These are biased ligands that can favor the activation of specific pathways over the others. Therefore, it is possible that mGluR5 allosteric

modulators may be more effective at treating HD symptoms than competitive agonist/antagonists by selectively activating pro-survival pathways and/or inhibiting neurotoxic pathways.

1.10 Group II Metabotropic Glutamate Receptors

Group II metabotropic glutamate receptors, mGluR2 and mGluR3 (mGluR2/3), show roughly 67% sequence homology and distinct yet overlapping brain distribution pattern. Through *in situ* hybridization studies, mGluR2 transcripts are found to be abundant in the hippocampal dentate gyrus, main and accessory olfactory bulbs, cerebellum, thalamus and cerebral cortex of the rat central nervous system (Ohishi et al., 1993a). Moderate levels of mGluR2 mRNA are also found in neuronal cells of the septum, amygdala, striatum, globus pallidus, ventral pallidum and subthalamic nucleus (Ohishi et al., 1993a). mGluR3 mRNA is found in many of the same brain regions, including the hippocampal dentate gyrus, main olfactory bulb, cerebellum, cerebral cortex, amygdala, striatum, globus pallidus and ventral pallidum (Ohishi et al., 1993b). However, mGluR3 is not expressed in the accessory olfactory bulb, septum and subthalamic nucleus (Ohishi et al., 1993b). Furthermore, mGluR2 and mGluR3 are located in different regions of the thalamus, with mGluR2 showing expression in the anterior, midline and intralaminar nuclei, whereas mGluR3 mRNA is detected in the thalamic reticular nucleus (Ohishi et al., 1993b). One of the most important differences between mGluR2 and mGluR3 distribution pattern is observed in glial cells. mGluR3 but not mGluR2 is expressed in astrocytes, the most prevalent glial cell type (Abd-Elrahman et al., 2023). In contrast, both receptors are present on microglia and oligodendrocytes (Abd-Elrahman et al., 2023; Spampinato et al., 2018). Finally, in the hippocampus, cortex and striatum, mGluR2/3 are predominantly localized to presynaptic terminals at the extrasynaptic sites and to glial wrappings around the synapses (Petralia et al., 1996; Shigemoto et al., 1997; Testa et

al., 1998). However, low levels of postsynaptic mGluR2/3 can be found in the cortex and in the granule cells of the dentate gyrus molecular layer (Petralia et al., 1996; Shigemoto et al., 1997).

mGluR2/3 are coupled to inhibitory $G\alpha_{i/o}$ proteins and their activation inhibits adenyl cyclase to limits the production of cAMP (**Figure 1.5**) (Niswender & Conn, 2010). One of the key downstream effectors of cAMP is protein kinase A (PKA), which plays a unique role in neuronal development, synaptic plasticity and neurogenesis. More importantly, activation of mGluR2/3 by agonists reduces postsynaptic glutamate excitatory postsynaptic potentials (Schoepp, 2001). Specifically, upon their activation, the β and γ subunit of G protein mediate the inhibition of voltage gated calcium channels and activate G-protein-coupled inward rectifying potassium channels (GIRK) to inhibit glutamate release into the synaptic cleft (**Figure 1.5**) (Ribeiro et al., 2017; Schoepp, 2001; Sharon et al., 1997). Activation of group II mGluRs, likely mGluR3, with selective agonists also reduces glutamate in the synapses by facilitating its uptake by astrocytes. This is mediated by mGluR3 positively modulating the expression of glial glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) in astrocytes (**Figure 1.5**) (Aronica et al., 2003). The ability of mGluR3 to modulate glutamate transporter expression in astrocyte is facilitated by its downstream signalling through the ERK/PI3K/NFκB pathway (Aronica et al., 2003). Furthermore, the activation of mGluR3 in astrocyte has been linked to increased production and release of neurotrophic factors such as transforming growth factor β (TGFβ) to protect neighboring neurons from excitotoxic cell death (**Figure 1.5**) (Bruno et al., 1998).

mGluR2/3 also plays an important role in regulating the activity of NMDA receptors in neurons (**Figure 1.5**). In pyramidal neurons of the prefrontal cortex, activation of mGluR2/3 increases PKC-dependent phosphorylation of NMDA receptor containing the NR2A subunits, leading to enhanced currents through NMDA receptors (Tyszkiewicz et al., 2004). Similarly,

activation of mGluR2/3 in hippocampal CA1 pyramidal neurons also mediates the phosphorylation of NR2A-containing NMDA receptors by Src family kinases (Trepanier et al., 2013). Specifically, mGluR2/3 signalling inhibits the cAMP-PKA pathway, which decreases the phosphorylation of C-terminal Src kinase (Csk) by PKA and consequently prevents Csk from phosphorylating Src and keeping it in an inactive conformation (Trepanier et al., 2013). mGluR2/3 also directly modulates the exocytosis of NMDA receptors at postsynaptic sites. It is proposed that activation of mGluR2/3 enhances the activity of Rab4 and its interaction with syntaxin 4 (Stx4) to promote the formation of SNARE complex containing Stx4 and synaptosome-associated protein 25 (SNAP-25) at the postsynaptic sites and increase the delivery of NMDA receptors to the postsynaptic membranes (Cheng et al., 2013). Moreover, mGluR2/3 activation directly increases the expression of NMDA receptors and potentiates NMDA-induced currents via modulating the activity of the Akt/GSK3β pathway (Xi et al., 2011). In addition to NMDA receptors, evidence also suggest that mGluR2/3 signalling can also influence the activity of AMPA receptors, as application of mGluR2/3 agonists to cultured prefrontal cortical neurons elevates the surface and total expression of AMPA receptors through ERK1/2 and GSK3β signalling pathways (**Figure 1.5**) (Wang et al., 2013). Interestingly, it has also been reported that mGluR2/3 antagonism can increase mTOR signalling, as measured by higher levels of phosphorylated mTOR, p70S6 kinase and 4E-BP1, as well as ERK1/2 phosphorylation in rat prefrontal cortex (Dwyer et al., 2012).

Therefore, mGluR2/3 can regulate a variety of signalling pathways at both presynaptic and postsynaptic terminals. They also have wide distribution through out the central nervous system and modulate different signalling pathway depending on the region and cell type. This makes them attractive pharmacological targets for treating many neuropsychiatric and neurodegenerative diseases, particularly those in which abnormal glutamate signalling plays a significant role.

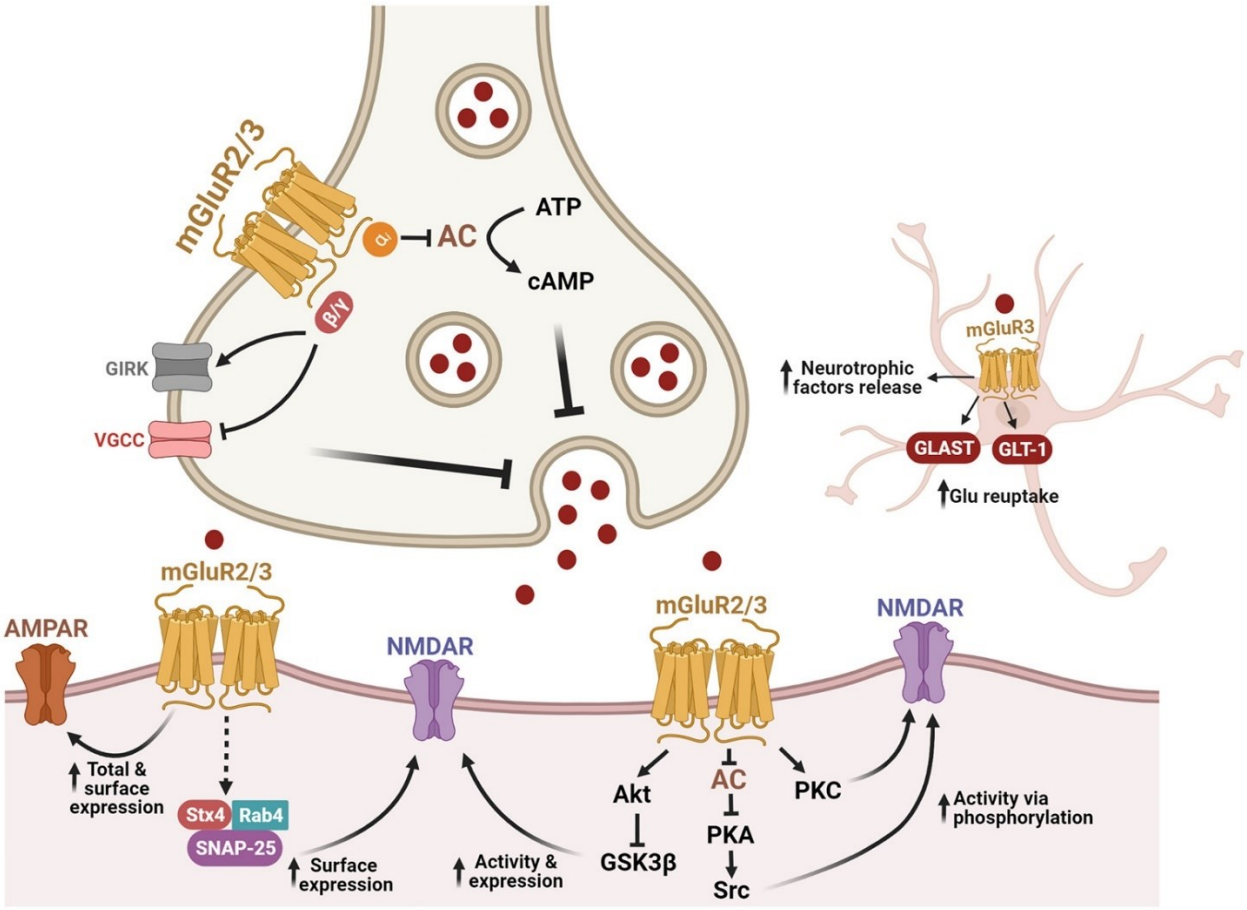

Figure 1.5 Overview of mGluR2/3 signalling. mGluR2/3 are predominantly located on presynaptic terminals but can also be found on postsynaptic terminals and in glial cells. Activation of presynaptic mGluR2/3 inhibits adenyl cyclase via $G_{\alpha i}$ and reduces the level of cAMP. $G_{\beta\gamma}$ subunits inhibit VGCC and activate GIRK to limit the release of glutamate from presynaptic neurons. In astrocytes, activation of mGluR3 upregulates surface expression of GLT1 and GLAST and increases the production of neurotrophic factors. Postsynaptic mGluR2/3 increase the phosphorylation of NMDA receptors through activation of PKC and Src. Postsynaptic mGluR2/3 also regulate the activity of the Akt/GSK3β pathway and increases the expression of both NDMA and AMPA receptors. Furthermore, mGluR2/3 can directly increase NMDA receptor exocytosis and upregulate its surface expression at postsynaptic sites through interaction with Rab4, SNAP-25 and Stx 4. Abbreviations: AC, adenyl cyclase; Akt, protein kinase B; GIRK, G-protein-coupled inward rectifying K^+ channels; GLAST, glutamate-aspartate transporter; GLT-1, glutamate transporter-1; GSK3β, glycogen synthase kinase 3β; PKC, protein kinase A; PKC, protein kinase C; SNAP-25, synaptosome-associated protein 25; Stx4, syntaxin 4; VGCC, voltage-gated calcium channels. The figure is retrieved from Li et al., 2022a.

1.11 Group II Metabotropic Glutamate Receptors in HD

As previously described, considerable evidence implicates excitotoxicity as one of the key factors in the pathogenesis of HD. Since mGluR2/3 are expressed on cortico-striatal presynaptic terminals and can modulate the levels of glutamate in the synapse through several different mechanisms, activation of mGluR2/3 to reduce the availability of extracellular glutamate and postsynaptic glutamate signalling has been proposed as a potential strategy to treat HD. In fact, a study reveals that mGluR2/3 ligand binding is lower in R6/2 transgenic HD mice compared to age-matched controls due to decreased expression of mGluR2/3 mRNA (Cha et al., 1998). This suggest that glutamate release may be dysregulated in HD due to the loss of mGluR2/3. Indeed, early in-vitro studies demonstrated that selective agonists of mGluR2/3, such as L-CCG-I, 4C3HPG and DCG-IV, can protect cultured cortical neurons from excitotoxic cell death (Bruno et al., 1994; Buisson & Choi, 1995; Buisson et al., 1996). Similarly, the more potent and selective mGluR2/3 agonists LY354740, LY379268 and LY389795 are all protective against toxicity induced by NMDA, kainate and staurosporine (Kingston et al., 1999). Furthermore, LY354740 partially prevents apoptosis of CA1 hippocampal neurons under ischemic conditions (Kingston et al., 1999). Interestingly, the neuroprotective effect of mGluR2/3 agonists is enhanced in mixed cultures containing both glial cells and neurons compared to pure neuronal cultures (Kingston et al., 1999). Inhibition of RNA and protein synthesis also neutralises the protective effects of LY354740 against NMDA-induced toxicity in vivo (Kingston et al., 1999). These results are consistent with the observation that the neuroprotective activity of group II mGluRs is dependent on the production of neurotrophic factors by glial cells (Bruno et al., 1998; Bruno et al., 1997). Moreover, it is shown that the neuroprotection offered by DCG-IV treatment is not dependent on the modulation of glutamate release (Buisson et al., 1996). However, there is also contradictory evidence that showed LY354740 at low concentrations can not prevent the death of cultured

cortical neurons upon NMDA exposure (Behrens et al., 1999). Raising the drug concentration to much higher levels results in partial protection against NMDA-induced cell death (Behrens et al., 1999).

While the mechanisms underlying the neuroprotective effects of mGluR2/3 agonists remains unclear, there is promising evidence that suggests mGluR2/3 activation can improve HD symptoms in preclinical studies. In R6/2 transgenic HD mice, administration of LY379268 at a low dosage (1.2 mg/kg) significantly increases survival time and attenuates pathological hyperactivity, but fails to rescue decline in motor coordination on the rotarod or reduce mutant huntingtin intranuclear inclusion (Schiefer et al., 2004). Increasing the concentration of LY379268 administered to its maximum tolerated dose (20 mg/kg) leads to a more diverse set of benefits, including improved the rotarod performance, rescued striatal neuron loss and increased survival time, in R6/2 HD mice (Reiner et al., 2012a). These improvements in R6/2 HD mice correlate with increased BDNF production in layer V neurons of the motor cortex , which projects to the striatum, and a rescue of enkephalin-containing striatal neurons (Reiner et al., 2012b). LY379268 treatment also enhances the expression of substance P (SP) in SP striatal neurons, but this does not correlate with the change in BDNF production, suggesting that LY379268 treatment benefits the two types of striatal projection neuron through distinct mechanisms (Reiner et al., 2012b). Interestingly, heterozygous BDNF knockout in R6/1 HD mice exacerbates the loss of enkephalinergic neurons but not SP neurons (Canals et al., 2004). Moreover, the infusion of exogenous BDNF rescues the loss of enkephalinergic neurons but has no effect on the number of SP neurons (Canals et al., 2004). Therefore, LY379268 treatment may improve HD symptoms in R6/2 mice through both decreasing cortico-striatal glutamate release and increasing the production of BDNF. mGluR3 is also

expressed on some neurons of the striatum, thus it is possible that LY379268 also acts directly on these neurons to protect them in HD (Testa et al., 1994).

Another mechanism by which mGluR2/3 activation can help reduce HD brain pathology is through promoting glutamate reuptake by glial cells. In post-mortem brains of HD patients, the uptake of glutamate is severely impaired, and the level of impairment inversely correlates with the length of CAG repeats (Hassel et al., 2008). Consistent with this observation, post-mortem brains of HD patients and several HD mouse models show deficits in GLT-1 expression and function (Huang et al., 2010; Lievens et al., 2001). Furthermore, the expression of mutant huntingtin in astrocytes reduces GLT-1 expression, impairs glutamate uptake and triggers HD-like neuronal dysfunction (Bradford et al., 2009; Faideau et al., 2010). As previously mentioned, mGluR3 is expressed on astrocytes and its activation increases the expression of both GLT-1 and GLAST, leading to increased glutamate uptake (Aronica et al., 2003). Therefore, it is possible that pharmacological activation of mGluR2/3 can also reduce glutamate over-exposure by facilitating its uptake from the synaptic cleft into astrocytes. In fact, continuous administration of LY379268 via implanted osmotic pumps has been shown to increase GLT-1 expression in the spinal cord of SOD1G93A mice (Battaglia et al., 2015). Moreover, LY379268 treatment also increases the expression glial derived neurotrophic factor (GDNF) in both SOD1G93A mice spinal cord and cultured striatal neurons (Battaglia et al., 2009; Battaglia et al., 2015).

In summary, excitotoxicity due to an overabundance of extracellular glutamate appears to play a key role in the preferential loss of MSNs that takes place in HD brain. Activation of mGluR2/3 can reduce the concentration of extracellular glutamate by preventing its release from presynaptic terminals and promoting its removal by glial cells. Moreover, mGluR2/3 signalling is linked directly to pro-survival cellular pathways, such as enhanced expression of BDNF, which

may also alleviate HD symptoms. Taken together, mGluR2/3 activators appear to be a very promising approach in mitigating HD pathology and symptoms.

1.12 Sex-Specific Differences in Neurodegenerative Diseases

In recent years, growing amount of evidence suggests that sex can influence the progression, age of onset and prevalence of multiple neurodegenerative diseases, including AD, PD and amyotrophic lateral sclerosis (ALS) (Hanamsagar & Bilbo, 2016). For instance, AD disproportionately affects women both in terms of occurrence and symptom severity (Ferretti et al., 2018). Currently, there are twice as many female AD patients as males. Within AD patients, hippocampal atrophy is observed to progress at a much faster rate in females than males (Ardekani et al., 2016). Consistent with this observation, the rate of cognitive decline is also higher in females than males (Lin et al., 2015). Furthermore, male AD patients outperformed female patients in several tasks designed to measure language and semantic abilities, visuospatial abilities and episodic memory (Laws et al., 2016). Interestingly, male AD patients exhibits higher mortality than female patients (Lopez-Lee et al., 2022). In contrast, PD is much more prevalent in men than women, with the ratio of male to female PD patients ranging from 1.3 to 3.7 (Baldereschi et al., 2000). Moreover, the age of PD onset is earlier in males by about two years and male patients experience more severe motor deficits and speech impairments (Haaxma et al., 2007; Miller & Cronin-Golomb, 2010). Similarly, ALS is also more prominent and onsets at an earlier age in man than in women (McCombe & Henderson, 2010). This is accompanied by higher expression of several ALS risk genes in the cortex, caudate and hippocampus of male patients than female patients (Gershoni & Pietrokovski, 2017). Recently, sex-dependent differences were also observed in HD patients, with female patients consistently showing more motor symptoms and self-reporting more depressive symptoms than males (Hentosh et al., 2021). One study also indicates

that female HD patients have a lower age of onset than male patients, but this has remained controversial since other studies report no sex-dependent effect on age of onset (Foroud et al., 1999; Hentosh et al., 2021).

The exact mechanism(s) underlying sex-dependent effects on the onset, progression and prevalence of neurodegenerative diseases is unclear. In post-mortem AD brains, it was found that amyloid-beta (Aβ) oligomers, a key pathological hallmark of AD, only interact with mGluR5 in male cortex and not in females (Abd-Elrahman et al., 2020a). Consistent with this observation, Aβ oligomers only interact with mGluR5 in male AD mice as part of a ternary complex that contains mGluR5, cellular prion protein and Aβ oligomers, which leads to pathological mGluR5 signalling (Abd-Elrahman et al., 2020a). This interaction is not detected in female AD mice and consequently, inhibition of mGluR5 with its NAM CTEP only improves AD symptoms in male mice and not in females (Abd-Elrahman et al., 2020a). Another study shows that microglial microRNAs involved in translational regulation are expressed in a sex-specific manner and removal of these microRNAs leads to sex-specific microglial response to tau-pathology in AD mice (Kodama et al., 2020). There is also evidence that suggests sex hormones can influence neurodegenerative disease symptoms and/or risks. For instance, the age of onset in female PD patients directly correlates with age of menopause, and hormone replacement therapy in postmenopausal women reduces the risk of developing PD (Currie et al., 2004; Frentzel et al., 2017; Song et al., 2020). Moreover, serum testosterone appears to prevent early Aβ deposition in female patients and slow down hippocampal neurodegeneration in male patients (Lee et al., 2017). It is possible that sex hormones modulate neurodegeneration by targeting glial cells, since: i) they express a wide variety of steroid receptors on their cell surface, ii) the expression of steroid receptors on glial cells is also often elevated in neurodegenerative conditions, and iii) level and type of sex hormones are different between males

and females, and the same hormones can also trigger sex-specific signalling pathways (Chowen & Garcia-Segura, 2021). Indeed, there is evidence showing that there are sex-specific differences in the activities of astrocytes, microglia and oligodendrocytes (Chowen & Garcia-Segura, 2021).

Unlike AD, PD and ALS, there is no significant difference in the prevalence of HD among males and females, likely due to its autosomal inheritance pattern. However, several studies using DNA samples from parent-offspring samples show that paternal transmission of CAG repeats often results in further expansions, whereas CAG repeat through maternal transmission mainly leads to contraction (Aziz et al., 2011; Kremer et al., 1995). Some potential explanation for this intergenerational CAG repeat stability include CAG repeats expansion occurs more frequently during spermatogenesis or massive CAG repeats are more detrimental in oocyte than in spermatocyte (Meoni et al., 2020). Furthermore, the majority of juvenile-onset HD cases are linked to paternal transmission, whereas maternally inherited cases are more frequently associated with later age of onset (Meoni et al., 2020). Men who inherited the disease from their mother also appears to have slower disease progression than those who inherited it from their father (Meoni et al., 2020).

As mentioned previously, two international cohort studies have shown that HD phenotype is more severe in women than men, especially regarding motor and cognitive functions (Foroud et al., 1999; Zielonka et al., 2013). Sex does not appear to influence neuropsychiatric symptoms, as several European studies found no significant sex-specific differences in anxiety and depressive symptoms (Dale et al., 2016; van Duijn et al., 2014). Interestingly, several studies in animal mouse models show different sex-specific effect compared to human data. For instance, in a study conducted using transgenic HD mice, there was a lack of motor impairments in female mice but not in males (Bode et al., 2008). Furthermore, the development of motor deficits in male mice is

accompanied by a decrease in the number of MSNs (Bode et al., 2008). Another study found that wheel running in R6/1 HD mice, a task well-established to delay their decline in hippocampal-dependent cognitive function and progression of motor symptoms, increased the expression of BDNF in the hippocampus of female but not HD mice (Zajac et al., 2010). However, sex-specific effects in HD mice seem to vary between different models. For example, one group showed that female homozygous HdhQ200 HD mice develop deficits in motor coordination at an earlier age than their male counterparts, whereas only male and not female heterozygous HdhQ350 HD mice have motor coordination impairments (Cao et al., 2018b; Cao et al., 2019).

Sex can also heavily influence the efficacy of therapeutic treatments for neurodegenerative conditions. For example, studies have found that men require higher dosages of levodopa, the main therapy for PD, than women due to differences in pharmacokinetic, pharmacodynamic and body weight (Kompoliti et al., 2002; Nyholm et al., 2010; Sharma et al., 2008). Specifically, women show higher levodopa plasma concentrations, slower levodopa clearance and greater bioavailability (Arabia et al., 2002; Kompoliti et al., 2002; Martinelli et al., 2003). In AD, it was found that the cholinesterase inhibitor rivastigmine delays the progression of cognitive impairments in women during the early stages, but provides more benefits for men in advanced dementia (Ferretti et al., 2018). Furthermore, in several cohort studies based in the US, UK and Finland, female AD patients are more likely to use antidepressants and anxiolytics than male patients (Ferretti et al., 2018). So far, there have not been any studies that look at sex-specific differences in the effectiveness of treatment for HD-associated symptoms. The dopamine receptor antagonist tetrabenazine has undergone several clinical trials as a potential treatment for HD chorea, and it showed similar efficacy and side effects between men and women (Meoni et al.,

2020). Similarly, olanzapine and risperidone are also equally effective in men and women at treating choreatic symptoms (Meoni et al., 2020).

Overall, sex has increasingly been considered as an important variable that can influence disease susceptibility, age of onset, symptoms progression and pathogenesis in a wide range of neurodegenerative conditions. Moreover, sex can lead to differences in the selection and efficacy of therapeutic treatment for different neurodegenerative diseases. However, the exact mechanism(s) underlying these differences are currently unclear and sex is still rarely considered during preclinical drug development. A better understanding of sex-specific differences in brain structure and function may help guide the development of innovative and personalized therapeutic option for treating neurodegenerative diseases.

1.13 Rationale and Hypothesis

Excessive glutamatergic signalling and the resulting excitotoxicity contribute to neurodegeneration in animal models of HD (Ribeiro et al., 2017; Zeron et al., 2002). mGluR2/3 and mGluR5 can modulate presynaptic glutamate release and postsynaptic glutamate signalling, respectively (Conn & Pin, 1997). **Therefore, we hypothesized that reducing glutamatergic signalling by activating mGluR2/3 or inhibiting mGluR5 could rescue behavioural deficits and improve neuropathology in zQ175 HD mice.** Moreover, increasing evidence suggests that sex is a critical factor that profoundly influence HD phenotype and neuropathology in both rodent models and human patients (Bode et al., 2008; Dorner et al., 2007; Hentosh et al., 2021). We have also reported sex-specific differences in glutamate signalling in AD mice (Abd-Elrahman et al., 2020a). **Thus, we hypothesized that sex could influence disease progression and the therapeutic potential of mGluR2/3 agonists or mGluR5 NAMs in HD mice.**

The specific aims of this thesis are: i) to investigate the effects of mGluR2/3 activation on the behaviour deficits and neuropathology of male and female zQ175 HD mice, ii) to examine whether mGluR5 antagonism differentially improves HD symptoms and brain pathology in male versus female zQ175 HD mice, and iii) to assess the impact of sex on HD progression in male versus female FDNQ175 mice.

Chapter 2

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

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

Si Han Li [1,2], Tash-Lynn L. Colson[1,2], Khaled S. Abd-Elrahman [1,2,3,#] and Stephen S. G. Ferguson[1,2,#,*]

[1]University of Ottawa Brain and Mind Research Institute, [2] Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, K1H 8M5, Canada.

[3]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Alexandria University, Alexandria, 21521, Egypt.

[#]co-senior authors

***Address correspondence to:**

Dr. Stephen S. G. Ferguson

University of Ottawa Brain and Mind Research Institute, Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Dr., Ottawa, Ontario, Canada, K1H 8M5.

Tel: (613) 562 5800 Ext 8889

sferguso@uottawa.ca

Authors Contributions: S.L, K.S.A-E and S.S.G.F were responsible for the conception and design of all experiments. S.L, K.S.A-E and T-L.L.C performed experiments and data analysis. S.L. and K.S.A-E wrote the manuscript and S.S.G.F edited the manuscript and supervised the study.

2.1 Abstract

Huntington's Disease (HD) is an autosomal dominant neurodegenerative disease that leads to progressive motor impairments with no available disease-modifying treatments. Current evidence indicates that exacerbated postsynaptic glutamate signalling 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 inhibits 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 GSK3β-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 signalling 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.

2.2 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 disturbances, cognitive impairments, behaviour difficulties and ultimately death (Martin & 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; DiFiglia et al., 1997; Furtado et al., 1996). 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 (Fan et al., 2009; Hassel et al., 2008; Ribeiro et al., 2011; Ribeiro et al., 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). 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 (McGeer & McGeer, 1976). Similarly, the N-methyl-D-aspartate receptor (NMDAR) agonist quinolinic acid causes striatal lesions that mimic the selective depletion of medium spiny neurons 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 signalling can ameliorate HD neuropathology and should be investigated as a treatment strategy.

mGluR2/3 are mainly located on cortico-striatal presynaptic terminals, and their activation reduces excessive glutamate release via negative feedback mechanisms (Conn & 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* (Battaglia et al., 1998; Buisson et al., 1996; 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 (Reiner et al., 2012a; Schiefer et al., 2004). There is also growing evidence that sex may influence HD phenotype and neuropathology in HD rodent models and patients (Bode et al., 2008; Dorner et al., 2007; Hentosh et al., 2021; Zielonka & Stawinska-Witoszynska, 2020). This is particularly important given that we and others have previously reported sex-specific differences in glutamate signalling in Alzheimer's disease and HD (Abd-Elrahman et al., 2020a; Padovan-Neto et al., 2019). 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 μM, but showed weak agonist activity towards mGluR6 at high nanomolar concentrations (Monn et al., 1999). LY379268 has also shown effectiveness in animal models of HD (Reiner et al., 2012a; Schiefer et al., 2004), 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 impairments and reduces mHTT aggregate pathology in both male and female heterozygous zQ175 mice. However, we find that LY379268 activates and inactivates divergent cell signalling 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 & Murphy, 2021).

2.3 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-GSK3β (S9, 9323s), anti-phospho-Akt (S473, 9271S) and mouse anti-GSK3β (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 trials 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 $MgCl_2$, 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 Na_3VO_4). 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., 2020b; 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 20× and the number of EM positive aggregates, 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 2 ROIs in the striatum were quantified (Abd-Elrahman et al., 2020b; Abd-Elrahman et al., 2017).

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.

2.4 Results

2.4.1 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 (**Figure 2.1A and 2.1B**). 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 (**Figure 2.1A and 2.1B**). 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 (**Figure 2.1C and 2.1D**). 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 (**Figure 2.1C and 2.1D**). 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 (**Figure 2.1C and 2.1D**). 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 (**Figure 2.1C and 2.1D**).

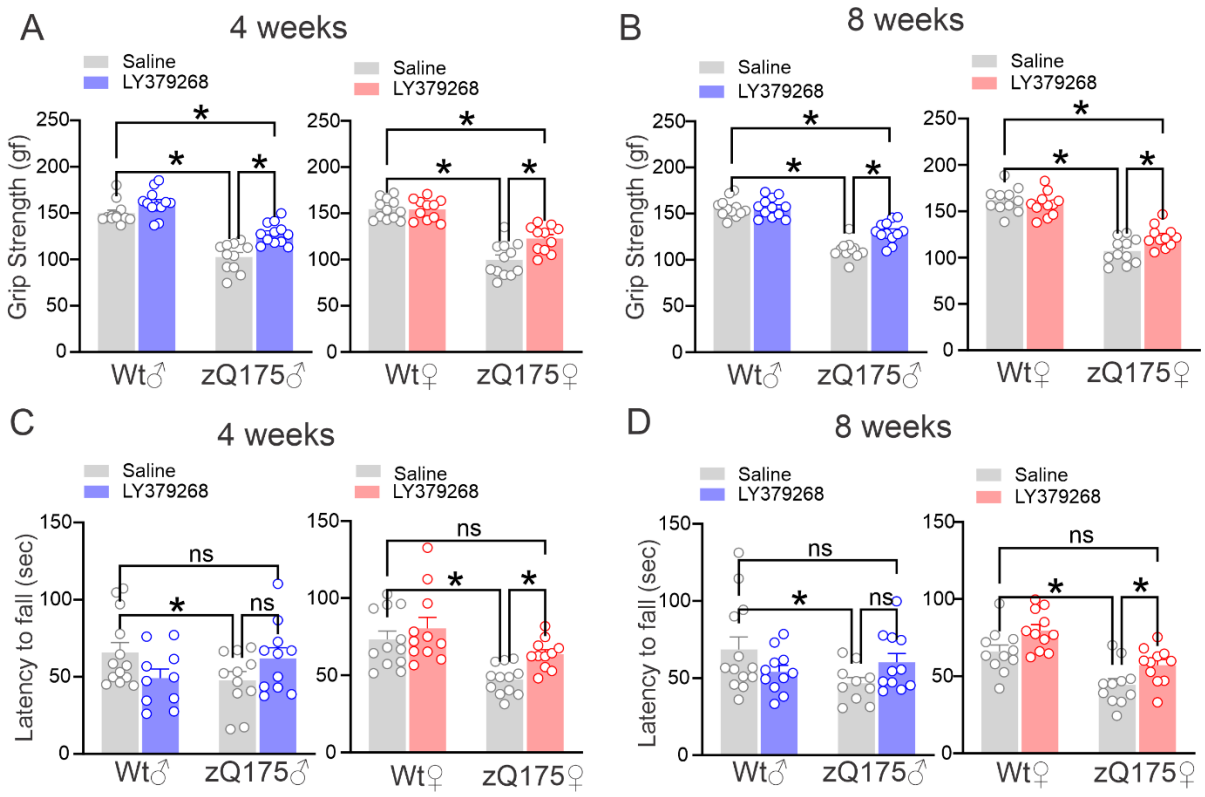

Figure 2.1. LY379268 improves grip strength and rotarod performance in male and female zQ175 mice.

Mean ± 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 ± 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.

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 (**Figure 2.2A and 2.2B**). 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 (**Figure 2.2A and 2.2B**). 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 (**Figure 2.2C and 2.2D**). 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 (**Figure 2.2C and 2.2D**). However, locomotor activity of LY379268-treated zQ175 mice remained reduced overall when compared with age- and sex-matched, saline-treated wild-types (**Figure 2.2C and 2.2D**). 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.

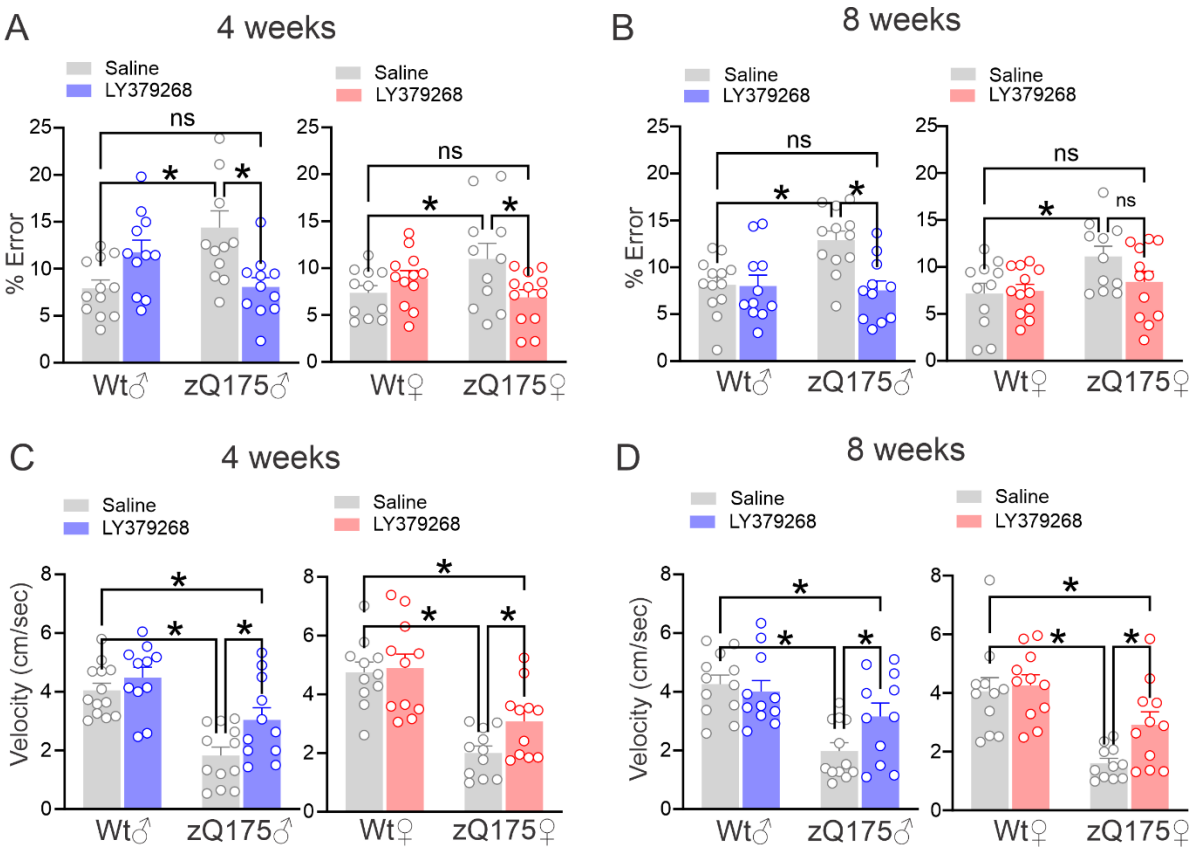

Figure 2.2. LY379268 improves locomotor activity and performance on the ladder rung test in male and female zQ175 mice.

Mean ± 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 ± 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.

2.4.2 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 (Abd-Elrahman et al., 2017; Ribeiro et al., 2014). This suggested that pathological glutamate signalling 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 cortico-striatal 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 (**Figure 2.3A**). 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 (**Figure 2.3B and 2.3C**). 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 (**Figure 2.3B and 2.3C**). 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.

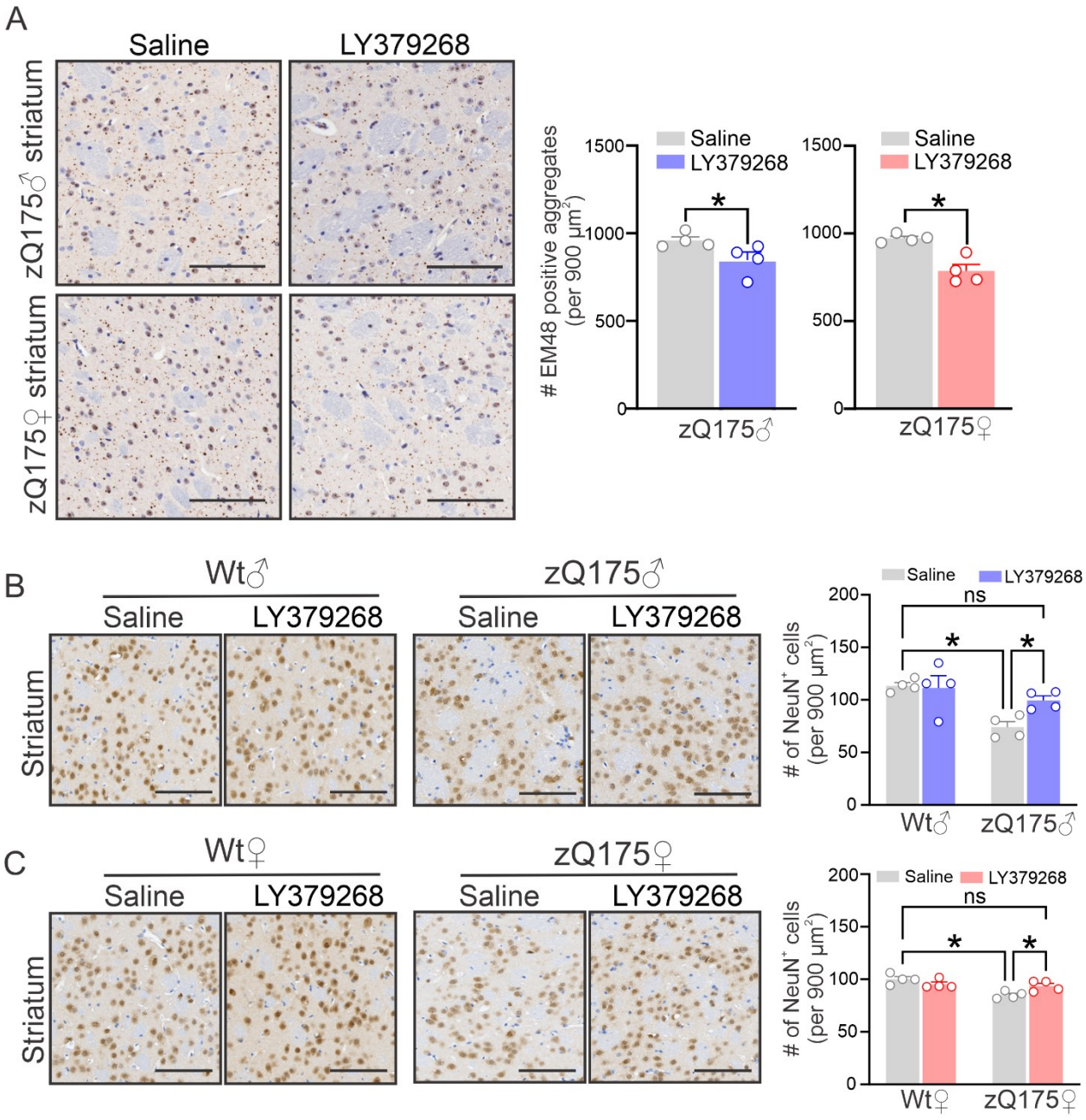

Figure 2.3. LY379268 reduces mHTT aggregates and neuronal loss in male and female zQ175 mice.

(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 900μm^2 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.

2.4.3 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 (Björkqvist et al., 2008; Perry et al., 2010; Tai et al., 2007). 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 (**Figure 2.4A and 2.4B**). 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 (**Figure 2.4A and 2.4B**). 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.

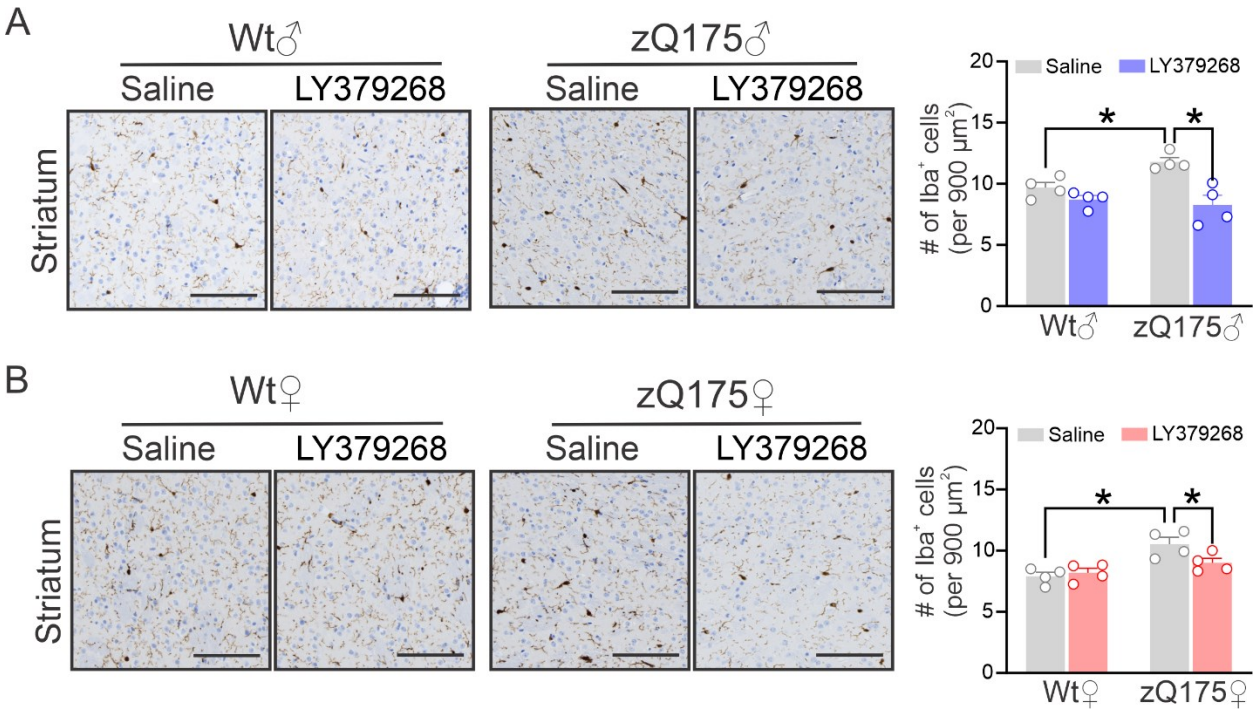

Figure 2.4. LY379268 reduces microglia activation in heterozygous male and female zQ175 mice.

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 900μm^2 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.

2.4.4 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 AD mice (Abd-Elrahman & Ferguson, 2019; Abd-Elrahman et al., 2017; Abd-Elrahman et al., 2018; Ibrahim et al., 2021). Specifically, mGluR5 induced the inhibitory phosphorylation of GSK3β 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 & Ferguson, 2019; Abd-Elrahman et al., 2017; Abd-Elrahman et al., 2018; 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 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 (**Figure 2.5A and 2.5B**). LY379268 treatment for 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 (**Figure 2.5A and 2.5B**). 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 (**Figure 2.5C and 2.5D**). In contrast, 8-week LY379268 treatment of male but not female zQ175 mice increased ATG14 expression compared to sex-matched saline-treated zQ175 mice (**Figure 2.5E and 2.5F**). 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 (**Figure 2.5G and 2.5H**). 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 signalling mediators and pathways.

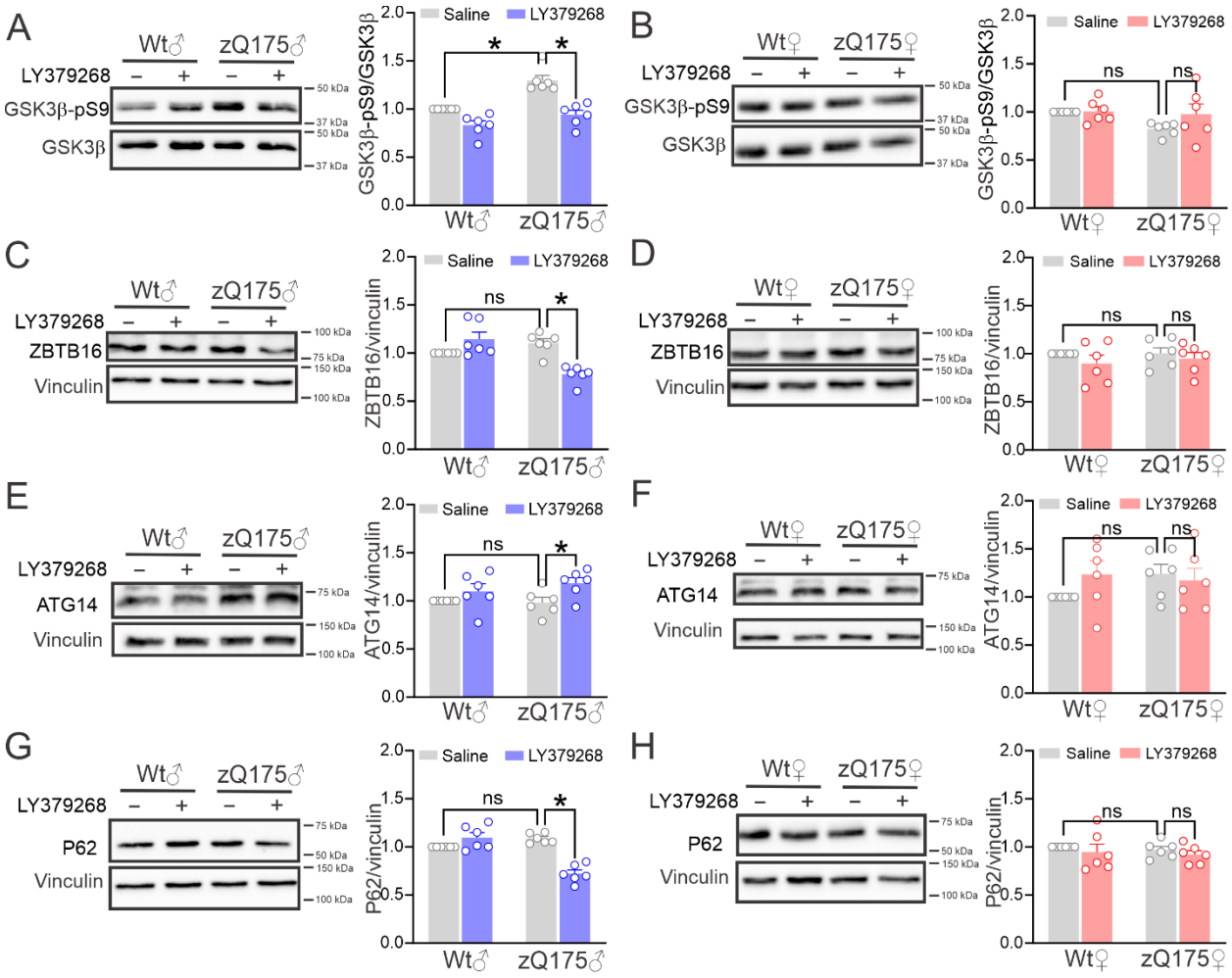

Figure 2.5. LY379268 activates the GSK3β/ZBTB16/ATG14 autophagy pathway in heterozygous male but not female zQ175 mice.

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 ± SEM 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 (LSD) comparisons.

2.4.5 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 GSK3β at S9 to potentially inhibit autophagy via ZBTB16-dependent mechanisms (Abd-Elrahman & Ferguson, 2019; Beaulieu et al., 2009; Hetman et al., 2002; Stambolic & Woodgett, 1994). 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. We found that Akt-pS473 and ERK1/2-PT202/Y204 phosphorylation were significantly increased in male, but not female, saline-treated zQ175 mice (**Figure 2.6A-D**). LY379268 treatment of male zQ175 mice restored Akt-pS473 and ERK1/2-pT202/Y204 phosphorylation to saline-treated male wild-type levels (**Figure 2.6A and 2.6C**). 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 (**Figure 2.6B and 2.6D**). 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 (**Figure 2.6B and 2.6D**). Thus, it was evident mGluR2/3 activation triggered sex-specific differences in the activation of both the ERK1/2 and Akt signalling pathways in heterozygous zQ175 mice, despite resulting in similar behavioral and pathological outcomes in both sexes.

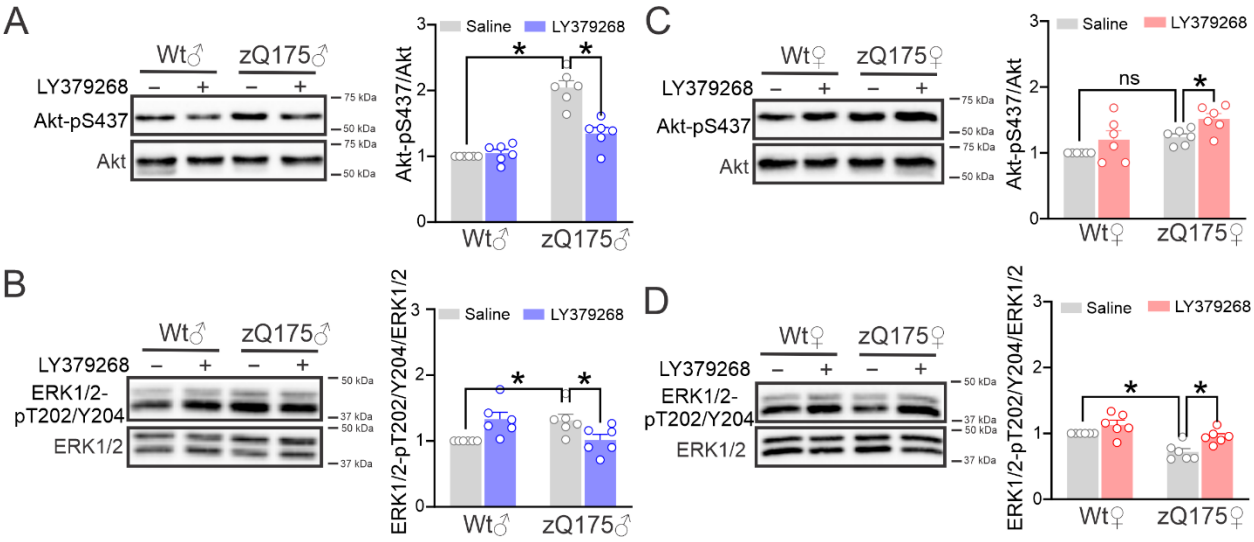

Figure 2.6. LY379268 alters Akt and ERK1/2 phosphorylation in heterozygous zQ175 mice in a sex selective manner.

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 ± 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.

2.5 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 (Abd-Elrahman et al., 2017; Ribeiro et al., 2014). 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 signalling/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 (Reiner et al., 2012a; Schiefer et al., 2004). 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 (Abd-Elrahman et al., 2017; Menalled et al., 2012; Smith et al., 2014). 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 (Abd-Elrahman et al., 2017; Menalled et al., 2012; Reilmann et al., 2001).

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 (Björkqvist et al., 2008; Silvestroni et al., 2009; Tai et al., 2007). 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 & 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 signalling 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 (Abd-Elrahman et al., 2017; Cortes & La Spada, 2014; Croce & Yamamoto, 2019; Li & Li, 2004). 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 & Ferguson, 2019; Abd-Elrahman et al., 2017). In fact, pathological glutamate signalling via mGluR5 induced an inhibitory phosphorylation of GSK3β that lead to ubiquitin-mediated degradation of the autophagy adaptor ATG14 via the ZBTB16-Cullin3-Roc1 E3-ubiquitin ligase complex (Abd-Elrahman et al., 2017; Ibrahim et al., 2021; Zhang et al., 2015). Here we showed that LY379268-mediated activation of mGluR2/3 in male Q175 mice also reduced 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 was likely that mGluR2/3 activation reduces synaptic glutamate leading to a reduction in pathological post-synaptic glutamate signalling resulting in the activation of autophagy and improved HD neuropathology in male zQ175 mice. However, we did not detect any changes in the GSK3β/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 signalling 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 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 signalling. 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 signalling mechanism(s) that do not involve the GSK3β/ZBTB16/ATG14 autophagy pathway. This finding further supports a sex-specific contribution of pathological glutamate signalling to the neuropathology of many neurodegenerative diseases.

While 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 signalling in neurons (Gines et al., 2003; Zhou et al., 2009). Additionally, agonist-dependent activation of group I mGluRs enhances Akt signalling in response to phosphoinositide 3-kinase (PI3K) activation (Guhan & Lu, 2004; Rong et al., 2003) 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 GSK3β at S9 and inhibit its catalytic activity (Beaulieu et al., 2009; Hetman et al., 2002; Stambolic & Woodgett, 1994). 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 signalling. This is consistent with the enhancement of ERK1/2 and Akt phosphorylation we have previously observed in male homozygous zQ175 brain samples (Abd-Elrahman & Ferguson, 2019; Abd-Elrahman et al., 2017). 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 signalling are not altered providing an explanation for the lack of change in GSK3β phosphorylation or ZBTB16-dependent autophagy. Rather,

ERK1/2 signalling 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 signalling 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 signalling 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 signalling remains unclear, we can not rule out that mGluR2/3 may heterodimerize with other GPCRs in a sex-selective manner to trigger differential cell signalling 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 signalling 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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Chapter 3

mGluR5 Antagonism Reduces Pathology and Differentially Improves Symptoms in Male and Female Heterozygous zQ175 Huntington's Mice

mGluR5 Antagonism Reduces Pathology and Differentially Improves Symptoms in Male and Female Heterozygous zQ175 Huntington's Mice

Si Han Li [1,2], Tash-Lynn L. Colson [1,2], Khaled S. Abd-Elrahman [1,2,3,#] and Stephen S.G. Ferguson [1,2,#,*]

[1]University of Ottawa Brain and Mind Research Institute, [2]Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, K1H 8M5, Canada.

[3]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Alexandria University, Alexandria, 21521, Egypt.

[#]co-senior authors

***Address correspondence to:**

Dr. Stephen S. G. Ferguson

University of Ottawa Brain and Mind Research Institute, Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Dr., Ottawa, Ontario, Canada, K1H 8M5.

Tel: (614) 562 5800 Ext 8889

sferguso@uottawa.ca

Authors Contributions: S.L, K.S.A-E and S.S.G.F were responsible for the conception and design of all experiments. S.L, K.S.A-E and T-L.L.C performed experiments and data analysis. S.L. and K.S.A-E wrote the manuscript and S.S.G.F edited the manuscript and supervised the study.

3.1 Abstract

Huntington's Disease (HD) is an inherited autosomal dominant neurodegenerative disorder that leads to progressive motor and cognitive impairment. There are currently no available disease modifying treatments for HD patients. We have previously shown that pharmacological blockade of metabotropic glutamate receptor 5 (mGluR5) signalling rescues motor deficits, improves cognitive impairments and mitigates HD neuropathology in male zQ175 HD mice. Mounting evidence indicates that sex may influence HD progression and we have recently reported a sex-specific pathological mGluR5 signalling in Alzheimer's disease (AD) mice. Here, we compared the outcomes of treatment with the mGluR5 negative allosteric modulator CTEP in both male and female symptomatic zQ175 mice. We found that female zQ175 mice required a longer treatment duration with CTEP than male mice to show improvement in their rotarod performance. Unlike males, chronic CTEP treatment did not improve the grip strength nor reverse the cognitive decline of female zQ175 mice. However, CTEP reduced the number of huntingtin aggregates, improved neuronal survival and decreased microglia activation in striatal tissue of both male and female zQ175 mice. Together, our results indicate that mGluR5 antagonism can reduce HD neuropathology in both male and female zQ175 HD mice, but sex has a clear impact on the efficacy of the treatment and must be taken into consideration for future HD drug development.

Keywords: neurodegenerative disease, huntingtin, GPCR, Sex differences, striatum, neuroglia, NeuN

3.2 Introduction

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disease characterized by the early loss of medium spiny neurons in the striatum (Martin & Gusella, 1986). HD symptoms typically manifests between the age of 30 – 50 and includes choreatic movements, dementia and behavioural difficulties (Roos, 2010). HD is caused by the expansion of a polyglutamine repeat in the N-terminal region of the huntingtin protein (MacDonald et al., 1993). Mutant huntingtin proteins (mHtt) with this expanded polyglutamine have been shown to be targeted for proteolysis and their cleavage at the N-terminus results in the formation of cytoplasmic and intranuclear aggregates that strongly correlate with HD symptoms and severity (DiFiglia et al., 1997). Indeed, longer polyglutamine repeats are associated with earlier disease onset and more severe symptoms (Andrew et al., 1993; Furtado et al., 1996). Despite this well-characterized etiology, disease modifying approaches to treat HD are lacking.

Glutamate is the major mediator of excitatory transmission in the brain and considerable evidence suggests glutamate-induced toxicity and reduction in glutamate uptake contribute to the selective loss of striatal neurons in HD (Hassel et al., 2008; Ribeiro et al., 2011; Ribeiro et al., 2017). Metabotropic glutamate receptor 5 (mGluR5) is a member of the G protein-coupled receptor (GPCR) superfamily that is highly expressed in the striatum and cortex, the two brain regions most affected in HD (Ribeiro et al., 2017; Shigemoto et al., 1993). We have previously reported that mutant but not wildtype huntingtin can disrupt mGluR5 signalling by interacting with it as a part of a protein complex that includes the huntingtin-binding protein optineurin (Anborgh et al., 2005). We have also demonstrated that genetic deletion of mGluR5 in a Q111 mutant huntingtin knock in mouse model reduces mutant huntingtin aggregate size and improves disease pathology (Ribeiro et al., 2014). The prolonged pharmacological blockade of mGluR5 signalling

with the negative allosteric modulator CTEP(2-chloro-4-[2-[2,5-dimethyl-1-[4-(trifluoromethoxy)phenyl]imidazol-4-yl]ethynyl]pyridine) also improves HD symptoms and promotes autophagic removal of mutant huntingtin aggregates in the brains of zQ175 HD mouse model (Abd-Elrahman & Ferguson, 2019; Abd-Elrahman et al., 2017). These findings indicate that targeted antagonism of mGluR5 may be effective for the treatment of HD. However, these studies were conducted exclusively in male HD mice and the effects of mGluR5 antagonism on HD pathology in female mice have not yet been investigated.

There is growing evidence that sex may influence HD phenotype and neuropathology in HD rodent models and patients (Bode et al., 2008; Dorner et al., 2007; Zielonka et al., 2013). We recently showed that activation of mGluR2/3 in male and female HD mice led to differential regulation of cell signalling pathways and there are sex-specific differences in cell signalling mechanisms contributing to the pathogenesis of HD (Li et al., 2021). More importantly, we have also reported sex-specific signalling of mGluR5 in AD mice (Abd-Elrahman et al., 2020a; Abd-Elrahman & Ferguson, 2022). Therefore, it is particularly important to study the disease modifying properties of CTEP and assess the contribution of pathological mGluR5 signalling to HD progression in female mice.

Here, we investigated whether targeted antagonism of mGluR5 using CTEP differentially improves HD symptoms and neuropathology in male versus female zQ175 HD mice. We find indeed that chronic treatment with CTEP differentially improves motor and cognitive deficits in males and females zQ175 mice. We also find that CTEP reduces mHtt aggregate pathology, neuronal loss and microgliosis in both male and female zQ175 mice. Our findings point to potential sex-specific differences in the contribution of mGluR5 to HD pathology.

3.3 Materials and Methods

Reagents

CTEP (1972) was purchased from Axon Medchem (Reston, USA). Rabbit anti-Iba1 (Abcam Cat# ab178847, RRID:AB_2832244) was from Abcam (Cambridge, USA). Mouse anti-NeuN (Millipore Cat# ABN78, RRID:AB_10807945) and anti-Huntingtin clone mEM48 (Millipore Cat# MAB5374, RRID:AB_177645) 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. Wildtype 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 wildtype (Wt) and heterozygous zQ175 (zQ175) mice. Groups of 22 male and female Wt and zQ175 mice were aged to 12 months of age and 12 mice from each group were treated with either DMSO or CTEP (2 mg/kg; dissolved in 10% DMSO and then mixed with chocolate pudding; final DMSO concentration was 0.1%) for 12 weeks. This drug dose was calculated weekly according to weight and was based on our previous studies in male HD mice and AD mice (Abd-Elrahman et al., 2020a; Abd-Elrahman et al., 2020b; Abd-Elrahman et al., 2017; Abd-Elrahman et al., 2018; de Souza et al., 2020; Hamilton et al., 2016). All groups were assessed in a battery of behavioral experiments after 4 weeks and 12 weeks of drug treatment. At the end of the 12-week treatment, mice were sacrificed by exsanguination, and the brains were collected and randomized for 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).

Rotarod test

Mice were introduced to the rotarod apparatus (IITC Life Science, 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).

Novel object recognition

Mice were placed in a square arena measuring 45 cm × 45 cm × 45 cm and tracked using an overhead camera fed to a computer in a separate room. Mice were allowed to explore the empty arena for 5 min, and 5 min later, two identical objects were placed in the arena 5 cm from the edge

and 5 cm apart. Mice were returned to the arena for 5 min and allowed to explore. The time spent exploring each object was recorded, and mice were considered to be exploring an object if their snout was within 1 cm of the object. Twenty-four hours after first exposure, the experiment was repeated with one object replaced with a novel object. The time spent exploring each object was recorded and analyzed using the Noldus EthoVision 10 software. Data were interpreted using the recognition index (time spent exploring the familiar object or the novel object over the total time spent exploring both objects multiplied by 100) and was used to measure the recognition memory [*TA* or *TB*/(*TA* + *TB*)] * 100, where *T* represents the time, *A* represents a familiar object, and *B* represents a novel object (Abd-Elrahman et al., 2017).

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 staining was done using Leica Bond III automatic stainer using BOND polymer Refine Detection Kit (Leica Biosystems Cat# DS9800, RRID:AB_2891238) from Leica Biosystems. Slide were scanned using a Leica Aperio Slide scanner at 20× and the number of EM positive aggregates, NeuN or Iba1 positive cells were counted in representative 300*300 μm^2 areas of the striatum. Experimenters were blinded to analysis and six sections per mouse were analyzed and for each section 2 ROIs in the striatum were quantified using the cell counter tool in Image J (Abd-Elrahman et al., 2020b; Abd-Elrahman et al., 2017; Abd-Elrahman et al., 2022; Li et al., 2021).

Statistical analysis

Means ± SEM are shown for each of independent experiment and $P < 0.05$ was used as the threshold for statistical significance. Statistical significance was assessed using GraphPad Prism 9 software and was determined by Two-way or Three-way Analysis of Variance (ANOVAs) as appropriate, followed by Tukey's Post Hoc test to determine the source of significant interactions. Statistical details of individual experiments are indicated in figure legends.

3.4 Results

3.4.1 CTEP treatment differentially rescues motor deficits in male and female heterozygous zQ175 HD mice.

To investigate potential sex-specific differences in the contribution of pathological mGluR5 signalling to HD progression and pathology, we first assessed whether targeted mGluR5 antagonism would improve motor deficits in symptomatic heterozygous male and female zQ175 mice. Twelve-month-old male and female wildtype and heterozygous zQ175 mice were treated with either vehicle or CTEP (2mg/kg) every 48 hours and their motor performance was assessed 4 weeks (13-month-old) and 12 weeks (15-month-old) after the initiation of treatment. The ANOVA indicated that vehicle-treated 13- and 15-month-old male and female heterozygous zQ175 mice showed significant impairment in fore limb grip strength compared to age- and sex-matched, vehicle-treated wildtype mice, F's [1, 80] = 343.8 and 323.4; $p < 0.0001$, for 13- and 15-month-old vehicle-treated wildtype and zQ175 mice, respectively (**Figure 3.1A and 3.1B**). After both 4 and 12 weeks of treatment, the forelimb grip strength varied as a function of the Treatment × Genotype × Sex interaction, F's [1, 80] = 5.298 and 16.39, $p = 0.0239$ and 0.0001, for 4 and 12 weeks, respectively. The follow-up tests of the simple effects of this interaction confirmed that CTEP treatment for either 4 or 12 weeks led to a statistically significant improvement in grip strength in male but not in female heterozygous zQ175 mice when compared to their sex-matched vehicle-treated heterozygous zQ175 mice (**Figure 3.1A and 3.1B**). However, as depicted in the figure, the forelimb grip force of CTEP treated male heterozygous zQ175 mice remained lower than that of sex- and age-matched, vehicle-treated wildtypes (**Figure 3.1A and 3.1B**). As such, the treated male animals did not achieve full recovery.

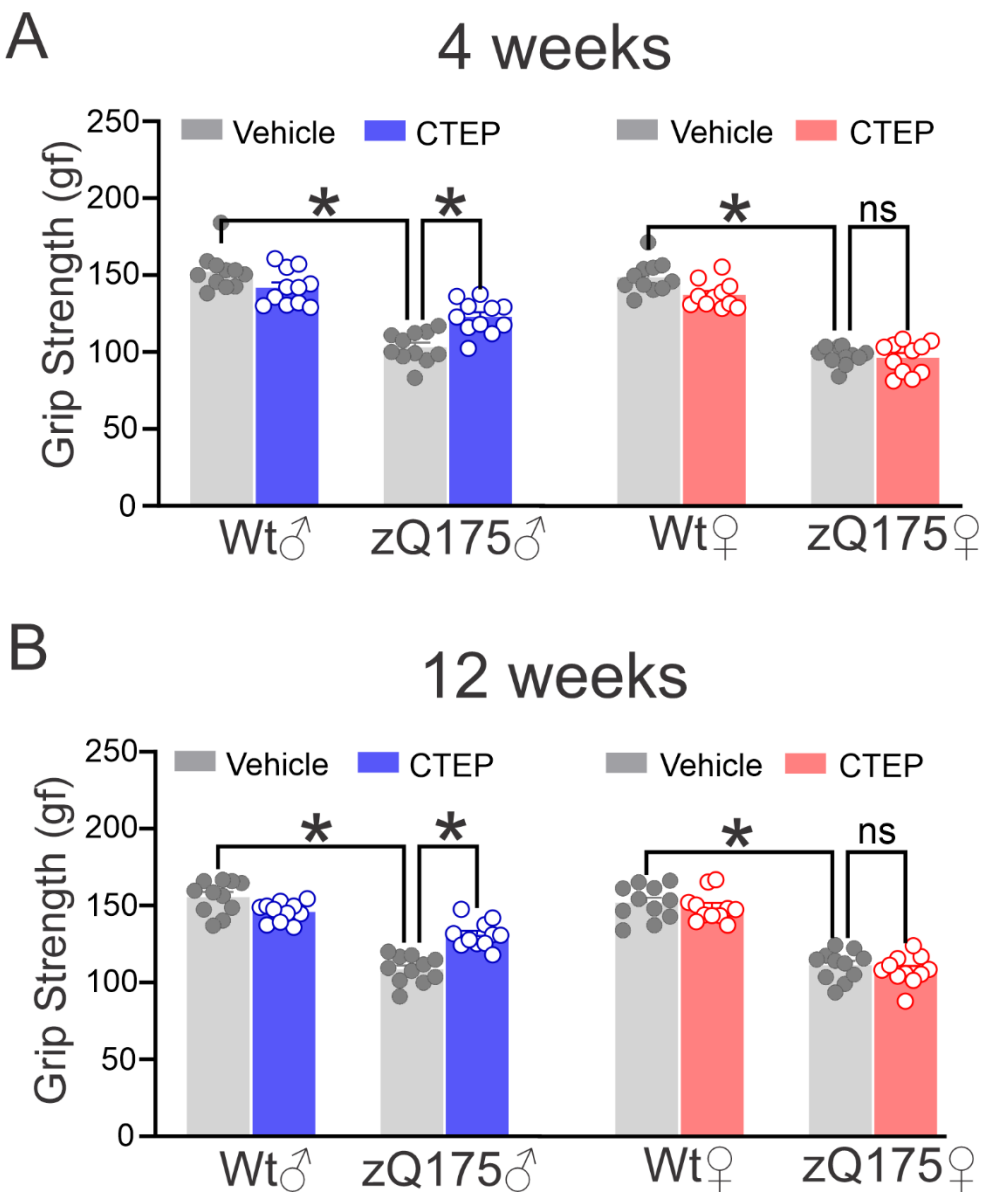

Figure 3.1. Effect of chronic administration of CTEP on grip strength in male and female zQ175 mice.

Mean ± SEM of grip strength [gram-force (gf)] after 4 weeks **(A)** and 12 weeks **(B)** of treatment with either vehicle or CTEP (2mg/kg/48hours) of 12-month-old heterozygous zQ175 (zQ175) and wildtype (Wt) male and female mice (n=11 for each group). *$P < 0.05$ by Three-way analysis of variance (ANOVA) and Tukey's multiple comparisons test.

Vehicle-treated (4 weeks and 12 weeks) male and female heterozygous zQ175 mice remained on the rotarod for a shorter time compared to age- and sex-matched, vehicle-treated wildtypes, F's [1, 80] = 36.88 and 72.85, $p < 0.0001$, for 13- and 15-month-old vehicle-treated wildtype and zQ175 mice, respectively (**Figure 3.2A and 3.2B**). After 4 weeks of CTEP treatment, the rotarod performance varied as a function of the Treatment × Genotype × Sex interaction, F [1, 80] = 4.705, $p = 0.0330$. Comparisons of the simple effects comprising this interaction indicated that four weeks of CTEP treatment improved the rotarod performance of male heterozygous zQ175 mice so that performance was significantly better than that of age- and sex-matched, vehicle-treated zQ175 counterparts. However, CTEP failed to elicit any significant improvement in female heterozygous zQ175 mice (**Figure 3.2A**). A comparable interaction was not apparent after 12 weeks of CTEP treatment, F [1, 80] = 0.5789, $p = 0.4490$. Specifically, after 12 weeks of CTEP treatment, both male and female heterozygous zQ175 mice showed significantly better rotarod performance relative to vehicle treated mice of the same sex, F [1, 80] = 21.49, $p < 0.0001$ (**Figure 3.2B**). In effect, CTEP improved the rotarod performance of both male and female heterozygous zQ175 mice after 12 weeks of treatment. However, as depicted in Figure 3.2B, the magnitude of this effect was larger in the females than in the males, although the interaction involving this variable was not significant. Collectively, these data indicated that CTEP treatment differentially rescues motor deficits in female and male heterozygous zQ175 mice and highlight how sex can influence the efficacy of potential drug candidates in reversing specific HD symptoms.

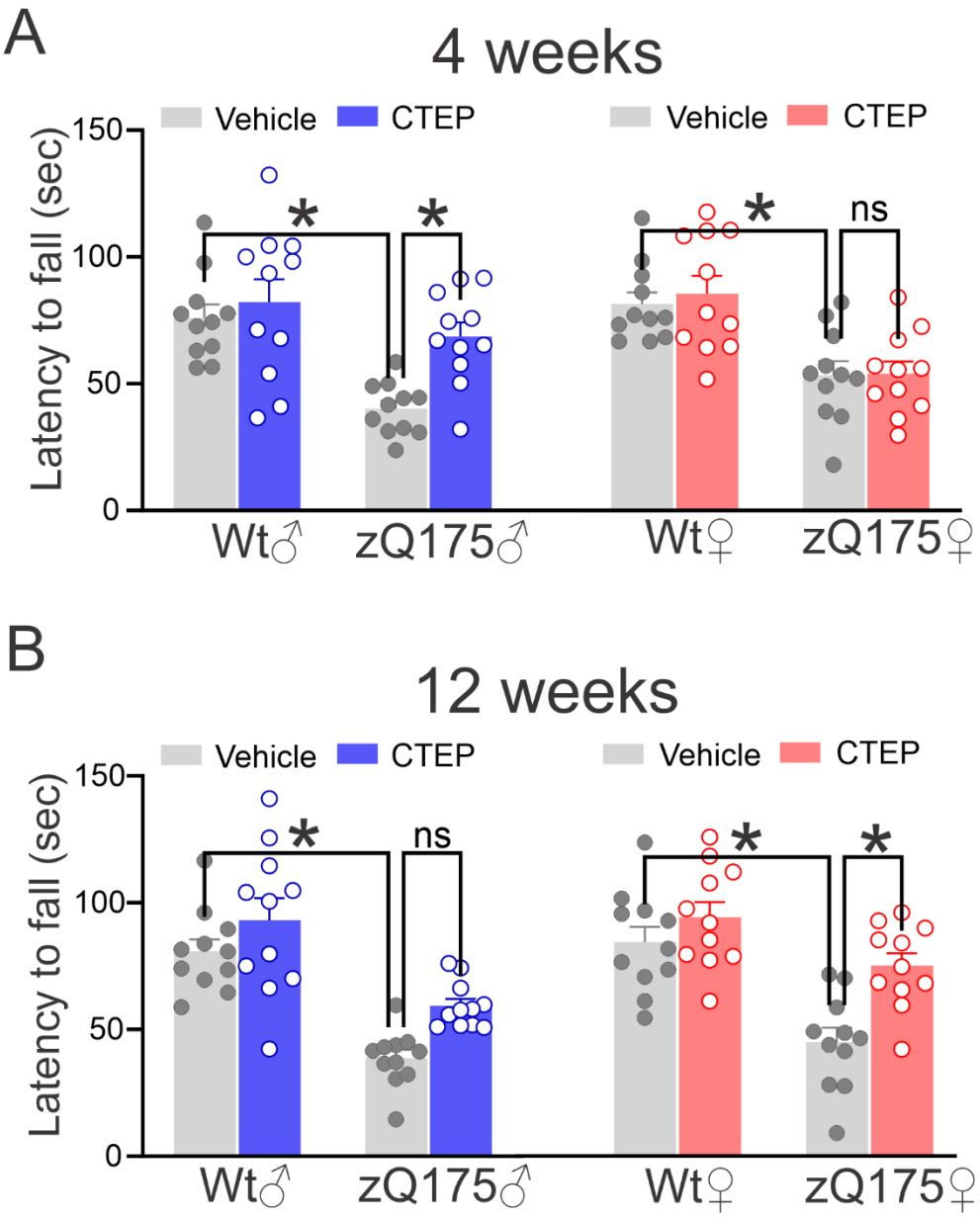

Figure 3.2. Effect of chronic administration of CTEP on rotarod performance in male and female zQ175.

Mean ± SEM of latency to fall (sec) from accelerating rotarod after 4 weeks **(A)** and 12 weeks **(B)** of treatment with either vehicle or CTEP (2mg/kg/48hours) of 12-month-old heterozygous zQ175 (zQ175) and wild-type (Wt) male and female mice (n = 11 for each group). *$P < 0.05$ by Three-way analysis of variance (ANOVA) and Tukey's multiple comparisons test.

3.4.2 CTEP treatment improves cognitive impairment in male but not female heterozygous Q175 mice.

HD was associated with cognitive impairment in addition to motor deficits (Lemiere et al., 2004). We previously reported that CTEP treatment for 12 weeks improved cognitive impairments in 15-month-old male heterozygous zQ175 mice (Abd-Elrahman et al., 2017). Thus, we assessed whether female heterozygous zQ175 exhibited memory impairment in the novel object recognition test at the same age and whether CTEP treatment could alleviate the impairment in female mice. Analysis of the recognition scores revealed a significant interaction between Genotype × Treatment, F's [1, 72] = 90.37 and 22.07, $p < 0.0001$ for male and female, respectively. The follow up tests confirmed that unlike wildtypes, where comparable performance was observed irrespective of treatment, at 15 months of age, both vehicle-treated male and female heterozygous zQ175 mice failed to distinguish between novel and familiar objects (**Figure 3.3A and 3.3B**). At the end of the 12 weeks of CTEP treatment, male heterozygous zQ175 mice regained their ability to discriminate between familiar and novel objects but female heterozygous zQ175 mice remained cognitively impaired (**Figure 3.3A and 3.3B**). Collectively, these data indicated that while both male and female heterozygous zQ175 mice present with cognitive deficits, mGluR5 antagonism does not rescue these deficits in HD mice on a female background.

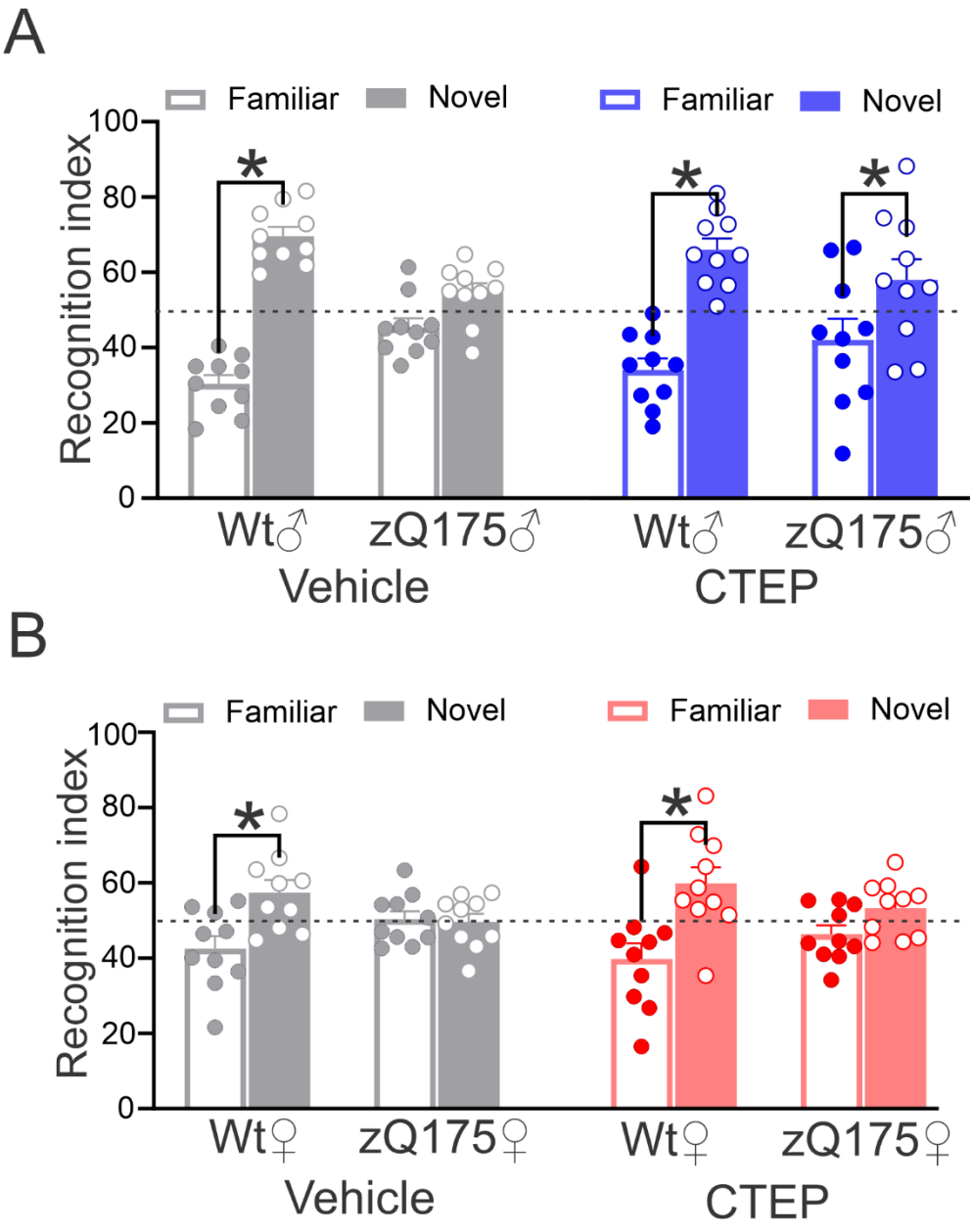

Figure 3.3. Effect of chronic administration of CTEP on novel object recognition in male and female zQ175 mice.

Mean ± SEM of the recognition index, for exploring a novel object versus a familiar object on the second day of novel object recognition test, after 12 weeks of treatment with either vehicle or CTEP (2mg/kg/48hours) of 12-month-old heterozygous zQ175 (zQ175) and wild-type (Wt) male **(A)** and female **(B)** mice (n = 10 for all groups). *$P < 0.05$ by two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test.

3.4.3 CTEP treatment reduced huntingtin aggregate number and neuronal loss in both male and female heterozygous zQ175 HD mice

The formation of intranuclear and cytoplasmic mutant huntingtin aggregates are the pathological hallmark of HD (DiFiglia et al., 1997). We have reported that genetic silencing and pharmacological blockade of mGluR5 reduced the number of mutant huntingtin aggregates in male Q111 and zQ175 HD mice, respectively (Abd-Elrahman et al., 2017; Ribeiro et al., 2014). Therefore, we examined whether chronic CTEP treatment can also reduce the number of mutant huntingtin aggregates in female zQ175 HD mice. After 12 weeks of CTEP treatment, the number of mutant huntingtin aggregates in the striatum of both male and female heterozygous zQ175 mice were significantly reduced compared to age- and sex-matched, vehicle-treated heterozygous zQ175 mice, $F [1, 16] = 23.19$, $p = 0.0002$ (**Fig. 3.4A and 3.4B**). Next, we examined whether the improvement in motor function and the decrease in aggregate accumulation were associated with the rescue of neuronal survival. The number of neuronal nuclei (NeuN)-positive cells in the striatum of vehicle-treated 15-month-old male and female heterozygous zQ175 mice was significantly lower than that of age- and sex-matched, vehicle-treated wildtype mice, $F [1, 32] = 48.82$, $p < 0.0001$ (**Figure 3.5A-C**). Twelve week-treatment with CTEP significantly increased the number of NeuN-positive striatal neurons of both male and female heterozygous zQ175 mice compared to age-and sex-matched, vehicle-treated heterozygous zQ175 mice, $F [1, 32] = 9.361$, $p =0.0045$, and to values that are not different from age- and sex-matched, vehicle-treated wildtype mice (**Figure 3.5A-C**). Collectively, these findings indicate that chronic CTEP treatment can reduce HD pathology and rescue neuronal loss in both male and female heterozygous zQ175 mice.

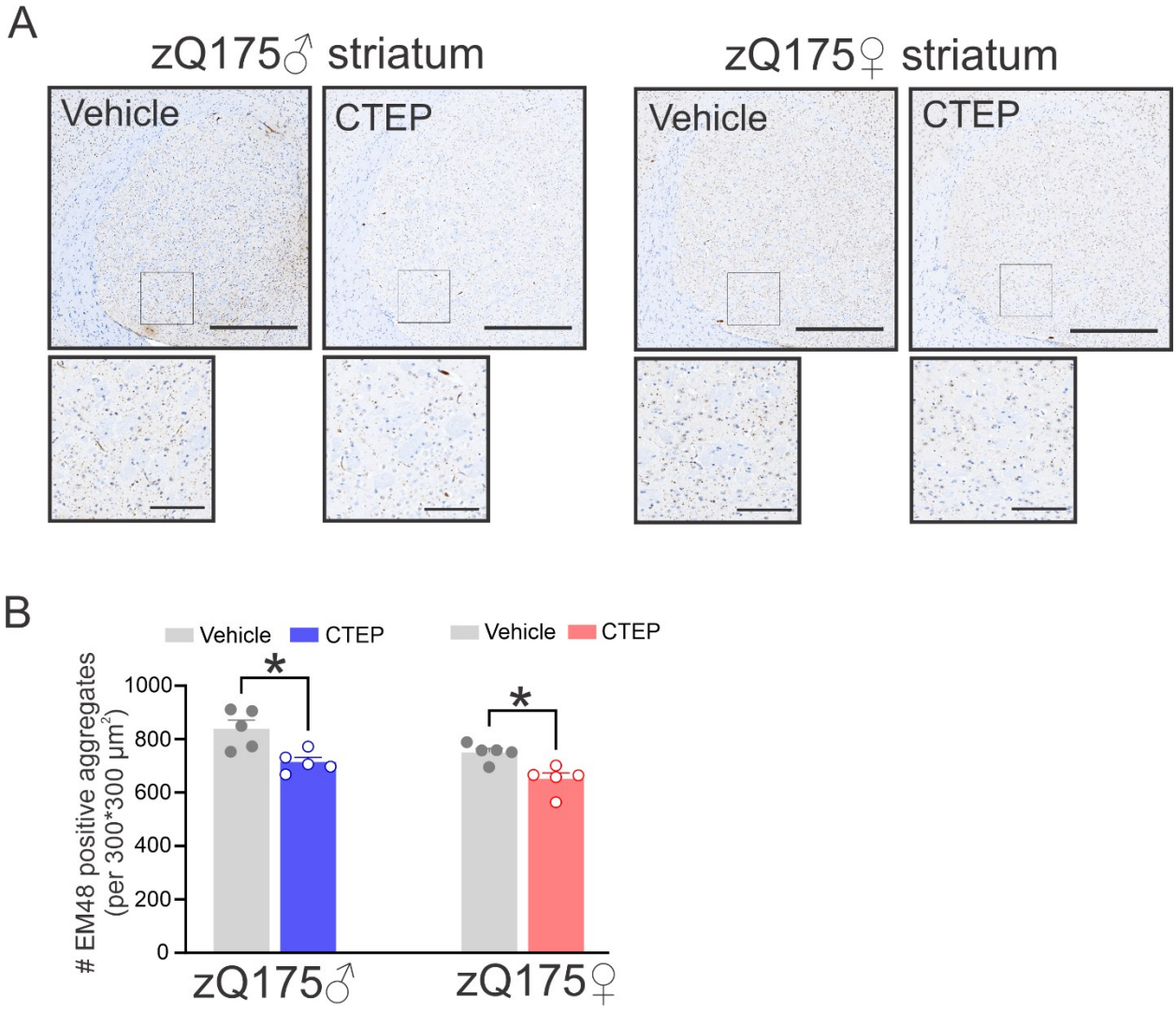

Figure 3.4. Effect of chronic administration CTEP on mutant huntingtin aggregates in male and female zQ175 mouse striatum.

Representative images of staining for mutant Htt aggregates using the antibody EM48 **(A)** and quantification of the number of huntingtin aggregates **(B)** in striatal brain slices from 15-month-old male and female heterozygous zQ175 mice after 12 weeks of treatment with either vehicle or CTEP (2mg/kg/48hours). Scale bar, 500 µm for whole striatum and 100 µm for magnified areas. Data are quantified from two different 300*300 μm^2 striatal regions of 6 sections per mouse and five independent mouse brains from each group were used for analysis. Data are mean ± SEM. *P < 0.05 by two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test.

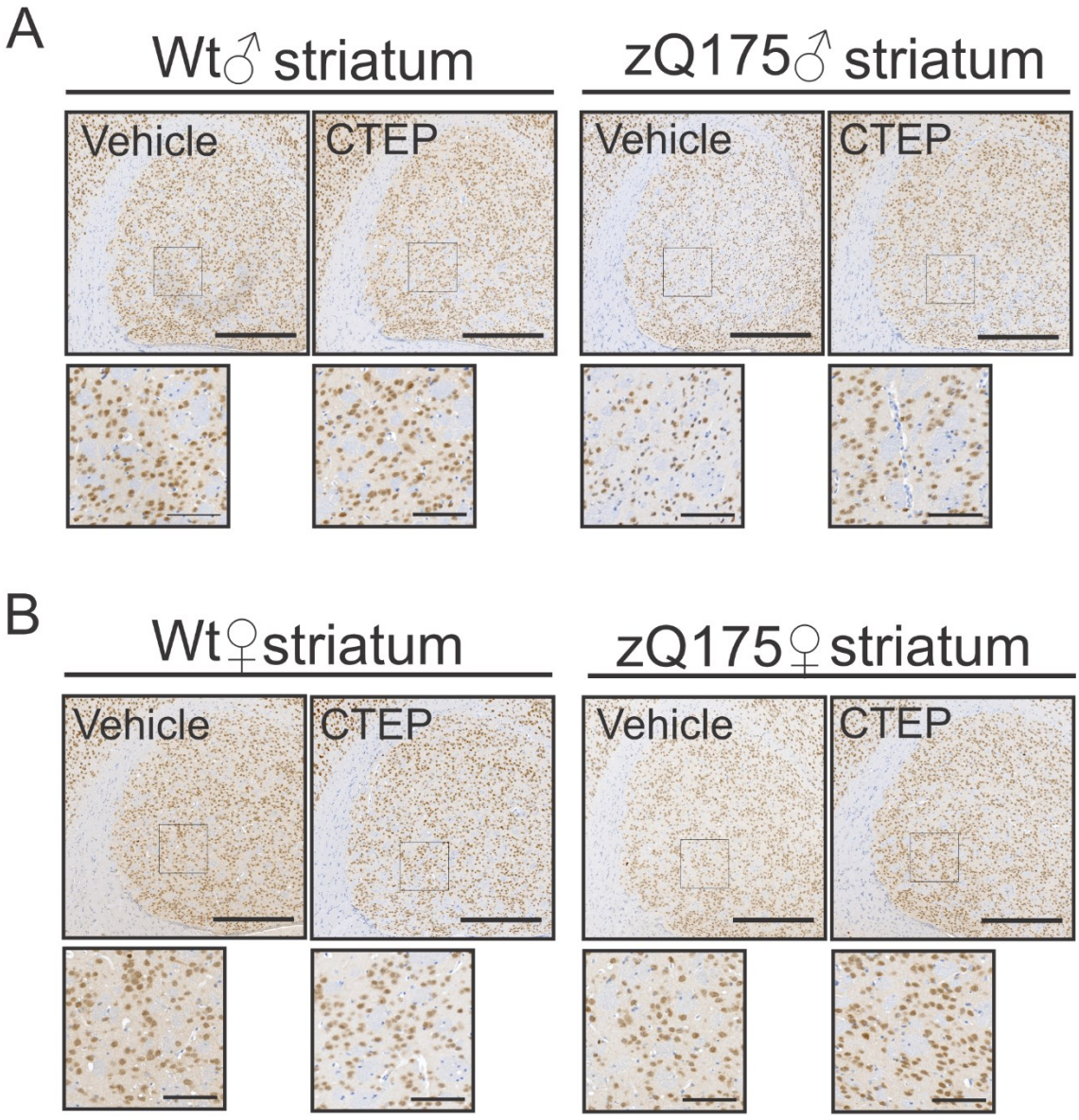
A
Wt♂ striatum
zQ175♂ striatum
Vehicle
CTEP
Vehicle
CTEP
B
Wt♀striatum
zQ175♀striatum
Vehicle
CTEP
Vehicle
CTEP
C
Vehicle
CTEP
Vehicle
CTEP
# of NeuN+ cells
(per 300*300 μm2)
200
150
100
50
0
*
*
*
*
Wt♂
zQ175♂
Wt♀
zQ175♀

Figure 3.5. Effect of chronic administration of CTEP on neuronal survival in male and female zQ175 mouse striatum.

Representative images of staining for neuronal nuclei (NeuN)-positive cells in striatal brain slices from 15-month-old male **(A)** and female **(B)** heterozygous zQ175 (zQ175) and wild-type (Wt) mice after 12 weeks of treatment with either vehicle or CTEP (2mg/kg/48hour). Scale bar, 500 μm for whole striatum and 100 μm for magnified areas. **(C)** quantification of the number of NeuN-positive cells in striatal brain slices from 15-month-old male and female zQ175 and Wt mice after 12 weeks of treatment with either vehicle or CTEP. Data are quantified from two different 300*300 μm^2 striatal regions of 6 sections per mouse and five independent mouse brains from each group were used for analysis. Data are mean ± SEM. $*P < 0.05$ by Three-way analysis of variance (ANOVA) and Tukey's multiple comparisons test.

3.4.4 CTEP treatment reduces microglial activation in heterozygous zQ175 HD mice

Microglia activation has been suggested to contribute to the pathogenesis of several neurodegenerative diseases, including AD, Parkinson's disease, Amyotrophic Lateral Sclerosis and HD (Abd-Elrahman et al., 2023; Perry et al., 2010). Activation of microglia has been observed in both pre-symptomatic HD gene carriers and symptomatic patients (Björkqvist et al., 2008; Pavese et al., 2006; Tai et al., 2007). Therefore, we assessed the number of activated microglia in the striatum of our mice by staining for ionized calcium-binding adapter molecule 1 (Iba1), a protein that is specifically expressed during microgliosis (Ito et al., 1998). The number of Iba1-positive cells was significantly higher in the striatum of 15-month-old vehicle-treated male and female heterozygous zQ175 mice compared to age- and sex-matched, vehicle-treated wildtypes, $F [1,32] = 41.48$, $p < 0.0001$ (**Figure 3.6A-C**). Twelve weeks of CTEP treatment reduced the number of Iba1-positive cells in the striatum of both male and female zQ175 mice, $F [1,32] = 6.573$, $p = 0.0153$ (**Figure 3.6A-C**). Once more, as depicted in Figure 3.6C, although there was a decrease of Iba1 positive cells in the striatum of CTEP-treated zQ175 mice, the extent of the decrease was relatively limited.

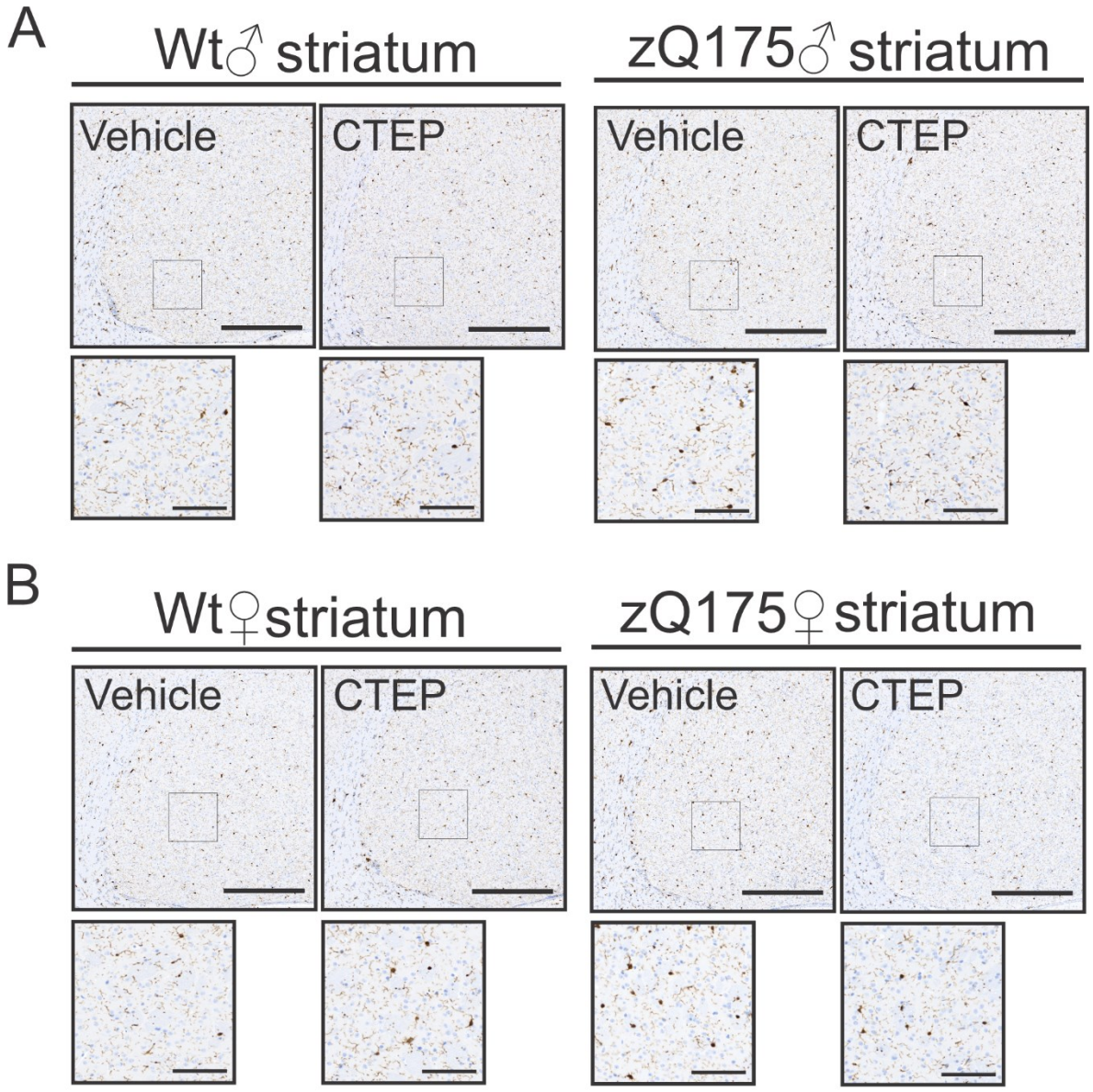
A
Wt♂ striatum
zQ175♂ striatum
Vehicle
CTEP
Vehicle
CTEP
B
Wt♀striatum
zQ175♀striatum
Vehicle
CTEP
Vehicle
CTEP
C
Vehicle
CTEP
Vehicle
CTEP
# of Iba1+ cells (per 300*300 µm2)
20
15
10
5
0
*
ns
*
ns
Wt♂
zQ175♂
Wt♀
zQ175♀

Figure 3.6. Effect of chronic administration of CTEP on microglia activation in male and female zQ175 mouse striatum.

Representative images of staining for ionized calcium binding adaptor molecule 1 (Iba1)-positive cells in striatal brain slices from 15-month-old male **(A)** and female **(B)** heterozygous zQ175 (zQ175) and wild-type (Wt) mice after 12 weeks of treatment with either vehicle or CTEP (2mg/kg/48hour). Scale bar, 500 μm for whole striatum and 100 μm for magnified areas. **(C)** quantification of the number of Iba1-positive cells in striatal brain slices from 15-month-old male and female zQ175 and Wt mice after 12 weeks of treatment with either vehicle or CTEP. Data are quantified from two different 300*300 μm^2 striatal regions of 6 sections per mouse and five independent mouse brains from each group were used for analysis. Data are mean ± SEM. *$P <$ 0.05 by Three-way analysis of variance (ANOVA) and Tukey's multiple comparisons test.

3.5 Discussion

Despite the discovery of its underlying genetic cause decades ago, the exact mechanism(s) underlying HD progression remain poorly understood and treatment options for HD patients are largely symptomatic. Glutamate signalling plays a significant role in the pathophysiology of HD, and the pharmacological blockade of mGluR5 NAMs delays disease progression in male zQ175 HD mice (Abd-Elrahman et al., 2017). However, several reports have emerged suggesting that sex can influence age of onset and disease progression in HD (Bode et al., 2008; Cao et al., 2018b; Roos et al., 1991; Zajac et al., 2010). Furthermore, sex-specific difference in mGluR5 signalling and response to mGluR5 NAMs have been reported in AD mice (Abd-Elrahman et al., 2020a; Abd-Elrahman & Ferguson, 2022). Here, we show that CTEP can indeed improve the performance of male HD mice in all motor and cognitive tasks but fails to elicit similar outcomes in female HD mice. We also demonstrate that CTEP reduces mHtt pathology, microgliosis and neuronal death in both sexes. Our findings point to distinct sex-specific differences in the outcomes of chronic mGluR5 blockade between male and female HD mice that warrants investigating the plausible underlying mechanism(s).

We have previously shown that male heterozygous zQ175 mice at 12 months of age have significant deficits in both motor and cognitive functions that can be reversed by 12-week treatment with CTEP (Abd-Elrahman et al., 2017; Li et al., 2021). Here, we find that both male and female heterozygous zQ175 mice presents with significant and comparable impairments in their grip strength and motor coordination that is consistent with previous findings by our group and others using the same mouse model (Abd-Elrahman et al., 2017; Li et al., 2021; Menalled et al., 2012; Smith et al., 2014). We also show that the short (4 weeks) and the long (12 weeks) treatment paradigms are to a great extent equally effective in reversing impairments in grip force

and motor coordination of male zQ175 mice during rotarod test. Despite both age-matched male and female HD mice showing deficits in grip strength and motor function, CTEP was not able to improve the performance of female zQ175 mice in most of the motor tasks. Specifically, CTEP did not improve grip strength and only longer treatment with CTEP was able to significantly improve the rotarod performance of female zQ175 mice. Sex-dependent differences in the onset of some motor symptoms were previously reported in another knock-in model of HD, HdhQ350/+ mice (Cao et al., 2018b). Moreover, the expression of the brain-derived neurotrophic factor (BDNF) was found to be severely affected in female R6/1 mice compared to age-matched males (Zajac et al., 2010). Therefore, it is possible that motor deficits present earlier in female compared to male zQ175 mice and extended treatment is required to reverse these impairments. Interestingly, impairment in precision grip control is an early predictor of disease onset and manifest in the pre-HD stage in patients (Rao et al., 2011).Thus, it is also possible that deficits in grip force manifests even earlier than limb coordination and an extended treatment paradigm (beyond 12 weeks) is required to detect a significant improvement in grip strength in female zQ175 mice.

Progressive cognitive decline is another debilitating symptom of HD and MRI study show that HD pathology spreads to the hippocampus, a brain region well-known to be important for learning and memory (Bird & Burgess, 2008; Lemiere et al., 2004; Rosas et al., 2003; van den Bogaard et al., 2011). Additionally, impaired neurogenesis and appearance of mHtt aggregates in the hippocampus have also been reported in animal models of HD (Abd-Elrahman et al., 2017; Morton et al., 2000; Simpson et al., 2011). Here, we show that CTEP reverses cognitive impairment in the novel object recognition test in male but not female zQ175 mice. Interestingly, such an observation is consistent with our most recent work showing that CTEP can reverse deficits in spatial and working memory in male but not female AD mice (Abd-Elrahman et al.,

2020a). mGluR5 signalling is differentially regulated between male and female AD mice due to sex-specific differences in the composition of the pathological scaffold formed between Aβ and mGluR5 (Abd-Elrahman et al., 2020a; Abd-Elrahman & Ferguson, 2022). Thus, it is possible that similar to Aβ, mHtt triggers a sex-specific pathological signalling of mGluR5 that alters the efficacy of mGluR5 NAMs in reversing memory and motor deficits in female HD mice.

Deposition of insoluble mHtt aggregates in the striatum is one of the distinguishing features of HD pathology and mGluR5 is highly expressed in striatum (DiFiglia et al., 1997; Shigemoto et al., 1993). We show that mGluR5 antagonism results in a significant reduction in the number and size of mHtt aggregates and rescues neuronal loss in the striatum of both male and female zQ175 mice. Since mHtt is known to alter transcriptional regulation and apoptosis (Cui et al., 2006; Kim et al., 1999; Rigamonti et al., 2000), it is likely that the reduction in mHtt following chronic mGuR5 inhibition reduces the loss of striatal neurons and nurture the neurotrophic capacity in HD brains. We have previously reported a similar reduction in apoptotic neuronal loss and mHtt aggregates in male zQ175 mice that was attributed to reactivation of a ZBTB16-dependent autophagy pathways that facilitate the clearance of mHtt aggregate from the striatum (Abd-Elrahman et al., 2017). However, ZBTB16 autophagic pathway is regulated in a sex-specific manner in zQ175 and AD mice and therefore it is likely that the mechanisms underling such reduction in mHtt load and neuronal loss after mGluR5 antagonism are different between both sexes (Abd-Elrahman et al., 2020a; Li et al., 2021). Further investigation in the mechanism(s) underlying such reduction in mHtt pathology in CTEP-treated female zQ175 mice is required in the future.

Activated microglia and elevated levels of pro-inflammatory cytokines have been found in the brains of HD patients and are thought to contribute to HD pathology (Björkqvist et al., 2008;

Silvestroni et al., 2009; Tai et al., 2007). mGluR5 is heavily expressed in microglia and the genetic deletion of mGluR5 in BACHD mouse model of HD triggers cortical microgliosis (Biber et al., 1999; Carvalho et al., 2019). We detected microgliosis in the striatum of both male and female zQ175 mice that was abrogated by CTEP suggesting that mGluR5 antagonism can be effective in reducing neuroinflammation in HD brains of both sexes. It is worth noting that pharmacological silencing of mGluR5 using CTEP in amyotrophic lateral sclerosis (ALS) and AD mice, two neurodegenerative diseases in which glutamate-mediated excitotoxicity plays a crucial role, reduced the number of activated microglia (Abd-Elrahman et al., 2020b; Milanese et al., 2021). Therefore, it is possible that in HD, glutamate excitotoxicity triggers microglial mGluR5 overactivation leading to microgliosis and exacerbation of neuroinflammation that can be abolished by mGluR5 NAMs.

mGluR5 NAMs remain a promising disease modifying treatment in HD since they are capable of reversing disease pathology in both sexes, but it is possible that extended treatment in females is required to accomplish a significant recovery in motor and cognitive symptoms. The mechanism(s) underlying the sex-specific difference in the efficacy of mGluR5 NAMs in zQ175 HD mice remains unclear. So far, no differences in the subcellular localization, expression, and function of mGluR5 between males and females have been reported in HD. However, mGluR5 can directly interact with mutant huntingtin in male Q111 HD mice leading to altered receptor signalling but such interaction has not been investigated in female HD mice (Anborgh et al., 2005; Ribeiro et al., 2010). Furthermore, membrane estrogen receptors is coupled to mGluR5 in female rat striatum and can activate mGluR5 signalling in the presence of estradiol (Grove-Strawser et al., 2010). Therefore, it is possible that mGluR5 interaction with mHtt in HD brain is either intrinsically different between both males and females or is influenced by the crosstalk between

mGluR5 and sex hormone receptors. Additionally, membrane mGluR5 forms heterodimers and it is possible that the composition of this dimers is regulated in a sex-specific manner leading to differential binding and/or efficacy of mGluR5 allosteric ligands (Lee et al., 2020; Prinster et al., 2005).

3.6 Conclusion

We demonstrate that mGluR5 contributes to HD pathophysiology in male and female zQ175 HD mice and that while mGluR5 NAMs can reverse neuropathology in both sexes, they are less efficacious in reversing symptoms in female compared with male mice. Thus, there are important sex-specific differences in the signalling pathways downstream of mGluR5 that contributes to the pathophysiology zQ175 HD mice that should be investigated in the future. We also emphasize the importance of designing individualized HD treatments that takes both the sex and disease stage of the patient into account.

Data Availability statement: All data generated or analyzed during this study are included in this published article.

Ethics statement: 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.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 4

Comparison of Huntington's Disease Phenotype Progression in Male and Female Heterozygous FDNQ175 Mice

Comparison of Huntington's Disease Phenotype Progression in Male and Female Heterozygous FDNQ175 Mice

Si Han Li[1,2], Tash-Lynn L. Colson[1,2], Jingwei Chen[1,2], Khaled S. Abd-Elrahman[4,5,6] and Stephen S. G. Ferguson[1,2,3,*]

[1]University of Ottawa Brain and Mind Research Institute, [2] Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, K1H 8M5, Canada.

[3]Department of Neuroscience, Carleton University, Faculty of Health Sciences, 1125 Colonel By Dr. Ottawa, Ontario, K1S 5B6.

[4]Department of Anesthesiology, Pharmacology and Therapeutics, and Djavad Mowafaghian Center for Brain Health, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.

[5]Department of Pharmacology and Therapeutics, College of Medicine and Health Science, Khalifa University, Abu Dhabi, United Arab Emirates

[6]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Alexandria University, Alexandria, 21521, Egypt.

***Address correspondence to:**

Dr. Stephen S. G. Ferguson

University of Ottawa Brain and Mind Research Institute, Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Dr., Ottawa, Ontario, Canada, K1H 8M5.

Tel: (614) 562 5800 Ext 8889

sferguso@uottawa.ca

Authors Contributions: S.L, K.S.A-E and S.S.G.F were responsible for the conception and design of all experiments. S.L, T-L.L.C and J.C performed experiments and data analysis. S.L. wrote the manuscript. K.S.A-E and S.S.G.F edited the manuscript and supervised the study.

4.1 Abstract

Huntington's Disease (HD) is an inherited autosomal dominant neurodegenerative disorder that leads to progressive motor and cognitive impairment due to the expansion of a polyglutamine (CAG) repeat in the N-terminal region of the huntingtin (Htt) protein. The creation of HD mouse models represents a critical step in the research for HD treatment. Among the currently available HD mouse models, the zQ175 knock-in mouse line is the first to display robust disease phenotype on a heterozygous background. The newer FDNQ175 mouse model is derived from the zQ175 mouse line and presents a more aggressive phenotype. Moreover, increasing evidence has implicated sex as a contributing factor in the progression of HD symptoms. Here, we compared the progression of HD phenotypes in male and female heterozygous FDNQ175 mice. We found that both male and female heterozygous mice showed deficits in forelimb grip strength and cognition as early as 6 months of age. However, female FDNQ175 mice were less vulnerable to HD-associated decline in limb coordination and movement. Neither male nor female FDNQ175 mice exhibited reduced locomotor activity in the open field or exhibit consistent differences in anxiety at 6-12 months of age. Both male and female FDNQ175 mice exhibited increased numbers of huntingtin aggregates with age and 8-month-old female FDNQ175 mice had significantly more aggregates than their male counterparts. Taken together, our results provide further evidence that sex can influence the progression of HD phenotype in preclinical animal models and must be taken into consideration for future HD research.

4.2 Introduction

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disorder characterized by the early loss of medium spiny neurons in the striatum (Martin & Gusella, 1986). HD symptoms typically manifests between the age of 30 – 50 and includes choreatic movements, dementia and behavioural difficulties (Roos, 2010). HD is caused by the expansion of a polyglutamine (CAG) repeat in the N-terminal region of the huntingtin (Htt) protein (MacDonald et al., 1993). Mutant Htt with this expanded polyglutamine tract has been shown to be targeted for proteolysis and the cleavage of Htt at the N-terminus results in the formation of cytoplasmic and intranuclear aggregates that strongly correlate with HD symptoms and severity (DiFiglia et al., 1997). Indeed, longer polyglutamine repeats are associated with earlier disease onset and more severe symptoms (Andrew et al., 1993; Furtado et al., 1996). Despite this well-characterized etiology, the underlying molecular mechanism(s) responsible for HD pathogenesis remains poorly understood and there is no disease modifying treatment currently available.

One important advance in HD research is the creation of genetic mouse models that replicate the clinical, pathological and biochemical changes observed in HD patients. These different mouse models present varying degrees of phenotype severity, pathology and onset. Based on the way in which mutant huntingtin is incorporated into the mouse genome, genetic models of HD can be broadly separated into three categories: i) transgenic mice that express toxic N-terminal fragments of human mutant Htt (mHtt) in addition to mouse wild-type Htt, ii) transgenic mice that express full length human mHtt alongside mouse wild-type Htt, and iii) knock-in mice with expanded CAG repeats in their endogenous Htt gene (Ferrante, 2009). Among the three categories, knock-in mouse lines represent the most genetically accurate models of HD, as they express the disease-causing mutant protein in the correct genomic context. zQ175 mouse is a knock-in mouse

model of HD that expresses a chimeric Htt protein containing exon 1 of mutated human Htt with roughly 188 CAG repeats. Importantly, zQ175 mice show robust HD phenotype on a heterozygous back ground, which closely recapitulates the autosomal dominant inheritance pattern of HD in humans (Menalled et al., 2012). More recently, the FDNQ175 mouse model was generated to increase phenotype severity and reduce testing time. These improvements are achieved by changing the background strain from C57BL/6 to FVB/N and removing the floxed neomycin resistance gene (Neo) upstream of the Htt locus, which increased the animal's susceptibility to neurodegeneration and elevated mutant Htt protein levels in the brain (Southwell et al., 2016).

The initial assessment of FDNQ175 mice suggests that it is a more suitable model for HD preclinical testing than zQ175 mice. However, the original study did not distinguish between the severity and progression of the phenotype in males and females. There is growing evidence that sex may influence HD phenotype and neuropathology in HD rodent models and patients (Bode et al., 2008; Dorner et al., 2007). Therefore, we examined and compared the progression of HD phenotypes in male versus female FDNQ175 mice. We found that female FDNQ175 mice appeared less vulnerable to HD-associated decline in motor function than male mice. Interestingly, female wild-type mice developed deficits in the novel object recognition test at 8 months of age, whereas male wild-type mice only showed deficits in the same test at 12 months of age. Moreover, female FDNQ175 mice appears to have more insoluble aggregates than male mice at younger ages, but they eventually reach a similar level by 12 months of age. Overall, these studies highlight significant differences in disease progression between male and female FDNQ175 mice.

4.3 Materials and Methods

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. Food and water were given *ad libitum*. Wild-type FVB/N mice were from Charles River Laboratory (#207) and FDNQ175 mice were gifts from Michael Hayden's laboratory (University of British Columbia). Mice were bred to establish littermate-controlled male and female wild-type and heterozygous FDNQ175 mice. Groups ($n \geq 14$) of male and female wild-type and heterozygous FDNQ175 mice were aged and tested in a series of behavioural experiments at 6, 8, 10 and 12 months of age. After 12 months, mice were sacrificed by live cervical dislocation and the brains were collected for biochemistry and immunohistochemistry. A second group of male and female wild-type and heterozygous FDNQ175 mice were raised and sacrificed by live cervical dislocation at 8 months of age and the brains were also collected for immunohistochemistry.

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. Male and female mice were tested separately to prevent change in behaviour. All testing was performed in the University of Ottawa Behavior and Physiology Core Facility.

Forelimb grip strength test

The grip strength of each mouse was measured using the Chatillon DEF II Grip Strength Meter (Columbus Instruments, Columbus, Ohio). 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 ~2.5 cm/s until they released the bar. Each mouse was tested 8 times with a break of 5 s in between each trial and the values of maximal peak force were recorded.

Open field test:

Mice were individually placed in the bottom-left corner of an opaque and illuminated (~300 lux) open field arena (45 cm × 45 cm × 45 cm) and allowed to explore for 10 min. Activity of the mice were recorded by an overhead camera connected to a computer outside the room. Total distance travelled, velocity and time spent in the center versus four corners were calculated using the Noduls Ethovision 17 software.

Rotarod test:

Mice were introduced to the rotarod apparatus (IITC, Woodlands Hills, CA, USA) on day one by placing them on the stationary rotarod for 3 minutes. Four trials 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 scored as 300 s. If mice fell in the first 10 seconds of a trial, the trial was repeated from the start, for up to three times. Data obtained from the four trials of the second day were used for analysis.

Horizontal ladder test:

The mice were first trained (1 trial) 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 then filmed crossing the ladder for 4-5 trials using high-definition camera. The number of successful and missed steps for each limb during the

2 best trials were quantified and interpreted as percentage error.

Novel object recognition

Mice were placed in a square arena measuring 28 cm × 28 cm × 35 cm and tracked using an overhead camera fed to a computer in a separate room. Mice were allowed to explore the empty arena for 5 min, and 5 min later, two identical objects were placed diagonally in the arena 5 cm from the edge and 5 cm apart. Mice were returned to the arena for 5 min and allowed to explore. The time spent exploring each object was recorded, and mice were considered to be exploring an object if their snouts were within 1 cm of the object. Twenty-four hours after first exposure, the experiment was repeated with one object replaced with a novel object. The time spent exploring each object was recorded and analyzed using the Noldus EthoVision 17 software. Data were interpreted using the recognition index (time spent exploring the familiar object or the novel object over the total time spent exploring both objects multiplied by 100) and was used to measure the recognition memory [TA or $TB/(TA + TB)$] * 100, where T represents the time, A represents a familiar object, and B represents a novel object (Abd-Elrahman et al., 2017).

Immunohistochemistry

One hemisphere of each brain sample was fixed in 4% paraformaldehyde for 48 hours 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 (Sigama-Aldrich, MAB5374) at 1:100 dilution for 30 minutes at room temperature and staining was done using Leica Bond III automatic stainer using BOND polymer Refine Detection Kit (Leica Biosystems Cat# DS9800) from Leica Biosystems. Slides were scanned using a Leica Aperio Slide scanner at 20X and the number of EM48 positive

aggregates were counted in representative 300 × 300 μm^2 areas of the striatum. Experimenters were blinded to analysis and six sections per mouse were analyzed and for each section 2 ROIs in the striatum were quantified using the cell counter tool in Image J (Abd-Elrahman et al., 2020b; Abd-Elrahman et al., 2017; Abd-Elrahman et al., 2021; Li et al., 2021).

Statistical analysis:

Means ± SEM for each independent experiment is shown in the various figure legends. GraphPad Prism software was used to analyze the data for statistical significance. Statistical significance was determined by a series of 2 (strain) × 2 (sex) or 2 (strain) × 2 (age) analyses of variance (ANOVAs), followed by Fisher's least significant difference comparisons for the significant main effects or interactions.

4.4 Results

4.4.1 Male and female heterozygous FDNQ175 mice develop deficits in grip strength at 6 months of age.

It was previously reported that heterozygous zQ175 mice develop deficits in motor function at approximately 8 months of age (Menalled et al., 2012). In comparison, FDNQ175 mice showed decline in motor function starting at 6 months of age (Southwell et al., 2016). In this study, we tested both male and female FDNQ175 mice to determine whether they develop HD-related motor impairments in a sex-specific manner. At 6 months of age, both male and female heterozygous FDNQ175 mice showed lower grip strength than age and sex-matched wild-type controls (**Figure 4.1A**). Furthermore, the grip strength of heterozygous FDNQ175 mice continued to deteriorate as they aged, with additional decline occurring from 6 to 10 months (**Figure 4.1B-F**). Interestingly, we did not observe a further drop in grip strength between the age of 10 and 12 months, suggesting that the loss of grip strength had already plateaued at the 10 months time point for FDNQ175 mice (**Figure 4.1B-F**). We also did not observe significant differences in grip strength between wild-type male and female mice nor between heterozygous male and female FDNQ175 mice at each time point tested in this study (**Figure 4.1G**). In conclusion, both male and female heterozygous FDNQ175 mice exhibited significant decline in their grip strength at 6 months of age. Moreover, their grip strength continued to decrease further up to 10 months of age. However, no evidence of any sex-specific differences in the development of grip strength dysfunction was observed in FDNQ175 mice.

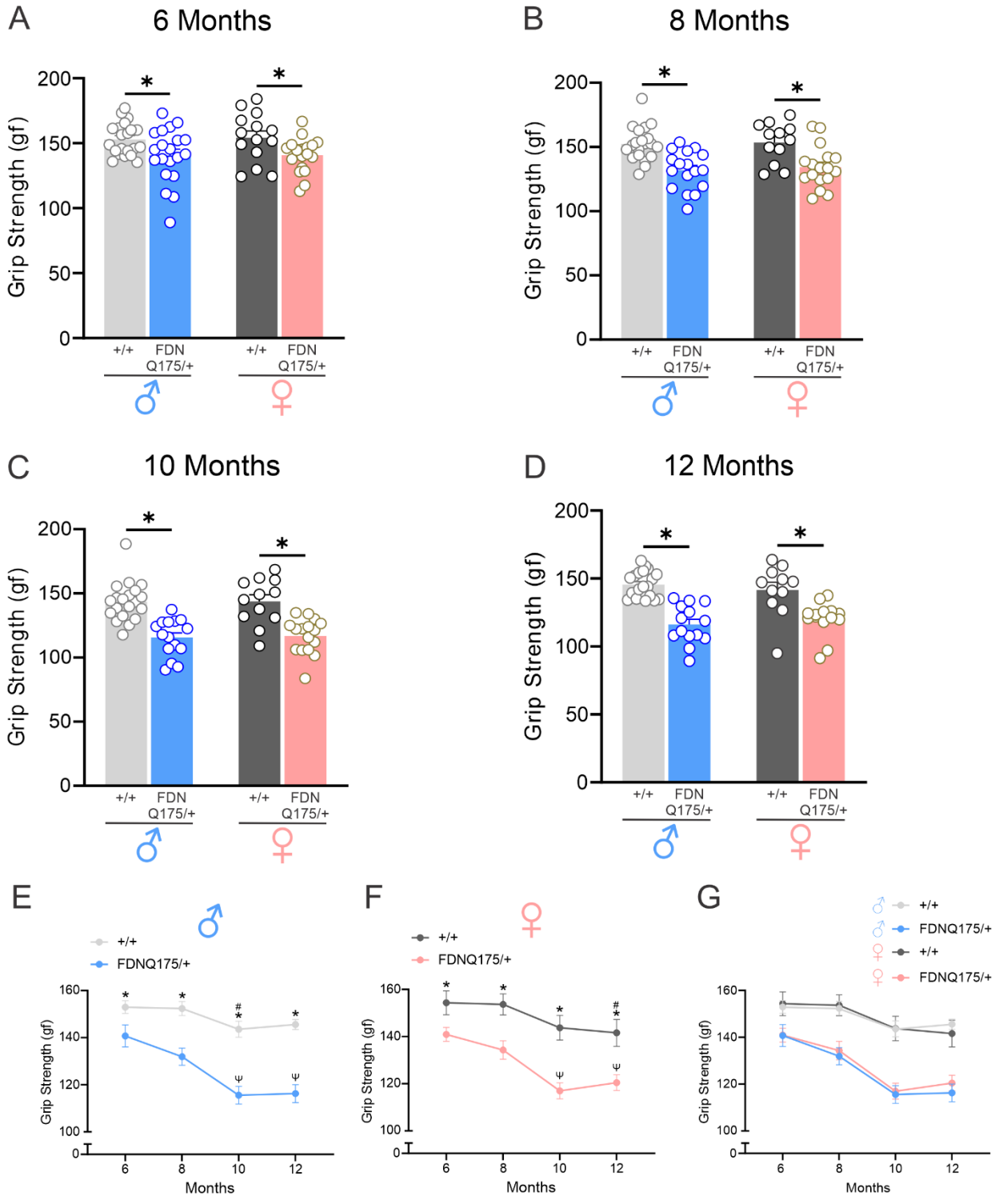

Figure 4.1. Fore limb grip strength of male and female wild-type and FDNQ175 mice from 6 to 12 months of age.

Mean ± SEM grip strength [gram-force (gf)] of wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice at 6 months (n = 14-21 per group) **(A)**, 8 months (n = 12-20

per group) **(B)**, 10 months (n = 12-20 per group) **(C)**, and 12 months (n = 11-20 per group) **(D)** of age. **(E)** Line graph summarizing changes in the mean ± SEM grip strength [gram-force (gf)] of wild-type (+/+; n = 20-21) and heterozygous FDNQ175 (FDNQ175/+; n = 14-20) male mice from 6 to 12 months of age tested at 2 month intervals. **(F)** Line graph summarizing changes in the mean ± SEM grip strength [gram-force (gf)] of wild-type (+/+; n = 11-14) and heterozygous FDNQ175 (FDNQ175/+; n = 14-19) female mice from 6 to 12 months of age tested at 2 month intervals. **(G)** Combined line graph summarizing changes in the mean ± SEM grip strength [gram-force (gf)] of wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice from 6 to 12 months of age tested at 2 month intervals. * indicates significant difference ($p < 0.05$) between age-matched and sex-matched wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) mice. $^{\#}$ indicates significant difference ($p < 0.05$) as compared with 6-month-old sex-matched wild-type (+/+) mice. $^{\Psi}$ indicates significant difference ($p < 0.05$) as compared with 6-month-old sex-matched heterozygous FDNQ175 (FDNQ175/+) mice.

4.4.2 Male and female heterozygous FDNQ175 mice develop deficits in limb coordination and placements in a sex-dependent manner.

We previously detected deficits in rotarod and horizontal ladder performance in both male and female heterozygous zQ175 mice at 13 months of age (Li et al., 2021, 2022b). Another study had shown that heterozygous zQ175 mice can develop impairments in rotarod performance as early as 7 months (Menalled et al., 2012). In the current study, we examined whether similar deficits would develop at an earlier age in the heterozygous FDNQ175 mouse model. The rotarod performance of male heterozygous FDNQ175 mice was significantly worse than that of male wild-type mice at 6 and 8 months of age (**Figure 4.2A**). In contrast, the rotarod performance of female heterozygous FDNQ175 mice remained comparable to age-matched female wild-type controls at the 6- and 8-month time points (**Figure 4.2A and 4.2B**). Starting from 10 months of age, both male and female heterozygous FDNQ175 mice showed significant deficits in rotarod performance compared to their respective age- and sex-matched wild-type controls (**Figure 4.2C and 4.2D**). Furthermore, the rotarod performance of male and female heterozygous FDNQ175 mice deteriorates between the age of 6 and 10 months (**Figure 4.2E and 4.2F**). Importantly, the rotarod performance of age-matched male and female heterozygous FDNQ175 mice were similar at all 4 time points tested (**Figure 4.2G**). Instead, female wild-type mice appeared to perform worse than age-matched male wild-type mice. Specifically, the rotarod performance of female wild-type mice also shows a downward trend after the 8-month time point and is significantly worse than that of age-matched male wild-type mice at the 10- and 12-month time points (**Figure 4.2C, 4.2D and 4.2G**). In fact, 12-month-old wild-type female mice showed significant decrease in their rotarod performance compared to their 6 months data (**Figure 4.2F**). However, female wild-type mice at 12 months of age still performed significantly better than age-matched female heterozygous

FDNQ175 mice (**Figure 4.2D and 4.2F**). Because female wild-type mice showed a lower starting level of performance on the rotarod than male wild-type mice, it is difficult to conclude whether the development of deficits in the rotarod test is sex-dependent in FDNQ175 mice.

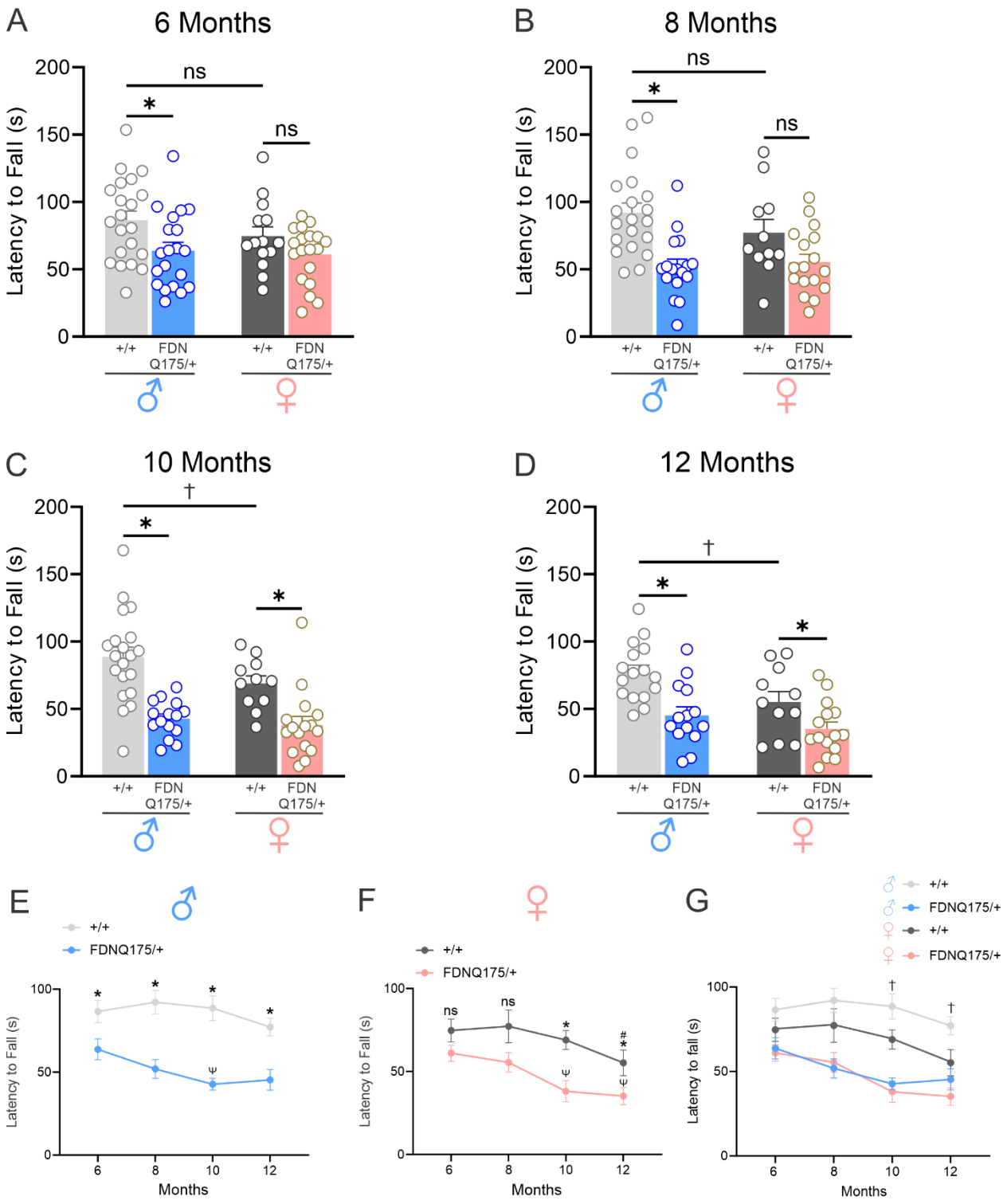

Figure 4.2. Rotarod performance of male and female wild-type and FDNQ175 mice from 6 to 12 months of age.

Mean ± SEM latency to fall (s) from accelerating rotarod of wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice at 6 months (n = 14-21 per group) **(A)**, 8 months

(n = 11-20 per group) **(B)**, 10 months (n = 11-20 per group) **(C)**, and 12 months (n = 11-16 per group) **(D)** of age. **(E)** Line graph summarizing changes in the mean ± SEM latency to fall (s) of wild-type (+/+; n = 16-21) and heterozygous FDNQ175 (FDNQ175/+; n = 14-20) male mice from 6 to 12 months of age tested at 2-month intervals. **(F)** Line graph summarizing changes in the mean ± SEM latency to fall (s) of wild-type (+/+; n = 11-14) and heterozygous FDNQ175 (FDNQ175/+; n = 15-19) female mice from 6 to 12 months of age at tested 2-month intervals. **(G)** Combined line graph summarizing changes in the mean ± SEM latency to fall (s) of wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice from 6 to 12 months of age at tested 2-month intervals. * indicates significant difference ($p < 0.05$) between age-matched and sex-matched wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) mice. $^{\#}$ indicates significant difference ($p < 0.05$) as compared with 6-month-old sex-matched wild-type (+/+) mice. $^{\Psi}$ indicates significant difference ($p < 0.05$) as compared with 6-month-old sex-matched heterozygous FDNQ175 (FDNQ175/+) mice. † indicates significant difference ($p < 0.05$) between age-matched male and female wild-type (+/+) mice.

At 6 months of age, neither male nor female heterozygous FDNQ175 mice made significantly more errors in their limb placements than age- and sex-matched wild-type controls during horizontal ladder traversal (**Figure 4.3A**). However, at 8 months of age, male heterozygous FDNQ175 mice developed deficits in their limb placements, as evidenced by their significantly higher number of mistakes on the horizontal ladder compared to male wild-type mice of the same age (**Figure 4.3B**). Impairments in limb placements were also observed in 10- and 12-month-old male heterozygous FDNQ175 mice compared to age-matched male wild-type controls (**Figure 4.3C and 4.3D**). Furthermore, the deficits in limb placement appeared to progress aggressively from the age of 6 months to 10 month and then plateaus (**Figure 4.3E**). In comparison, the number of mistakes made by female heterozygous FDNQ175 mice during horizontal ladder traversal also showed an upward trend between the age of 6 months and 10 months (**Figure 4.3F and 4.3G**). However, there is no statistically significant difference between the horizontal ladder performance of female heterozygous FDNQ175 mice and age-matched female wild-type mice across all four time points tested (**Figure 4.3A-D and 4.3F**). In conclusion, HD-related deficits in limb coordination and placements appeared to be less pronounced in female heterozygous FDNQ175 mice than males based on the results of the horizontal ladder test.

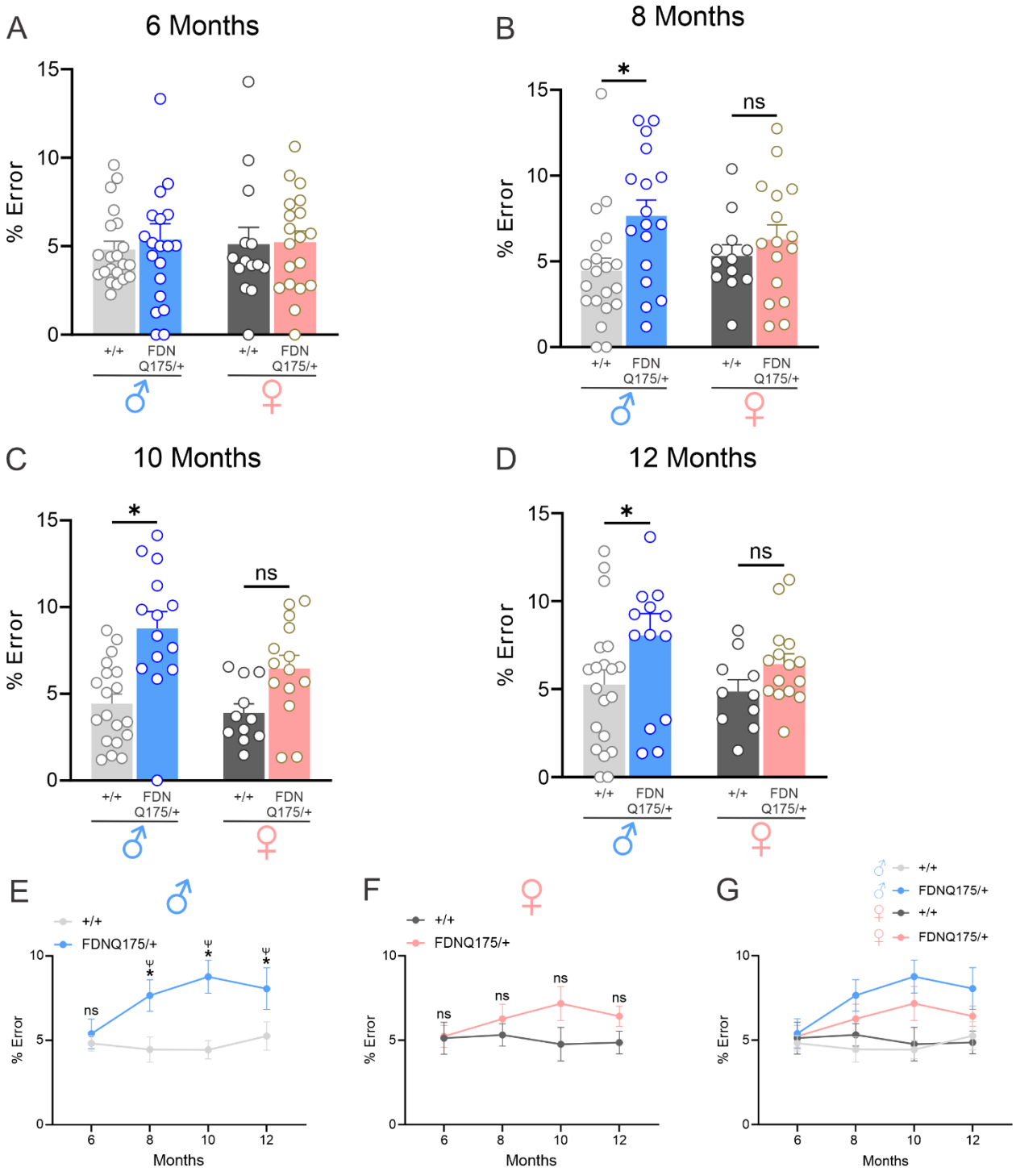

Figure 4.3. Horizontal ladder rung test performance of male and female wild-type and FDNQ175 mice from 6 to 12 months of age.

Mean ± SEM percent error (% error) in limb placement while crossing the horizontal ladder of wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice at 6 months (n = 14-21 per group) **(A)**, 8 months (n = 12-20 per group) **(B)**, 10 months (n = 11-19 per group) **(C)**,

and 12 months (n = 10-20 per group) **(D)** of age. **(E)** Line graph summarizing changes in the mean ± SEM percent error (% error) in limb placement of wild-type (+/+; n = 19-21) and heterozygous FDNQ175 (FDNQ175/+; n = 14-20) male mice from 6 to 12 months of age tested at 2-month intervals. **(F)** Line graph summarizing changes in the mean ± SEM percent error (% error) in limb placement of wild-type (+/+; n = 10-14) and heterozygous FDNQ175 (FDNQ175/+; n = 15-19) female mice from 6 to 12 months of age tested at 2-month intervals. **(G)** Combined line graph summarizing changes in the mean ± SEM percent error (% error) in limb placement of wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice from 6 to 12 months of age tested at 2-month intervals. * indicates significant difference ($p < 0.05$) between age-matched and sex-matched wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) mice. $^{\#}$ indicates significant difference ($p < 0.05$) as compared with 6-month-old sex-matched wild-type (+/+) mice. $^{\Psi}$ indicates significant difference ($p < 0.05$) as compared with 6-month-old sex-matched heterozygous FDNQ175 (FDNQ175/+) mice.

4.4.3 Heterozygous FDNQ175 mice show similar locomotor activity as wild-type mice.

In addition to decline in motor functions, decrease in locomotor activity has been observed in several mouse models of HD, including the R6/2 and zQ175 mouse lines (Abd-Elrahman et al., 2017; Li et al., 2021, 2022b; Lüesse et al., 2001). Therefore, we examined the locomotor activity of heterozygous FDNQ175 mice at different ages. Locomotor activity in an open field arena, as measured by average velocity, remained similar between sex-matched wild-type and heterozygous FDNQ175 mice at 6 months of age (**Figure 4.4A**). In contrast to their decline in grip strength and rotarod performance, heterozygous FDNQ175 mice did not show decreased locomotor activity as they aged. Indeed, with the exception of female mice at 8 months, both male and female heterozygous FDNQ175 mice showed similar levels of locomotor activity as age- and sex-matched wild-type controls across all time points tested in this study (**Figure 4.4A-G**). Interestingly, female heterozygous FDNQ175 mice did show higher levels of locomotor activity than age-matched male heterozygous FDNQ175 mice at 6 months and 8 months of age (**Figure 4.4A, 4.4B and 4.4G**). Moreover, male wild-type mice showed a trend of decreasing locomotor activity, and their average velocity at 12 months of age is significantly lower compared to their own 6-month data and 12-month-old female wild-type mice (**Figure 4.4E and 4.4G**). We also assessed anxiety-like behaviour in FDNQ175 mice by calculating the time spent in four corners of the arena, as more anxious mice have a tendency to spend more time in the corners of the testing arena. Within the time frame we tested, both male and female heterozygous FDNQ175 mice spent similar amount of time as age- and sex-matched wild-type controls in the four corners on average (**Figure 4.5A-G**). Taken together, heterozygous FDNQ175 mice of both sexes did not appear to develop discernible deficits in locomotor activity or anxiety-like behavior up to 12 months of age.

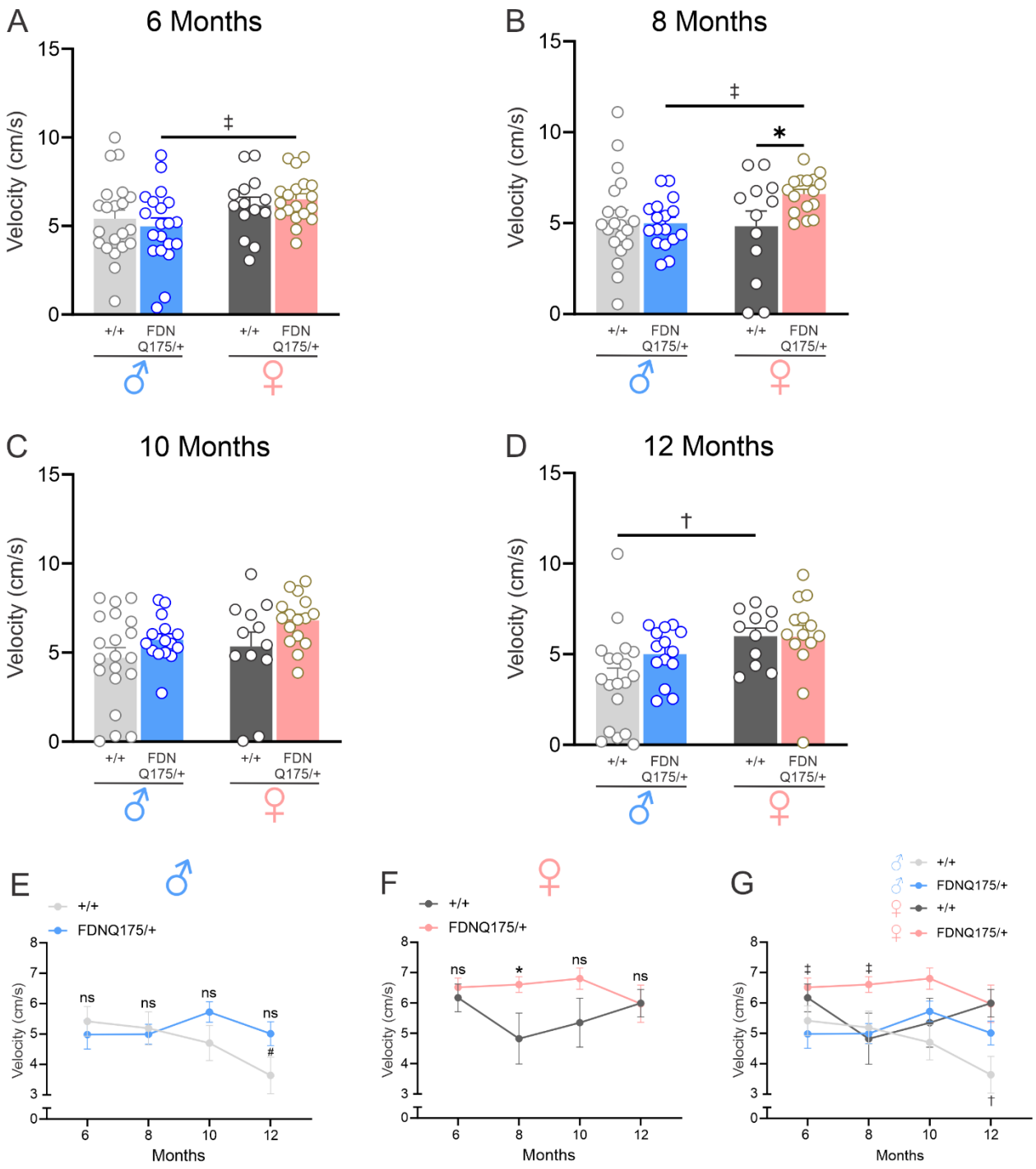

Figure 4.4. Locomotor activity of male and female wild-type and FDNQ175 mice from 6 to 12 months of age.

Mean ± SEM average velocity (cm/s) of wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice at 6 months (n = 14-21 per group) **(A)**, 8 months (n = 12-20 per group) **(B)**, 10 months (n = 12-20 per group) **(C)**, and 12 months (n = 11-19 per group) **(D)** of

age in an open-field arena. **(E)** Line graph summarizing changes in the mean ± SEM average velocity (cm/s) of wild-type (+/+; n = 19-21) and heterozygous FDNQ175 (FDNQ175/+; n = 14-20) male mice from 6 to 12 months of age tested at 2-month intervals. **(F)** Line graph summarizing changes in the mean ± SEM average velocity (cm/s) of wild-type (+/+; n = 11-14) and heterozygous FDNQ175 (FDNQ175/+; n = 14-19) female mice from 6 to 12 months of age tested at 2-month intervals. **(G)** Combined line graph summarizing changes in the mean ± SEM average velocity (cm/s) of wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice from 6 to 12 months of age tested at 2-month intervals. * indicates significant difference ($p < 0.05$) between age-matched and sex-matched wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) mice. # indicates significant difference ($p < 0.05$) as compared with 6-month-old sex-matched wild-type (+/+) mice. † indicates significant difference ($p < 0.05$) between age-matched male and female wild-type (+/+) mice. ‡ indicates significant difference ($p < 0.05$) between age-matched male and female heterozygous FDNQ175 (FDNQ175/+) mice.

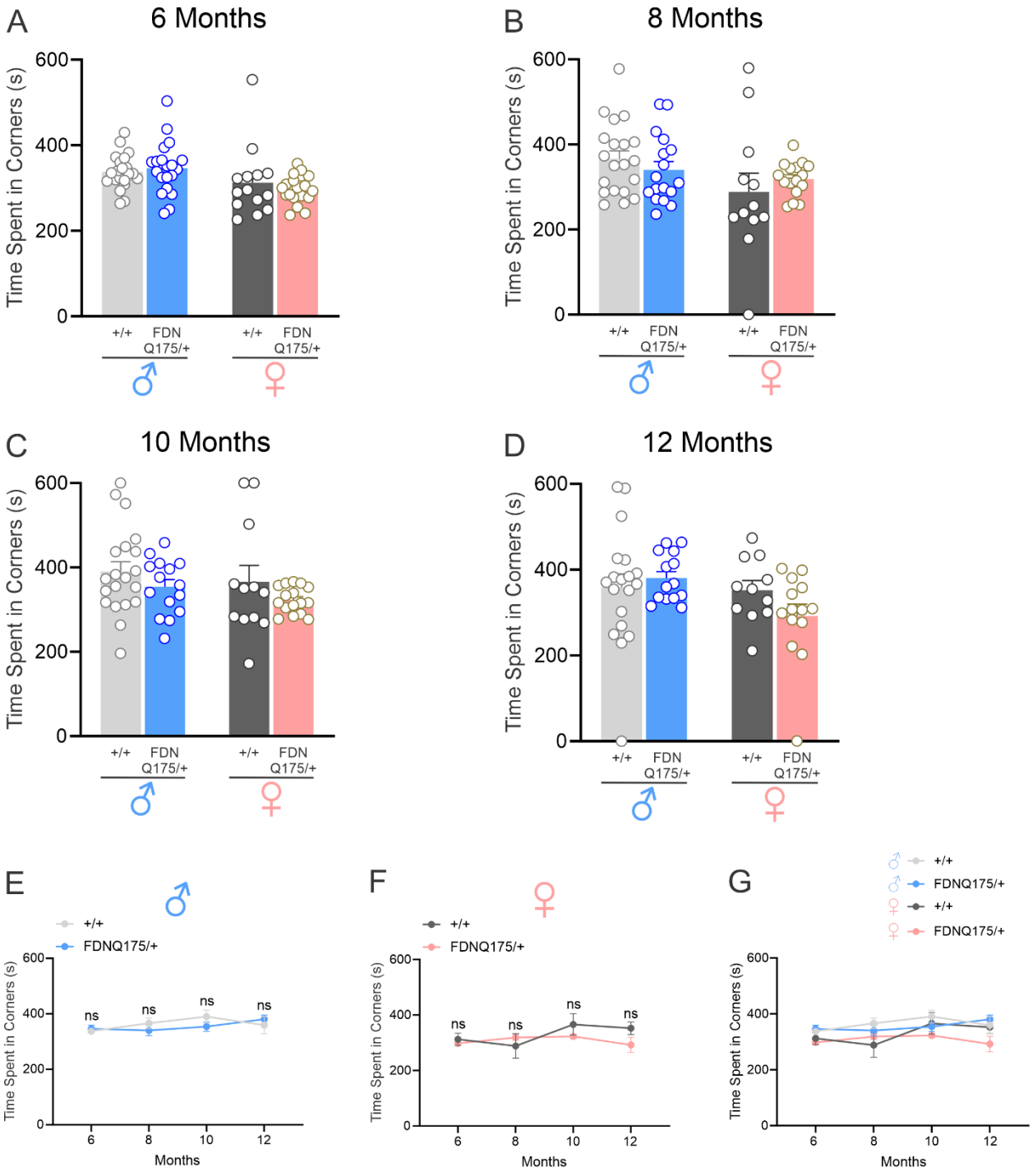

Figure 4.5. Time spent in four corners by male and female wild-type and FDNQ175 mice from 6 to 12 months of age.

Mean ± SEM total time spent in four corners (s) of an open-field arena by wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice at 6 months (n = 14-21 per group) **(A)**, 8 months (n = 12-20 per group) **(B)**, 10 months (n = 12-20 per group) **(C)**, and 12 months (n

= 11-19 per group) **(D)** of age. **(E)** Line graph summarizing changes in the mean ± SEM total time spent in four corners (s) of wild-type (+/+; n = 19-21) and heterozygous FDNQ175 (FDNQ175/+; n = 14-20) male mice from 6 to 12 months of age tested at 2-month intervals. **(F)** Line graph summarizing changes in the mean ± SEM total time spent in four corners (s) of wild-type (+/+; n = 11-14) and heterozygous FDNQ175 (FDNQ175/+; n = 14-19) female mice from 6 to 12 months of age tested at 2-month intervals. **(G)** Combined line graph summarizing changes in the mean ± SEM total time spent in four corners (s) of wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice from 6 to 12 months of age tested at 2-month intervals (ns: nonsignificant)

4.4.4 Both female wild-type and heterozygous FDNQ175 mice develop deficits in cognitive function.

Cognitive impairments have been well documented in HD patients and can often appear prior to formal diagnosis by motor symptoms (Paulsen et al., 2017). Similarly, cognitive deficits have been observed in mouse models of HD (Abd-Elrahman et al., 2017; Southwell et al., 2018). In this study, we examined the development of cognitive decline in the heterozygous FDNQ175 mouse line. At 6 months of age, both male and female heterozygous FDNQ175 mice failed to distinguish between novel and familiar objects, whereas age-matched wild-type mice showed significantly higher preference to explore the novel object on day 2 of the novel object recognition test (**Figure 4.6A**). Furthermore, both male and female heterozygous FDNQ175 continue to show inability to recognize the novel object in tests conducted at 8, 10 and 12 months of age (**Figure 4.6B-D**). Unexpectedly, wild-type mice used in the study also exhibited decline in their cognitive functions. At 12 months of age, male wild-type mice no longer show preference towards the novel object (**Figure 4.6D**). This surprising decline is observed even earlier in female wild-type mice as they explored both the novel and familiar objects equally starting at 8 months of age (**Figure 4.6B**). The early loss of cognitive function in the novel object recognition test in wildtype mice limited our ability to examine the sex-specific progression of cognitive decline in FDNQ175 mice, especially with regard to females.

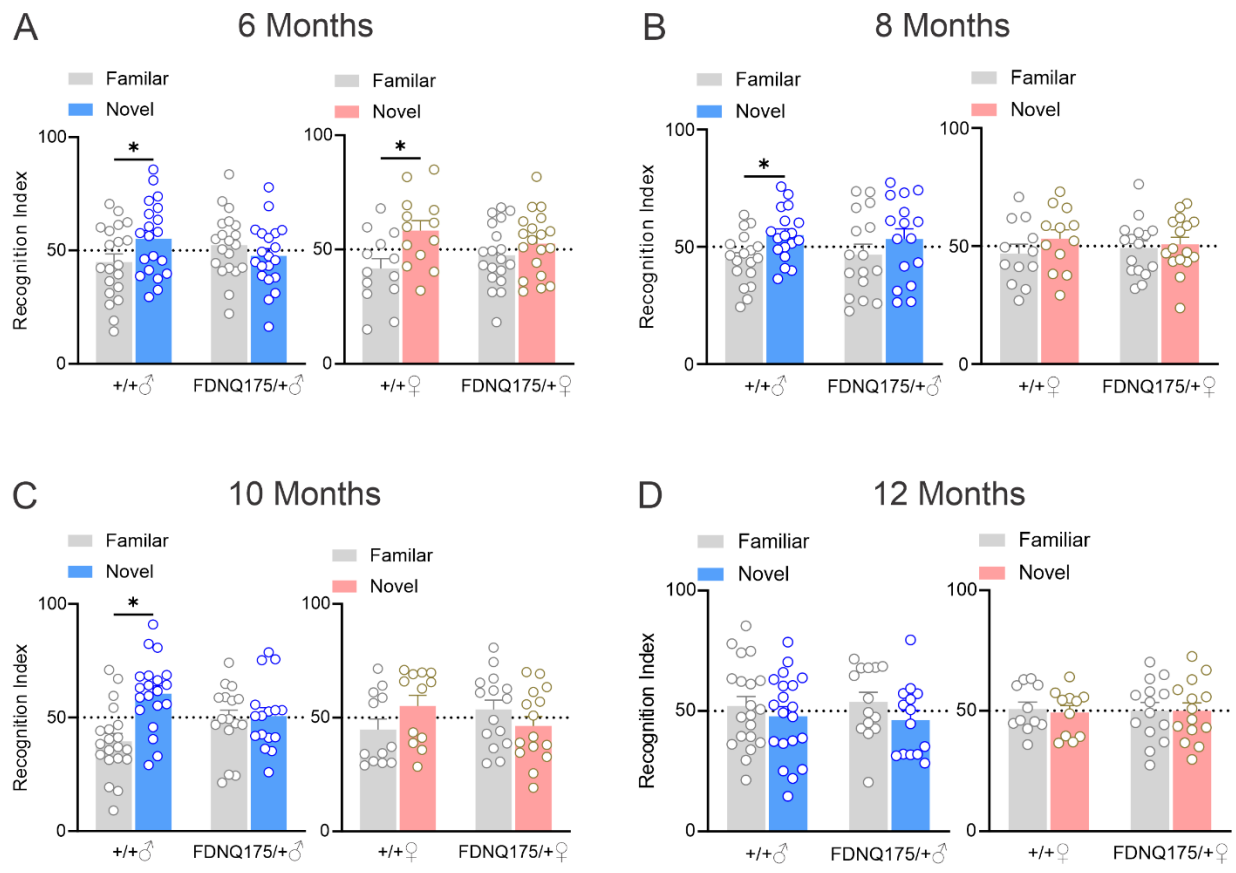

Figure 4.6. Novel object recognition in male and female wild-type and FDNQ175 mice from 6 to 12 months of age.

Mean ± SEM of the recognition index, for exploring a novel object versus a familiar object on the second day of novel object recognition test, of wild-type (+/+) and heterozygous FDNQ175 (FDNQ175/+) male and female mice at 6 months (n = 13-20 per group) **(A)**, 8 months (n = 12-18 per group) **(B)**, 10 months (n = 12-20 per group) **(C)**, and 12 months (n = 11-20 per group) **(D)** of age. * indicates significant difference ($p < 0.05$) between recognition index of the familiar and the novel object.

4.4.5 FDNQ175 mice show progressive increase in huntingtin aggregate deposition.

The formation of cytoplasmic and intracellular aggregates is the key pathological hallmark of HD and these insoluble aggregates have been shown to disrupt normal cellular functions by trapping critical proteins into their matrix (DiFiglia et al., 1997; Landles & Bates, 2004). We have previously detected insoluble mHtt aggregates in 15-month-old male and female heterozygous zQ175 mice (Abd-Elrahman et al., 2017; Li et al., 2021, 2022b). Given the progressive nature of HD in humans, we examined whether the number of mutant huntingtin aggregates in the striatum of FDNQ175 mice increases as they age by staining for EM48 positive aggregates in 8- and 12-month-old mice. Both male and female FDNQ175 mice showed wide-spread mutant huntingtin aggregate deposition in the striatum at 8 months of age. The number of mutant huntingtin aggregates in 12-month-old male and female FDNQ175 mice is significantly higher than that of sex-matched 8-month-old FDNQ175 mice (**Figure 4.7A and 4.7B**). Interestingly, at 8 months of age, female heterozygous FDNQ175 mice have significantly higher number of mutant huntingtin aggregates in the striatum than male heterozygous FDNQ175 mice (**Figure 4.7A and 4.7B**). However, the level of insoluble aggregates was similar between male and female FDNQ175 mice at 12 months of age (**Figure 4.7A and 4.7B**). Overall, we detected an age-dependent increase in the level of mutant huntingtin aggregates in FDNQ175 mice.

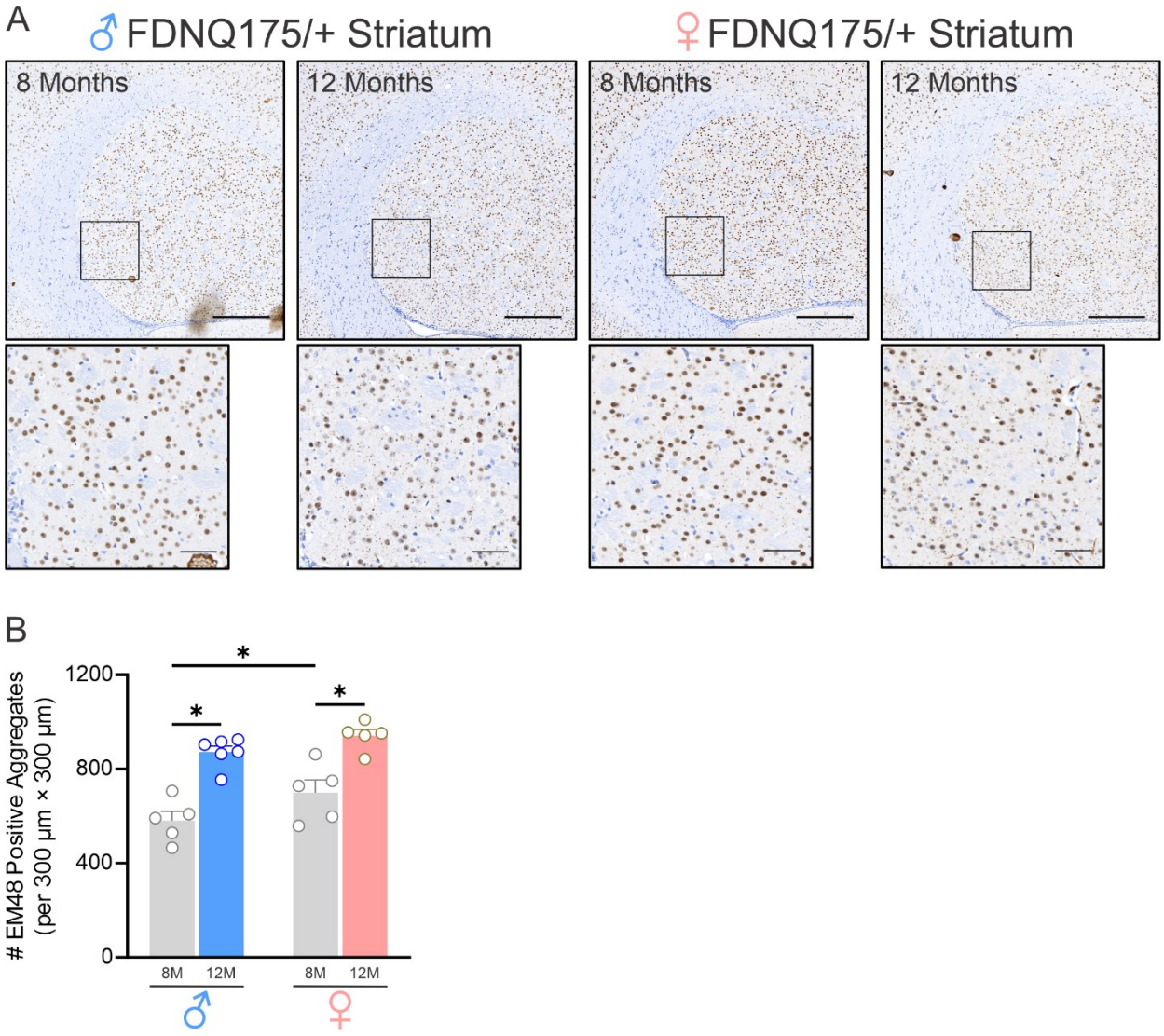

Figure 4.7. Deposition of mutant huntingtin aggregates increases with age in both male and female FDNQ175 mice.

Representative images of staining for mutant huntingtin aggregates using the antibody EM48 **(A)** and quantification of the number of huntingtin aggregates **(B)** in striatal brain slices from 8- and 12-month-old male and female FDNQ175 mice. Scale bar, 300 µm for whole striatum and 50 µm for magnified areas. Data were quantified from two different 300 × 300 μm^2 striatal regions of 6 sections per mouse and five independent mouse brains from each group were used for analysis. * indicates significant difference ($p < 0.05$).

Discussion

The creation of HD mouse models represents a critical step in the research and development of effective treatments for this devastating neurodegenerative disease. Among the different available mouse models, the R6/2 line is the most commonly used for preclinical drug research due to its rapid and aggressive phenotypic progression. However, the R6/2 mouse only expresses exon 1 of the human mutant huntingtin gene and its early disease onset makes it more suitable for modelling juvenile HD. On the other hand, the zQ175 knock-in mouse model of HD represents a more genetically accurate replication of the human condition since its mutant huntingtin expression is under the control of the endogenous promoter and it shows robust phenotype on a heterozygous background (Menalled et al., 2012). The novel FDNQ175 mouse line attempts to improve on the zQ175 model by removing the neo cassette to increase mutant huntingtin expression and using the FVB/N background strain to increase susceptibility to neurodegeneration (Southwell et al., 2016). In this study, we performed longitudinal assessment of the behavioral deficits and neuropathology in heterozygous FDNQ175 mice of both sexes. We found that both male and female FDNQ175 mice showed lower grip strength and cognitive deficits starting 6 months of age. However, we did not detect hypoactivity and elevated anxiety in FDNQ175 mice. More interestingly, female FDNQ175 mice appeared less susceptible to decline in motor function than male mice but showed higher levels of insoluble mHtt aggregates at a younger age.

Growing evidence suggest that sex is a potential contributing factor in the pathophysiology of neurodegenerative diseases (Abd-Elrahman et al., 2017; Li et al., 2021, 2022b). Here, we tested both motor and disease pathology progression in FDNQ175 mice to determine whether sex influences the onset and development of HD symptoms in this mouse model. We observed that

deficits in limb placements and coordination, as measured by the horizontal ladder test, but not grip strength, developed in a sex-dependent manner. This is consistent with the trend observed in Hdh350/+ mice, where gait abnormalities and reduction in motor coordination are detected in only males and not females, but the loss of grip strength is not sex-dependent (Cao et al., 2018b). Interestingly, female heterozygous FDNQ175 mice have higher levels of insoluble mHtt aggregates in the striatum than male heterozygous FDNQ175 mice at 8 months of age, when only male but not female heterozygous FDNQ175 mice showed significantly worse horizontal ladder test performance. Some studies have suggested that insoluble aggregates can play a protective role by sequestering the more toxic protein species, most notably the N-terminal fragments of mutant huntingtin, and thus reducing their toxicity (Arrasate et al., 2004). At 12 months of age, the levels of mHtt aggregates reached a similar level in male and female striatum. It would be interesting in the future to examine whether female heterozygous FDNQ175 mice begin to perform worse than female wild-type mice in the horizontal ladder test after 12 months of age. This could help determine whether insoluble mHtt aggregates play a neuroprotective or neurotoxic role in HD, which remains debatable.

The lack of sex-specific differences in the decline of grip strength may be contributed to the fact that grip strength is found to be controlled, to a large extent, by globus pallidus internus (GPi) and the subthalamic nucleus (STN) (Prodoehl et al., 2009). It is possible that HD pathology in these two basal ganglia nuclei is different from that of the striatum, which could explain why the onset of grip strength deficits are the same between male and female mice despite the sex-dependent changes observed in their striatum.

Anxiety and cognitive decline are key symptoms of HD and can even appear years before disease onset (Ghosh & Tabrizi, 2018). Here, we did not detect anxiety-like behaviour or reduced

locomotor activity in FDNQ175 mice up to 12 months of age, although female FDNQ175 mice had higher levels of locomotor activity than male FDNQ175 mice at younger ages. In a previous study conducted with these mice, decreased open field locomotor activity was not detected in heterozygous FDNQ175 mice up to 9 months of age (Southwell et al., 2016). Here, we showed that even 12-month-old heterozygous FDNQ175 mice do not have reduced locomotor activity. On the other hand, the onset of cognitive deficits in both male and female FDNQ175 mice is consistent with the previous report in which 6-month-old heterozygous FDNQ175 mice did not show a preference for the novel object (Southwell et al., 2016). However, our male and female wild-type mice showed an unexpected early decline in cognitive function at 12 months and 8 months of age, respectively. The surprising decline in the cognitive function of wild-type mice may be caused by inherent vulnerabilities of the FVB/N strain. It was previously reported that FVB/N mice are at risk of sudden death and nervous system lesion due to a pathological syndrome called "space cadet" (Hennemann, 2003; Rosenbaum et al., 2007). The brain lesions are characterized by neurodegenerations in the thalamus, cerebral cortex and hippocampus (Hennemann, 2003). More importantly, female FVB/N mice are shown to be more predisposed to this pathological condition than males (Hennemann, 2003). Therefore, it is possible that the early decline in the cognitive function of our female wild-type controls is caused by the aforementioned syndrome, as the hippocampus is a brain region well-known to be important for learning and memory (Bird & Burgess, 2008).

Overall, it appears that sex does have a significant influence on the decline of motor coordination and limb function associated with HD. One area worth exploring in the future is the action of sex hormones in HD. Notably, membrane estrogen receptors are coupled to metabotropic glutamate receptor 5 (mGluR5) in female rat striatum and can activate mGluR5 signalling in the

presence of estradiol (Grove-Strawser et al., 2010). We have previously demonstrated that mGluR5 interacts with mHtt and its downstream signalling is implicated in HD pathogenesis through complex mechanisms that are still not fully understood (Abd-Elrahman et al., 2017; de Souza et al., 2022; Doria et al., 2013). Furthermore, estrogen has been found to alter the probability of glutamate release, the activity of NMDA receptors and the excitability of medium spiny neurons, all of which are critical factors of excitotoxicity in HD (Cao et al., 2018a; Smejkalova & Woolley, 2010; Weaver et al., 1997). In fact, it was discovered that estrogen regulates the probability of glutamate release through distinct mechanisms in male and female rats (Oberlander & Woolley, 2017). Therefore, glutamatergic signalling in HD brains could be heavily influenced by the activity of sex hormone receptors, leading to sex-specific differences in HD symptoms and progression.

One clear obstacle in determining the influence of sex in HD pathogenesis and progression is the somewhat contradictory data obtained from HD mouse models versus HD patients. Among HD patients, women tend to suffer from more severe motor and cognitive deficits (Foroud et al., 1999; Hentosh et al., 2021; Zielonka et al., 2013). In contrast, studies done using HD mouse models suggest that female HD mice are actually more resilient (Bode et al., 2008; Cao et al., 2018b). For instance, circadian disruption and behaviour fragmentation were found to be less severe in female BACHD mice than males (Kuljis et al., 2016). Furthermore, male mouse striatum has a lower level of extracellular ascorbate, which correlated with a more severe phenotype, and a higher level of disruption in neuroprotective nitric oxide synthase activity than female (Dorner et al., 2007; Padovan-Neto et al., 2019; Rice, 2000). The discrepancies we see between human and mouse could be caused by a variety of factors, including subtle differences between human and mouse huntingtin gene, dissimilarities in the gene's promoter and the extremely long polyglutamine tracts in some models that far exceed the average length reported in humans (Ferrante, 2009). It would

be interesting in the future to explore these plausible causes and their effects on neuroprotective or neurotoxic mechanisms in HD patients, which could explain the sex-specific differences seen in humans. Other non-human primate models of HD have been developed, such as the rhesus macaque model that expresses polygutamaine-expanded mHtt (Yang et al., 2008). Compared to mice, it is possible that higher primates will better replicate the disease physiology and pathophysiology observed in humans and will be more suitable for studying sex-specific differences in HD (Yang et al., 2008).

In conclusion, we have shown that heterozygous FDNQ175 mice develop robust HD phenotypes in various behavioural tests at 6 months of age. Furthermore, consistent with data from other HD mouse models, female FDNQ175 mice appeared to be less vulnerable to HD-related impairments in limb functions than male mice. The mechanism(s) underlying the differences between male and female FDNQ175 mice remains unclear. We have previously demonstrated that pharmacological agents targeting post-synaptic mGluR5 signalling show different efficacies in male versus female mouse models of neurodegenerative diseases, suggesting that glutamate signalling plays a role on sex-specific differences and should be explored in the future (Abd-Elrahman et al., 2020a; Li et al., 2021, 2022b). Here, we provide further evidence that sex is a major contributing factor in the pathophysiology of HD and emphasize the importance of taking it into account when designing novel approaches for treatment.

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Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate: 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.

Consent for publication: Not applicable.

Competing interests: The authors have declared that no conflict of interest exists.

Chapter 5

General Discussion

HD received its name after being described in vivid details by George Huntington in the 19th century. Since then, research surrounding this progressive, inherited and autosomal-dominant neurodegenerative disease has progressed significantly. One of the most important discoveries in HD research is the identification of its cause: an abnormal expansion of CAG repeats in exon 1 of the huntingtin gene, leading to the production of mutant huntingtin proteins with a long polyglutamine stretch (MacDonald et al., 1993). Considerable effort has subsequently gone into studying the action of mutant huntingtin protein in neurons and trying to understand the molecular mechanisms underlying the pathogenesis of HD. However, despite everything we have learned about this disease, there is still no disease-modifying treatment available for HD patients. The overall aim of this thesis is to investigate the potential of mGluR5 and mGluR2/3 as drug targets for the treatment of HD. Moreover, we also explored the effects of sex on glutamatergic signalling and symptom progression in HD mouse models. Specifically, data presented in chapters 2, 3 and 4 of this thesis summarize my effort to answer three questions:

1. Does the activation of mGluR2/3 improve HD symptoms and brain pathology in zQ175 mouse models of HD?
2. Does the inhibition of mGluR5 using the NAM CTEP improves HD symptoms and brain pathology in a sex-specific manner?
3. Is the progression of HD symptoms different between males and females in the FDNQ175 mouse model?

In chapter 2, we demonstrate that activation of mGluR2/3 with the agonist LY379268 rescues motor deficits and reduces brain pathology in the zQ175 mouse model of HD. Notably, treatment with LY379268 for 4 weeks or 8 weeks improved the grip strength, rotarod performance, locomotor activity and limb coordination of both male and female zQ175 mice. Furthermore, the

rescue of motor deficits is accompanied by reductions in mutant huntingtin aggregates, neuronal loss and microglial activation. Interestingly, LY379268 treatments activated and inactivated divergent cell signalling pathways in male and female zQ175 mice, suggesting that mGluR2/3 downstream signalling are regulated in a sex-specific manner in HD brains. In chapter 3, we showed that treatment of zQ175 mice with the mGluR5 NAM, CTEP, improved motor deficits in both male and female mice, but only rescued cognitive deficits in male, not female mice. Despite the sex-specific differences in the rescue of cognitive deficits, CTEP is effective at reducing mutant huntingtin aggregates, neuronal loss and microglia activation in both male and female mice. In chapter 4, we highlighted the effect of sex on HD progression by showing that female FDNQ175 HD mice are less vulnerable to decline in limb function but have higher levels of mutant huntingtin aggregates in the striatum at younger ages. Together, this work provided evidence that supports targeting mGluR2/3 and mGluR5 as a strategy to treat HD while also highlighting the importance of taking sex into consideration when developing therapeutic strategies for HD.

5.1 Therapeutic potential of targeting mGluR5 and mGluR2/3 in HD

Excitotoxicity as a result of glutamate overexposure has long been viewed as a potential trigger of the selective neurodegeneration observed in HD brains. Some compelling evidence includes the disproportionate loss of NMDA receptors in the striatum and the creation of early HD mouse models through direct injection of NMDA receptor agonists into the striatum. Within the striatum, mGluR2/3 and mGluR5 are expressed on presynaptic and postsynaptic terminals, respectively. More importantly, their downstream signalling plays a critical role in regulating glutamate signalling at the opposite end of the synapse, making them possible therapeutic target for the treatment of HD.

At cortico-striatal presynaptic terminals, activation of mGluR2/3 inhibits the release of glutamate into the synapses. This decrease in the availability of extracellular glutamate could help to reduce the activation of NMDA receptors on postsynaptic terminals and consequently reduce excitotoxicity. Moreover, lower levels of glutamate could also decrease the activation of mGluR5, which is implicated in several neurotoxic pathways, including sensitizing NMDA receptors and triggering the release of Ca^{2+} from intracellular storages (Ribeiro et al., 2017). Importantly, activation of NMDA receptors also potentiates mGluR5, creating a positive feed-back loop between these two receptor and greatly increase the levels of Ca^{2+} inside neurons (Alagarsamy et al., 1999). Therefore, simultaneously limiting the activity of both NMDA receptors and mGluR5 by reducing the availability of glutamate may prove to be more effective than trying to target either receptors alone. However, it is important to consider that mGluR2 and mGluR3 may play distinctive roles within the brain. For instance, there are some studies showing that knocking out mGluR2, but not mGluR3, abolished the antipsychotic effects of mGluR2/3 dual agonists LY379268 and LY404039 (Fell et al., 2008; Woolley et al., 2008). On the other hand, mGluR3, but not mGluR2, is expressed on astrocytes where its activation upregulates the expression of glutamate transporters GLAST and GLT1 to promote glutamate reuptake (Aronica et al., 2003; Ohishi et al., 1993b). Furthermore, activation of mGluR3 but not mGluR2 increases the production of glial derived neurotrophic factor (GDNF) in striatal neurons, which can prevent nigrostriatal damage induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection (Battaglia et al., 2009). Therefore, it is possible that the beneficial effects of LY379268 we observed in zQ175 mice is facilitated by either mGluR2 or mGluR3 alone rather than the combined action of both receptors. In fact, one study showed that the mGluR2-specific PAM LY566332 can actually exacerbate Aβ-induced neurodegeneration in neuronal cultures, whereas LY379268 is protective

against Aβ-induced neurodegeneration, suggesting that mGluR3 is the receptor primarily responsible (Caraci et al., 2011). With the recent advancements in mGluR subtype-specific PAMs, it may now be possible to elucidate the unique signalling pathways downstream of mGluR2 and mGluR3 activation. This is a critical step in determining whether one or both receptors are effective drug targets for the treatment of HD, as current evidence indicate that depending on the context, mGluR2 and/or mGluR3 can activate distinct or even antagonistic pathways.

Compared to mGluR2/3, mGluR5 may play a more direct role in regulating glutamate signalling within MSNs. However, it has been difficult to delineate the exact role of mGluR5 in HD as both antagonism and agonism of mGluR5 have shown neuroprotective effects (Budgett et al., 2022). On one hand, treatment with several mGluR5 PAMs rescued motor, cognitive and memory impairments in the BACHD mouse model of HD (Doria et al., 2015; Doria et al., 2013; Doria et al., 2018). These improvements in behavioural deficits are accompanied by the activation of pro-survival pathways, including Akt, ERK1/2 and BDNF, and a reduction in the deposition of mutant huntingtin aggregates (Doria et al., 2015). Interestingly, administration of the mGluR5 PAMs in these studies were done after the emergence of behaviour deficits but before the development of overt neurodegeneration, suggesting that mGluR5 improves the memory of BACHD mice independent of neuroprotective pathways (Doria et al., 2018). On the other hand, antagonism of mGluR5 through pharmacological inhibition also produced beneficial effects in HD mouse models. As demonstrated in this thesis and by earlier studies, CTEP-treated zQ175 HD mice showed improvements in motor function and a reduced number of mutant huntingtin aggregates in the brain (Abd-Elrahman et al., 2017; Ribeiro et al., 2014). The administration of CTEP in these two studies were done after the occurrence of significant striatal atrophy in zQ175 mice, which can be observed at roughly 4 months of age (Heikkinen et al., 2012; Peng et al., 2016).

Therefore, it is possible that mGluR5 signalling is altered with age, with pro-survival pathways being dominant at younger ages and then transition to neurotoxic ones at older ages. In fact, an earlier study by our laboratory showed that mGluR5 signalling is attenuated in young but not old Q111 HD mice (Ribeiro et al., 2010). We have also demonstrated that CTEP loses its efficacy in old AD mice, indicating that pathological mGluR5 signalling may shift as the disease progresses (Abd-Elrahman et al., 2020b). Another study showed that one application of the group I mGluR agonist, 3,5 dihydroxyphenylglycine (DHPG), to primary cortical cell cultures amplifies NMDA-induced neurodegeneration, whereas two consecutive applications of DHPG actually protects against NMDA-induced neurotoxicity (Bruno et al., 2001). These studies clearly show that mGluR5 can switch between its pro-survival and pro-death signalling state based on variety of factors, such as age, disease progression and the timing of previous activation, which will critically influence the efficacy of potential neuroprotective drugs that targets mGluR5. For instance, mGluR5 antagonists and/or NAMs may be effective neuroprotective agents in conditions where the majority of mGluR5 are in a pro-death signalling state, while mGluR5 agonists and/or PAMs are neuroprotective if most of the mGluR5 are in pro-survival signalling states. The recent emergence of biased allosteric modulators that can exert pathway-specific effects on GPCR signalling may offer a novel approach to target mGluR5 for the treatment of HD (Slosky et al., 2021). However, this will require a deeper understanding of their mechanisms and pharmacology, as well as efficient strategies for their discoveries (Slosky et al., 2021).

Based on previous studies and data presented in chapters 2 and 3 of this thesis, one potential strategy for treating HD that warrants exploration in the future is combining the activation of mGluR2/3 with the antagonism of mGluR5. This may prove to be particularly important for promoting the removal of glutamate from the synaptic cleft by astrocytes since mGluR3 and

mGluR5 are expressed on astrocyte where their activation have opposite effects on several pathways critical for astrocyte function (Aronica et al., 2003). In culture, activation of mGluR5 enhances astrocyte proliferation but downregulate the expression of glutamate transporters, whereas activation of mGluR3 produces the opposite effects (Aronica et al., 2003; Ciccarelli et al., 1997). Therefore, simultaneous activation of mGluR2/3 and inhibition of mGluR5 may synergistically enhance the clearance of extracellular glutamate by astrocytes. Another potential way of utilizing this strategy in the future is to target mGluR2/3 and mGluR5 at the same time but in different brain regions, as mGluR2/3 and mGluR5 have different expression patterns and the same receptor can also signal through different pathways depending on their location (Ohishi et al., 1993a, 1993b; Shigemoto et al., 1993). For example, while mGluR2/3 are predominantly expressed on presynaptic terminals within the striatum, they can be found on the postsynaptic side in the prefrontal cortex where their activation actually elevates the activity of NMDA receptors through several different mechanisms (Tyszkiewicz et al., 2004; Xi et al., 2011). Thus, administration of mGluR2/3 agonists may exacerbate excitotoxicity within the prefrontal cortex and should be avoided, whereas the striatum should continue to receive treatment. In addition, we can consider the idea of adopting different treatments based on the progression of HD. Since HD-induced neurodegeneration eventually spread from the striatum to the cortex and hippocampus (McColgan & Tabrizi, 2018), we can target mGluR2/3 and/or mGluR5 in the striatum first, and then expand treatments to other brain regions based on the specific needs of individual patients. However, these potential therapeutic strategies will require safer and more effective methods of administration to target specific brain regions without spilling over.

Overall, there is great potential in targeting mGluR2/3 and mGluR5 as ways to treat HD, especially given the fact that mGluR2/3 agonists and mGluR5 NAMs have been found to be safe

and well tolerated in several clinical studies (Adams et al., 2013; Patil et al., 2007; Quiroz et al., 2016; Youssef et al., 2018). However, we still need significantly deeper understanding of their signalling mechanisms and their functions in different brain regions.

5.2 Mechanisms underlying sex-specific differences in HD and mGluR signalling.

Sex has increasingly been considered as an important factor that influences the progression, prevalence and severity of neurodegenerative diseases such as AD, PD and ALS. Recent evidence indicate that female HD patients may suffer from more symptoms than males (Hentosh et al., 2021). The exact mechanisms that cause the discrepancies between males and females are currently unclear. There is ample amount of evidence showing that there are sex-specific differences in glutamatergic transmission under pathological conditions (Hentosh et al., 2021). We also demonstrated in chapters 2 and 3 of this thesis that targeting mGluR2/3 leads to differential regulation of signalling pathways and targeting mGluR5 appears less efficacious in reversing symptoms in female compared with male mice, respectively. According to studies in rodent models, sex-specific differences are present for both iGluR and mGluR expression. Under basal conditions, female rats show enhanced phosphorylation of the GluA2 subunit at serine 880 and increased levels of GluA2-containing AMPA receptor in post synaptic densities, leading to larger AMPA receptor-mediated synaptic response in the hippocampus (Monfort et al., 2015). Similarly, female rats also express higher levels of NMDA receptor subunits in the hippocampus and prefrontal cortex, which may explain why NMDA-receptor antagonists MK-801 and ketamine elicited stronger responses in female rats than male rats (McDougall et al., 2017; Wang et al., 2015; Wozniak et al., 1998). In addition, female rats are shown to have higher levels of mGluR2/3 and mGluR5 than male rats in several brain regions (Wang et al., 2015). Given that NMDA receptors, AMPA receptors, mGluR5 and mGluR2/3 have been implicated in the pathogenesis of HD, it is

likely that the differences in the expression of these receptors between males and females contribute to the sex-specific differences observed in HD. In the future, it is important to examine whether the expression of mGluR5 and NMDA receptors is higher in female zQ175 HD mice than males. This may help explain why, in chapter 3, we found that the same concentration of CTEP failed to elicit similar outcomes in male and female zQ175 mice.

The reported sex-specific differences in receptor expression levels could be partially responsible for the reduced effectiveness of CTEP in female zQ175 mice. Yet, they do not explain why several studies, as well as our data described in chapter 4, show that female HD mice appear to be more resilient to behavioural deficits and neuropathology (Bode et al., 2008; Cao et al., 2018b; Dorner et al., 2007). The actions of sex hormones are often hypothesized to underlie sex differences observed in neurodegenerative diseases. 17β-estradiol has been shown to protect against NMDA-induced excitotoxicity by inhibiting NMDA receptors (Weaver Jr et al., 1997). In rats, administration of 17β-estradiol reduced oxidative stress and cell death induced by 3-nitropropionic acid (Túnez et al., 2006). Therefore, it is possible that higher levels of 17β-estradiol in female HD mice help to protect against excitotoxicity and thus slowing down the progression of HD symptoms compared to male mice. Unfortunately, attempts to understand sex-specific differences in HD are further complicated by the fact that data obtained from HD mouse models and HD patients are somewhat contradictory. The mechanisms underlying the differences we see between human and mouse remains elusive. However, the estrogen levels in women change through several major hormone transition periods, including puberty, pregnancy, postpartum, perimenopause and postmenopause (Barth et al., 2015). It may be impossible for rodent models to replicate these major changes, and since estrogen can influence various signalling pathways implicated in HD, the effect of estrogen in HD patients may not be mimicked in rodents. In fact,

it was discovered that aging laboratory rodent models can spontaneously enter an abnormal polyfollicular anovulatory state with constant estrus and sustained levels of plasma 17β-estradiol, which is vastly different from natural menopause transition in women (Diaz Brinton, 2012). Furthermore, while all rodents eventually reach the anestrus state, their transition from constant estrus to anestrus is accompanied by significant changes in brain gene expression (Diaz Brinton, 2012). Therefore, we can not eliminate the possibility that this abnormal state introduces alterations to HD progression in rodents, leading to their inaccurate replication of the human condition.

There is also a variety of other factors that can influence glutamatergic signalling and neurodegeneration in male and female brains. One study reported an age-dependent decrease in glutamate concentration in the basal ganglia of men but not women (Sailasuta et al., 2008). This age-dependent and sex-dependent drop in extracellular glutamate levels may contribute to the more severe HD symptoms observed in female patients, since the decrease in glutamate availability may help counteract excitotoxicity to a degree in men suffering from HD. Moreover, several studies in rodents suggested that the intrinsic excitability of MSNs and the synaptic inputs they receive, such as those from the cholinergic or dopaminergic system, show sex- and region-specific differences in the striatum (Cao et al., 2018a). Since excitotoxicity in MSNs is thought to play a critical role in HD neurodegeneration, sex differences in MSN excitability may profoundly alter the effectiveness of treatments that aim to counteract excitotoxicity. Finally, the presence of mutant huntingtin can potentially affect the signalling mechanisms downstream of mGluRs. An early study by our group showed that mGluR5 interacts with mutant huntingtin and this interactions appears to severely impair mGluR5-stimulated IP3 formation via PKC-mediated desensitization (Anborgh et al., 2005; Ribeiro et al., 2010). However, these studies did not

distinguish between male and female mice. Since we discovered that pathological mGluR5 signalling in AD mice is regulated by its sex-dependent interaction with Aβ oligomers and cellular prion protein (Abd-Elrahman et al., 2020a), it may be worth exploring whether the interaction between mutant huntingtin and mGluR5 in HD mice is similarly regulated in a sex-dependent manner. We also reported sex-selective regulation of several cellular pathways following LY379268 treatment in chapter 2. It remains to be determined whether this is the direct result of sex differences in mGluR2/3 signalling or if the reduction in glutamate release following their activation leads to sex-specific changes downstream of other mGluRs or iGluRs.

5.3 Role of autophagy in HD

The exact role of insoluble mutant huntingtin aggregates remains a debated topic. Nevertheless, considerable effort has gone into investigating whether promoting aggregate clearance via autophagic pathways could lead to a therapeutic approach for combating HD. Normal Htt has been found to have an active role in autophagy, as the loss of Htt function in mouse CNS leads to protein accumulation as a result of impaired autophagic degradation (Ochaba et al., 2014; Wong & Holzbaur, 2014). In fact, one study found that huntingtin functions as a scaffold to mediate the binding of p62 to integral autophagosome component microtubule-associated protein 1A/1B-light chain 3 (LC3) and interacts with ULK1 to promote its activation (Rui et al., 2015). Therefore, the loss of wild-type Htt in HD could result in defective autophagy-mediated degradation and an accumulation of toxic mutant huntingtin. In chapters 2 and 3 of thesis, we showed that the activation of mGluR2/3 and inhibition of mGluR5 can reduce the deposition of mutant huntingtin aggregates in the striatum of zQ175 mice. In male mice, this is facilitated, in part, by the disinhibition of the novel GSK3β-ZBTB16-ATG14 and the canonical ULK1 autophagic pathways (Abd-Elrahman et al., 2017). In female mice, the mechanism(s) underlying

this decrease in mutant huntingtin aggregates is not clear. First, there were no significant changes in the GSK3β-ZBTB16-ATG14 autophagic pathway following LY379268 treatment. Second, we have showed that the disinhibition of ULK1 activity following CTEP treatment is due to normalization of Akt signalling in male zQ175 mice, but we did not detect enhanced Akt signalling in zQ175 female mice, and CTEP treatment actually increased the level of phosphorylated Akt in female zQ175 mice. Thus, it is possible that mutant huntingtin aggregates are cleared through other mechanisms. For instance, reducing the level of IP3 or inhibiting IP3 receptors can activate autophagy (Criollo et al., 2007). This is of particular interest in the context of HD, as both mGluR2/3 activation and mGluR5 inhibition can reduce the levels of IP3 in MSNs, which in turn can activate autophagy through this mechanism to promote the clearance of mutant huntingtin aggregates. Another study indicated that elevated levels of cAMP can prevent the degradation of autophagy substrates by enhancing the activity of calpain (Williams et al., 2008). Activation of mGluR2/3 inhibits the production of cAMP as they are coupled to $G\alpha_{i/o}$ (Niswender & Conn, 2010). Therefore, the reduction in mutant huntingtin aggregates we observed in chapter 2 could also be facilitated via this autophagic pathway, independent of the ZBTB16- and ULK1-dependent pathways.

Another area worth exploring in the future is the role of autophagy in the progression of HD neuropathology. In chapter 4, we found that young female FDNQ175 mice has significantly higher levels of mutant huntingtin aggregates in their striatum than males. Interestingly, some data from human and rat cells suggest that males have higher basal levels of autophagy than females due to their higher expression of autophagy related proteins (Addis et al., 2014; Congdon, 2018). In times of neuronal stress, female sex hormones can also limit autophagy activation via Akt and

ERK pathways (Congdon, 2018). Thus, the lower levels of autophagy in females may slow down the clearance of mutant huntingtin aggregates.

Overall, upregulate autophagy to promote the clearance of mutant huntingtin aggregates have been considered as a potential therapy to treat HD. Early studies using compounds such as rapamycin, lithium, trehalose, and rilmenidine yielded beneficial results in animal models, but they all possess side effects. In contrast, mGluR2/3 agonists and mGluR5 NAMs have been found to be safe and well tolerated in multiple clinical trials. Therefore, they may show more promise as therapeutics to activate autophagy, which can be useful for the clearance of pathological proteins, including mutant huntingtin, tau, α-synuclein, ataxin-3, and SOD1 (Martin et al., 2015). In chapters 2 and 3, we only tested compounds in symptomatic zQ175 mice. The treatments may be more efficacious if administered in pre-symptomatic mice to achieve constant mutant huntingtin clearance and prevent aggregate overload. Since wild-type huntingtin is involved in regulating autophagy, it is also important to identify therapeutic target that could help remedy the loss of huntingtin and restore proper autophagy functions. We should also explore other avenues of mutant huntingtin removal. For instance, huntingtin is subject to degradation by the ubiquitin-proteasome system, and thus activating this proteolytic pathway can also promote mutant huntingtin clearance (Martin et al., 2015). Mutant huntingtin stability is also regulated by post-translational modifications, in particular, sumoylation competes with ubiquitination and prevents proteosomal degradation of mutant huntingtin. Therefore, pharmacological agents that can block sumoylation may assist the removal of mutant huntingtin and reduce toxicity. Ultimately, a combination of therapies may be required to achieve the greatest extent of mutant huntingtin clearance and the best therapeutic outcomes.

5.4 Role of mGluR5 in microglial activation in HD

Inflammation in neurodegenerative diseases such as AD, PD and HD are characterized by microglia activation and cytokine production, but there is little infiltration of peripheral immune cells. A recent post-mortem study revealed that HD has a distinct profile of inflammatory cytokines, with the striatum having higher levels of interleukin (IL)-1β, tumor necrosis factor α (TNF-α), IL-6, IL-8 and matrix metallopeptidase 9 (MMP9) (Silvestroni et al., 2009). Activation of microglia can be observed in pre-symptomatic HD gene carriers several years prior to disease onset and correlates with pathology in HD patients (Crotti & Glass, 2015). More importantly, the expression of mutant huntingtin in microglia confers a cell-autonomous increase in proinflammatory gene expression and leads to hyperactive response to stimuli (Björkqvist et al., 2008; Crotti et al., 2014). Similar to the increased microglia cell counts in the caudate putamen of HD patients, we observed increased numbers of microglia in the striatum of zQ175 mice, which was reduced by treatment with CTEP. mGluR5 is expressed in microglia where their signalling plays a key role in regulating microglia activity. The exact role of microglial mGluR5 has not been explored in detail. In the context of excitotoxicity, injection of ibotenic acid, an NMDA receptor agonist, led to significant and persistent microglial activation, which is accompanied by increased mGluR5 expression in microglia (Drouin-Ouellet et al., 2011). However, it appears that activation of microglial mGluR5 under normal conditions can help reduce microglial activation and inflammatory cytokine production (Byrnes et al., 2012; Byrnes et al., 2009a; Byrnes et al., 2009b). In neurodegenerative diseases, the signalling of microglial mGluR5 is much more difficult to unravel, as it can be highly disease-dependent. In AD and PD, activation of mGluR5 can help reduce microglia-induced neuroinflammation (Bellozi et al., 2019; Zhang et al., 2021). In the SOD1G93A mouse model of amyotrophic lateral sclerosis, genetic silencing and pharmacological blockade of mGluR5 reduced

microglia activation (Bonifacino et al., 2017; Milanese et al., 2021). With the current available information, it is difficult to determine whether CTEP reduces the number of microglia directly by blocking specific microglial mGluR5 signalling pathways or indirectly by reducing overall neurodegeneration or both. It is widely believed that substances released from dying cells trigger microglial activation, which consequently secretes inflammatory molecules, leading to further changes in the dying cells (Möller, 2010). In chapter 3, we observed a significantly higher number of NeuN-positive cells in the striatum of CTEP-treated zQ175 mice. It is possible that the decreased number of microglia in CTEP-treated zQ175 mice is the result of improved neuronal survival following mGluR5 antagonism. In fact, studies have consistently shown that activation of mGluR5 with an agonist or PAM reduces microglial activation (Byrnes et al., 2009a; Farso et al., 2009; Loane et al., 2009; Loane et al., 2014; Piers et al., 2011). Therefore, we can not rule out the possibility that CTEP treatment is driving a proinflammatory microglial response and we are only seeing reduction in microglial numbers due to its neuroprotective effects in other cell types and consequently reducing the levels of molecules that can trigger microglial activation. For instance, CTEP could block astrocytic mGluR5-mediated calcium oscillations, which have been shown to propagate as a wave among astrocytes and contribute to distant microglial activation (Bradley et al., 2011; Liu et al., 2011).

Targeting neuroinflammation has been shown to be beneficial across multiple neurodegenerative diseases. mGluR5 clearly plays an important role in modulating neuroinflammatory processes. However, in order to select the most effective mGluR5 ligands to treat diseases, we will need to better understand the role of mGluR5 in each cell type and the relative contribution of each cell type to disease pathology. For now, it remains unclear whether mGluR5 needs to be activated, inhibited or biased towards specific signalling pathways to properly

modulate neuroinflammation. Recently, our lab has generated AD mice with site-specific knockout of mGluR5 in astrocytes and microglia, which could greatly help us elucidate the role of glial mGluR5 signalling in neuroinflammation associated with neurodegenerative diseases.

5.5 Role of mGluR2/3 in microglial activation in HD

In chapter 2, we showed that activation of mGluR2/3 can reduce microglial numbers in the striatum of zQ175 mice. Interestingly, this appears to be facilitated by the activity of mGluR3 and not mGluR2, as multiple studies suggest that selective activation of mGluR2 actually exacerbates microglial-mediated neurotoxicity (Pinteaux-Jones et al., 2008; Taylor et al., 2005). This is not the only instance in which mGluR2 and mGluR3 exhibited differential functions. In mixed neuronal cultures, selective activation of mGluR2 amplified Aβ-induced neurotoxicity, whereas dual activation of mGluR2 and mGluR3 is neuroprotective (Caraci et al., 2011). Moreover, selective antagonism of mGluR3 abolished the protective activity of LY379268 against Aβ (Caraci et al., 2011). LY379268 treatment could help reduce the number of microglia through two mGluR3-mediated mechanisms. First, the activation of mGluR3 could facilitate glutamate reuptake by astrocytes, which is found to be reduced in HD (Hassel et al., 2008; Lievens et al., 2001; Yao et al., 2005). Glutamate regulates microglia activity by activating microglial AMPA or kainate receptors, which can trigger microglia proliferation, morphological changes, migration, phagocytosis and the secretion of inflammatory mediators (Zhang et al., 2020). Similarly, activation of microglial NMDA receptors has been shown to trigger neuroinflammation and neuronal cell death (Kaindl et al., 2012). Therefore, the higher numbers of microglia in zQ175 HD mice could be caused by elevated levels of glutamate and LY379268 treatment reduces microglial numbers by normalizing extracellular glutamate concentrations. Second, mGluR3 stimulates the production of neurotrophic factors such as TGF-β and GDNF (Battaglia et al., 2009; Bruno et al.,

1998; Caraci et al., 2011). This could help improve neuronal survival and prevent further activation of microglia.

The molecular mechanisms underlying the opposing actions of mGluR2 and mGluR3 is not fully clear. Some have suggested that the neuroprotective effect of mGluR3 activation is associated with its ability to regulate gene expression in astrocytes, where mGluR2 is not expressed (Aronica et al., 2003; Battaglia et al., 2009; Bruno et al., 1998; Caraci et al., 2011). However, the neurotoxic pathways downstream of mGluR2 activation remains elusive. Nevertheless, current evidence suggests that selective activation mGluR3 is a viable strategy to reduce neuroinflammation. It may also be worth looking into group III mGluRs as potential therapeutic targets, as treatment with the mGluR4-specific PAM ADX88178 reduced pro-inflammatory responses in primary microglia (Ponnazhagan et al., 2016).

5.6 Potential contribution of microglia to sex-specific differences in HD.

Multiple studies have identified sex-specific differences in microglia gene expression, cell number and morphology (Guneykaya et al., 2018; Lenz et al., 2013; Mouton et al., 2002). These microglial sex differences are likely modulated by sex hormones, especially in adult mice. Since microglia are known to express hormone receptors in normal and injured brains, it is possible that these sex differences in microglia may lead to differential neural responses to pathological stimuli in male and female brains (García-Ovejero et al., 2002; Sierra et al., 2008). In fact, studies have shown sexually dimorphic microglial responses to traumatic brain injury, ischemic stroke and depress behaviour in animal models (Kerr et al., 2019; Liu et al., 2019; Villapol et al., 2017). Furthermore, female microglia transplanted into male mice are neuroprotective against ischemia, whereas male microglia transplanted into female mice did not produce the same result (Villa et al.,

2018). Therefore, we can not rule out the possibility that male and female microglia could also respond differently to the toxic actions of mutant huntingtin, leading to sex-specific differences in the progression of HD symptoms and brain pathology. Future studies will be needed to understand how sex hormones and changes in their circulating levels during aging influence the action of microglia. This may help explain the sex differences observed in HD and other neurodegenerative disorders.

5.7 Conclusion

HD has debilitating symptoms, but there is still no disease-modifying treatment to help its victims. Within this thesis, we demonstrated that inhibition of mGluR5 and activation of mGluR2/3 can improve HD symptoms and brain pathology via activation of autophagy and pro-survival pathways in preclinical mouse models. In addition, we provide evidence showing that sex can influence both the progression of HD symptoms and the efficacy of potential drug treatment. Given that pharmacological agents targeting either mGluR5 or mGluR2/3 have been found to be safe and well-tolerated in clinical trials, our findings argue for testing them as potential therapeutic treatments for HD. Of course, this will need to take into account the role of sex-specific differences in HD.

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