Role of Optineurin in Metabotropic Glutamate Receptor 5-regulated Autophagy

by

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Legend

AD	Alzheimer's disease
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	AMP-activated protein kinase
Atg12-5-16L1 complex	Autophagy-related protein 12-5-16L1 complex
Atg13	Autophagy-related protein 13
Atg14	Autophagy-related protein 14
BDNF	Brain-derived neurotrophic factor
Ca^{2+}	Calcium
DAG	Diacylglycerol
DHPG	3,5-dihyroxyphenylglycine
ERK1/2	Extracellular signal-regulated kinases 1 & 2
GSK3β	Glycogen synthase kinase 3 beta
HD	Huntington's disease
IP_3	Inositol-1,4,5-triphosphate
KO	Knockout
LC3	Microtubule-associated protein 1A/1B-light chain 3
LTD	Long-term synaptic depression
mGluR5	Metabotropic glutamate receptor 5
mHTT	Mutant Huntingtin
mTOR	Mammalian target of rapamycin

NMDA	N-Methyl-d-Aspartate
OPTN	Optineurin
PD	Parkinson’s disease
PI3K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
PLC	Phospholipase C
TBK1	TANK-binding kinase 1
ULK1	Unc-51-Like kinase 1
VPS34	Vacuolar protein sorting 34
WT	Wild-type
ZBTB16	Zinc finger and BTB domain-containing protein 16

Abstract

Autophagy is a conserved, regulated mechanism that is responsible for the degradation of misfolded proteins and recycling of cellular components. Defects in autophagy were linked to multiple pathological conditions, including neurodegenerative diseases. Metabotropic glutamate receptor 5 (mGluR5) is Gq-coupled receptor that has been shown to regulate autophagy via the mTOR/ULK1/Atg13 and GSK3β/ZBTB16/Atg14 pathways in two neurodegenerative diseases, Alzheimer's disease and Huntington's disease. Moreover, optineurin (OPTN), an autophagy receptor, has recently been shown to play a role in mGluR-mediated signaling but its exact role in regulating autophagy downstream of mGluR5 remains largely unknown. CRISPR/Cas9 genome editing was used to knockout OPTN in a mouse striatal cell line (ST*Hdh*$_{Q7/Q7}$) and cre/lox recombination technology was used to globally knockout OPTN in C57BL/6 mice. Protein expression levels were measured in several autophagy markers, including p62, LC3β, GSK3β, ULK1, ZBTB16, and VPS34, as well as, in cell survival marker, ERK1/2 following treatment with either DHPG (mGluR1/5 agonist) or HBSS in both wild-type and OPTN knockout groups. The activation of mGluR5 resulted in an increased phosphorylation of ERK1/2 in WT cells/tissue, however had no effect on OPTN knockout groups. Furthermore, OPTN knockout groups displayed an increase in phosphorylation of ULK1-S757, LC3β and p62, and inhibition of autophagy via the ULK1/Atg13 pathway. As well, our findings show a decrease in phosphorylation of GSK3β-S9, and ZBTB16 and an increase in VPS34 levels, indicating that autophagy is inhibited via the GSK3β/ZBTB16/Atg14 pathway. Overall, these findings provide further evidence for the critical role of OPTN in mGluR5 signaling via canonical and non-canonical pathways.

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1. Introduction

1.1 Neurodegenerative Diseases

Neurodegenerative diseases are classified as incurable and debilitating diseases, which results in the progressive degeneration and death of nerve cells. This causes a wide range of problems involving movement and mental functioning (Gitler et al., 2017). Examples of neurodegenerative diseases include, Alzheimer’s disease, Huntington’s disease and Parkinson’s disease (Ross and Poirier, 2004).

Huntington’s disease (HD) is caused by a CAG trinucleotide repeat, causing an expansion of the *Huntingtin (htt)* gene and an abnormal polyglutamine extension in the amino-terminal region of the Huntingtin (HTT) protein (MacDonald et al, 1993). This expansion results in a mutated HTT protein, which has been found to contain a toxic neuronal characteristic, primarily in the striatum, causing the accumulation of toxic aggregates and neuronal cell death (Snowden, 2017; Labbadia and Morimoto, 2013; Shirasaki et al., 2012). HD patients are described as having involuntary movements, cognitive disorder and neuropsychiatric change along with the unavoidable death of the patient 15-20 years within the onset of symptoms (Snowden, 2017; Li and Li, 2004).

Alzheimer’s disease (AD) is a late-onset dementing illness, which promotes neuron degeneration primarily in the forebrain and hippocampus (Gaugler et al., 2016). While the cause remains largely unknown, it is recognized that AD involves two major kinds of protein aggregates (Jouanne et al., 2017). Extracellular aggregates, known as β-amyloid plaques, form when amyloid precursor protein (APP) is improperly cleaved by the γ-secretase complex (O’Brien and Wong, 2011). Multiple of these misfolded β-amyloid proteins begin the process of aggregation to form the β-amyloid oligomers,

which are extremely toxic and result in neuron degeneration. The β-amyloid oligomers have the ability to aggregate further into amyloid plaques, which are also known for their neurotoxic characteristic (Reiss et al., 2018). There are also intracellular aggregates known as neurofibrillary tangles, which form when the microtubule-associated protein tau, is misfolded in a specific way (Congdon and Sigurdsson, 2018; Chong et al., 2018). This results in the inability to properly transport nutrients and other important substances within the nerve cell (Wu et al., 2017). AD patients are described as having progressive loss of memory, task performance speech, and recognition of people/objects (Ross and Poirier, 2004).

Parkinson's disease (PD) is characterized by the degeneration of dopaminergic neurons in the substantia nigra and striatum, due to an accumulation of Lewy body aggregates (Forno,1996; Dickinson, 2018). Lewy bodies form when the α-synuclein protein undergoes misfolding, due to various reasons including mutations involving several different genes, and other environmental factors (Valente et al., 2004). These misfolded α-synuclein proteins aggregate into higher-order structures, known as protofibrils and then ultimately, Lewy bodies (Irwin et al., 2013). Parkinson's disease is most commonly known for its effect on movement. Specifically, PD patients are defined as having tremors, rigidity, slow movements, as well as postural and autonomic instability (Ross and Poirier, 2004).

It is clear that neurodegenerative diseases represent a large group of illnesses that represent a major threat to human health, as there currently are no known disease-modifying therapies (Gitler et al., 2017). Although each disease has a different cause and mechanism, a common theme is the accumulation of a toxic aggregate, which

ultimately triggers neuron degeneration and death (Ross and Poirier, 2004). A potential method of degrading and clearing these toxic aggregates could be a process known as autophagy. Advancements in autophagy induction could lead to many promising developments of therapeutic approaches.

1.2 Autophagy

Autophagy is a process, by which mammalian cells perform cellular degradation by sequestering portions of cytosol and organelles into a vesicle, known as an autophagosome, and delivering them into a degradative organelle, known as a lysosome. Together, this creates a phagolysosome, which breaks down and recycles the cellular waste (Yang and Klionsky, 2010). Many genes have been identified as playing a role in the autophagy pathways, including but not limited to: autophagy-related genes (ATG), Tor complex 1 and 2 (TORC1 and TORC2), uncoordinated 51-like kinases 1 and 2 (ULK1 and ULK2), Beclin-1 (BECN1), LC3/GAPARAP, Ras/PKA (Yang and Klionsky, 2010). The autophagy process is originally initiated by the inhibition of mTOR or the activation of AMPK, which leads to the dephosphorylation of the ULK complex (Parzych and Klionsky, 2014; Yang and Klionsky, 2009). This results in the phosphorylation of the PI3K CIII complex, which contains the autophagy protein, ATG14, and VPS34, and begins the formation of the autophagosome (Yang and Klionsky, 2010). Following this, protein aggregates targeted for autophagy, known as ubiquitin chains, are assembled into “p62” protein bodies, and bind to the ubiquitin binding domain of the autophagy receptor. The autophagy receptor then transports the ubiquitinated misfolded proteins to the autophagosome, where the autophagy receptor

binds to LC3, which has been recruited to the autophagosome, via its LC3-interacting region (Tanida et al., 2008). This leads to the closure of the autophagosome, which then fuses with the lysosome to form the autolysosome. The vesicle is then brought for breakdown and degradation (reviewed in Nixon, 2013; Sword et al., 2004) (Figure 1).

Disruptions in autophagy have been known to be linked to many neurodegenerative diseases including, Huntington's, Alzheimer's and Parkinson's disease (Guo et al., 2018). These three neurodegenerative diseases all display a similar characteristic, where there is an accumulation of toxic aggregates in certain regions of the brain, which then ultimately leads to neuron degeneration and death (Ross and Poirier, 2004). Along with these higher than normal levels of toxic aggregates, the actual mechanism to breakdown and degrade these aggregates has also been hindered (Figure 2).

Huntington's disease is caused by an abnormal polyglutamine extension, resulting in a mutated Htt protein. It is known that Htt plays a key role in autophagosome transport (Steffan, 2010), however mutant Htt disrupts the axonal transport of autophagosomes (Zheng et al., 2010). Although Htt is not necessary for autophagosome formation or cargo binding, the disruption in autophagosome transportation is associated with the inability to degrade engulfed mitochondrial fragments. This suggests that the disruption of autophagosome transportation leads to ineffective autophagosome maturation (Yvette and Holzbaur, 2014). This could potentially be due to the inhibition of autophagosome and lysosome fusion along the axon. The molecular motor dynein is regulated by the Htt protein, and it has been shown that mutant Htt disrupts dynein motor activity, resulting in the impairment of autophagosome and lysosome fusion (Ravikumar et al., 2005). Moreover, it has been

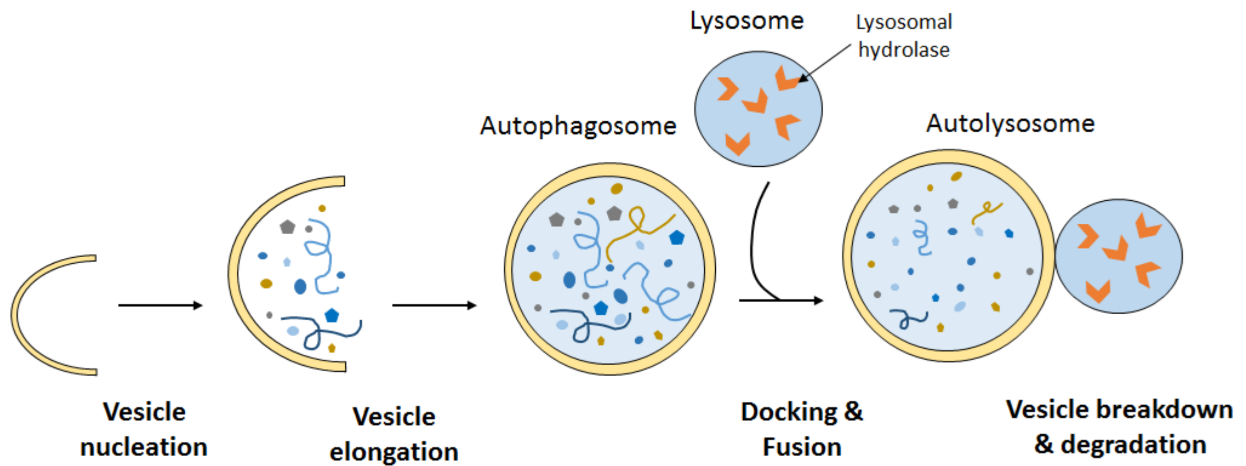
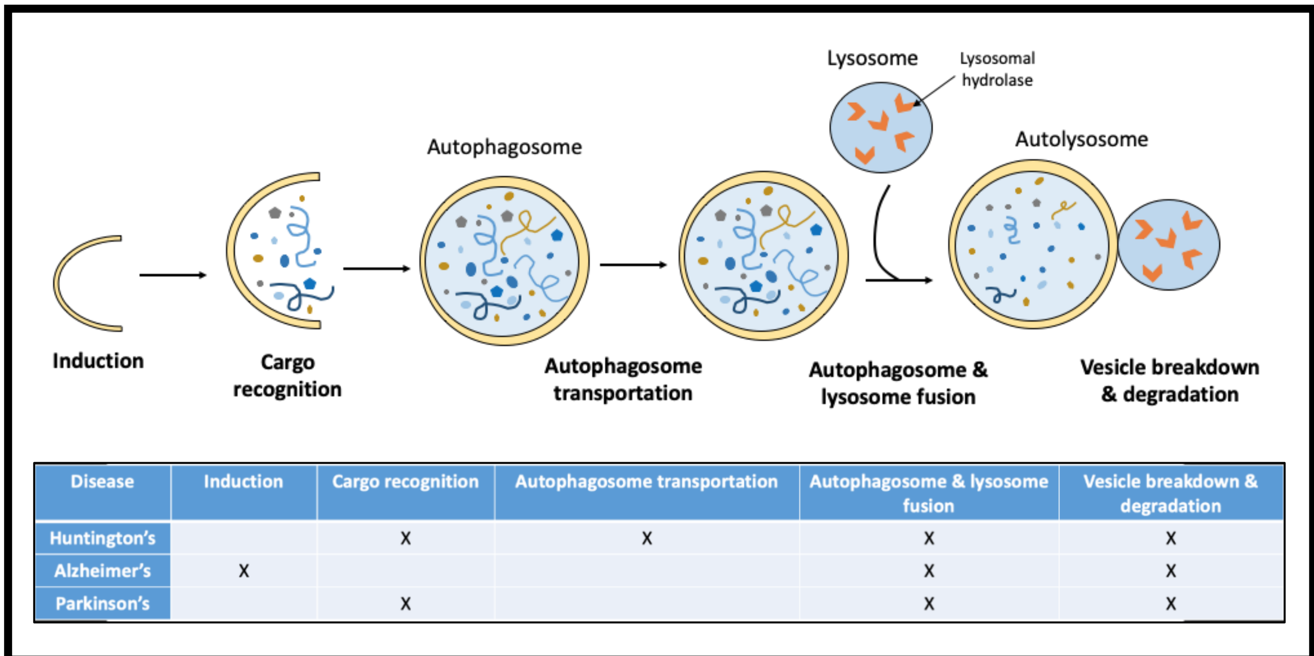


Figure 1. Autophagy process. Schematic representation of the autophagy process, where the autophagosome forms, binding with cargo marked for degradation. The autophagosome then fuses with the lysosome and is brought for vesicle breakdown and degradation.



Disease	Induction	Cargo recognition	Autophagosome transportation	Autophagosome & lysosome fusion	Vesicle breakdown & degradation
Huntington's		X	X	X	X
Alzheimer's	X			X	X
Parkinson's		X		X	X

Figure 2. Disruption of autophagy process in neurodegenerative diseases. Schematic representation of the autophagy process followed by table showing which steps of the autophagy process are disrupted in Huntington's, Alzheimer's and Parkinson's disease.

shown that in the presence of mutant Huntingtin, there is an impairment of cargo recognition, which may be due to the abnormal relationship between p62 and mutant Htt (Martinez-Vicente et al., 2010). It has been shown that in the presence of mutant Htt, p62 levels are significantly higher and demonstrate enhanced p62 binding to autophagic vesicles. (Martinez-Vicente et al., 2010). Additionally, polymorphisms in autophagy-related genes may contribute to an earlier onset of Huntington's disease. A polymorphism in the Atg7 gene that substitutes alanine for valine (V471A) was correlated with a 4-year earlier onset of the disease (Metzger et al., 2010).

Alzheimer's disease is characterized by extracellular aggregates, known as β-amyloid plaques, and intracellular aggregates, known as neurofibrillary tangles. An increase in β-amyloid has revealed an overactivation of the PI3K/Akt pathway. This results in the hyperphosphorylation of mTOR, an impairment of autophagy induction and an overall inhibition of autophagy (Tramutola et al., 2015; Congdon & Sigurdsson et al., 2018). It has also been shown that key autophagy-related proteins, including Beclin-1, are decreased in Alzheimer's disease, causing a disruption in neuronal autophagy (Pickford et al., 2008). Not only has it been discovered that there is an impairment involving the induction of autophagy, but there is also an impairment of the clearance of autophagosome vesicles found in dystrophic neurites, which are a common pathologic feature of Alzheimer's disease (Boland et al., 2008; Sanchez-Varo et al., 2012; Benzing et al., 1993). Similar to Huntington's disease, this is due to a failure of autophagosomes to fuse with lysosomes (Bordi et al., 2016; Piras et al., 2016). This defect in the autophagosome-lysosome pathway may contribute further to the development of neurofibrillary tangles found in AD patients (Piras et al., 2016). Recently, it has been

discovered that autophagy is already impaired in asymptomatic AD patients, suggesting that the disruption in autophagy may be an early event in the disease progression (Tramutola et al., 2015). If a deficiency in autophagy is one the first effects observed in AD patients, this could greatly contribute to β-amyloid deposition in the brain.

Parkinson's disease (PD) is characterized by the degeneration of dopaminergic neurons in the substantia nigra and striatum, due to an accumulation of Lewy body aggregates (Forno,1996; Dickinson, 2018), which form when the α-synuclein protein undergoes misfolding (Valente et al., 2004). Similar to other neurodegenerative diseases, postmortem brain samples from Parkinson's disease patients and cultured cells intoxicated with parkinsonian neurotoxins revealed an accumulation of autophagosomes, suggesting there is an impairment in the clearance of autophagosome vesicles (Anglade et al., 1997; Zhu et al., 2007). This increase in autophagosome vesicles is a direct result of dysfunctional lysosomal activity found in PD patients. It has been shown there is a significant decrease in the number of lysosomes within dopaminergic neurons, and those that still remain display an abnormal membrane permeabilization (Dehay et al., 2010). A protein involved in lysosomal ATPase, ATP13A2, has been found to be mutated in autosomal recessive forms of Parkinson's disease (Djarmati et al., 2009; Ramiraz et al., 2006) along with GBA, a gene responsible for encoding lysosomal hydrolase (Abeliovich and Gitler, 2016). Depletion of both ATP6AP2 and VPS13C result in lysosomal dysfunction and is associated with autosomal recessive Parkinsonism (Abeliovich and Gitler, 2016; Lesage et al., 2016). Furthermore, a mutation in the VPS35 protein, which causes autosomal

dominant Parkinson's disease, leads to a defect in autophagosome formation (Zavodszky et al. 2014).

Alterations in various aspects of the autophagy process, such as induction, cargo recognition, autophagosome transportation and autophagosome-lysosome fusion, have been associated with several neurodegenerative diseases (Figure 2). Several results have revealed that enhanced autophagy initiation significantly improves the neuropathology and cell death (Ravikumar et al., 2004; Son et al., 2012). Unfortunately, however, it is very difficult to pharmacologically target autophagy (Vakifahmetoglu-Norberg et al., 2015).

1.3 Glutamate and its Receptors

Glutamate is the major excitatory neurotransmitter in the brain and holds many important functions in the brain, including but not limited to, neuronal development, learning, memory, and synaptic plasticity (Meldrum, 2000). The two major subtypes of glutamate receptors include ionotropic and metabotropic (Figure 3). The ionotropic glutamate receptors are characterized as ligand-gated ion channels, which support prompt excitatory transmission (Traynelis et al., 2010). This subtype of receptor includes, N-Methyl-d-Aspartate (NMDA), α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) and kainite receptors (Dingledine et al., 1999). NMDA receptors are generally found on the post-synaptic terminal and are permeable to Ca^{2+} (Sheng and Hoogenraad, 2007). NMDA receptors can be composed from seven subunits, including NR1, NR2A-D and NR3A-B (Kritis et al., 2015). Generally, they exist as tetrameric complexes, containing two essential NR1 subunits paired with two NR2

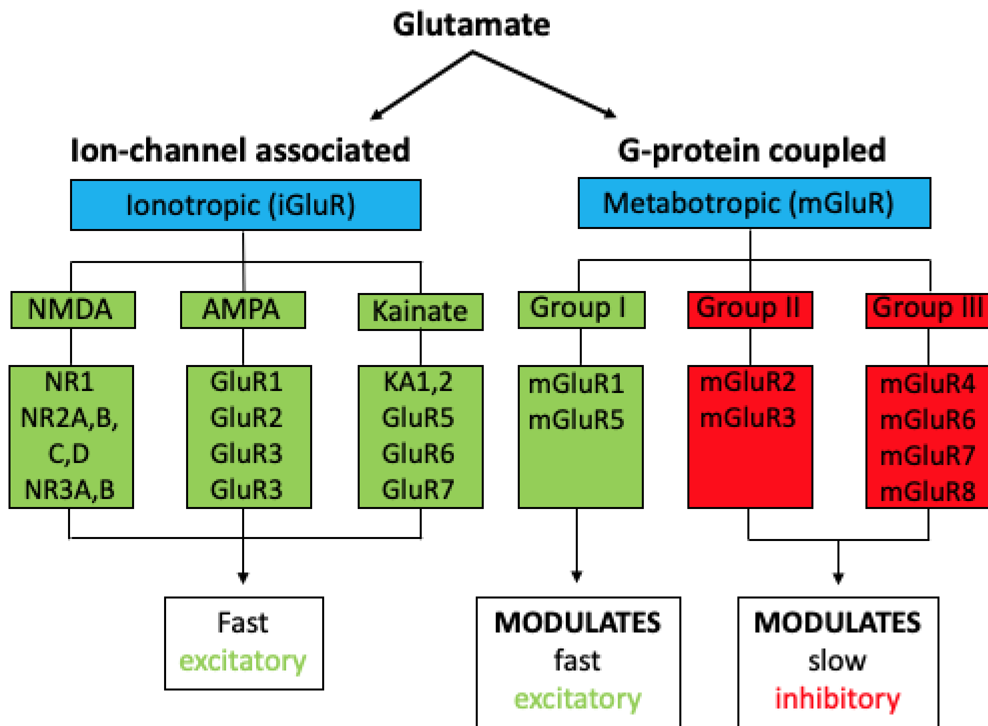


Figure 3. Glutamate and its receptors. Chart representing glutamate and its receptors, including ionotropic and metabotropic receptors.

subunits and their function is dependent on the combination of these subunits (Sheng and Hoogenraad, 2007). The NR2D subunit has been shown to exert the slowest receptor kinetics, meaning that it opens and closes the slowest. This allows more Ca^{2+} into the cell, which is necessary for learning and storing information in our memories. The NR2C subunit has been shown the exert the second slowest receptor kinetics, followed by NR2B, while NR2A exhibits the fastest receptor kinetics (Erreger et al., 2004). NMDA receptors containing NR2B subunits are found abundantly at the synapse in early stages of development, however they migrate to become extra-synaptic NMDA receptors as NR2A containing receptors are progressively added over time. There are many binding sites which regulate NMDA receptor activity, including glutamate, glycine, magnesium, zinc and polyamines (Kritis et al., 2015). Glutamate, which binds to the NR2 subunits, and glycine, which binds to the NR1 subunits, are both essential in activating NMDA receptors (Kohr, 2006). Once activated, the magnesium ions that are blocking the ion channel are expelled outside of the cell and the ion channel is opened, allowing an influx of Ca^{2+} into the cell (Mehta et al., 2013). The non-NMDA receptors include AMPA and kainite receptors. AMPA receptors are generally found on the post-synaptic terminal, whereas the kainite receptors can be found both on the pre-synaptic and post-synaptic terminals (Chen and Gouaux, 2019; Lerma, 2003). AMPA and kainite receptors are both more permeable to Na^{+} and K^{+} rather than Ca^{2+} (Kostandy, 2012). AMPA receptors are composed of four subunits, known as GluR1-4, and are often paired with NMDA receptors (Santos et al, 2009). Activation of AMPAR allows an influx of Na^{+} into the cell, resulting in the depolarization of the cell and the activation of the Ca^{2+} channels, such as NMDA receptors (Kostandy, 2012). Kainite receptors are

composed of five subunits, including KA1-2 and GluR5-7, and mainly play a modulatory role in the synaptic transmission of glutamate (Jane et al., 2009; Lerma, 2003).

The other major glutamate receptor subtype family, known as metabotropic glutamate receptor (mGluR), is categorized as a G protein-coupled receptor and is divided into 8 different subtypes, known as mGluR1 to mGluR8. mGluRs are responsible for modulating slow synaptic transmission through changes in intracellular secondary messenger levels, regulation of ion channels or the stimulation of G protein independent pathways (Ferraguti and Shigemoto, 2006; Gerber et al., 2007; Pin et al., 2003; Ribeiro et al., 2011). Metabotropic glutamate receptors can be divided into 3 groups based on their sequence similarity, pharmacology (agonist selectivity) and signal transduction (intracellular second messengers) (Goudet et al, 2008). Group 1 mGluRs constitute mGluR1 and mGluR5 receptors; group 2 mGluRs include mGluR2 and mGluR3; and lastly, group 3 mGluRs comprise mGluR4, mGluR6, mGluR7, and mGluR8 (Blackshaw et al., 2011). Group 1 mGluRs are coupled to phosphoinositide hydrolysis and can be selectively activated by 3,5-dihyroxyphenylglycine (3,5-DHPG) (Ferraguti and Shigemoto, 2006). The activation of group 1 mGluRs through the $G_{q/11}$ pathway results in the downstream activation of phospholipase C (PLC), which initiates the formation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). This subsequently causes the stimulation of calcium release from neuronal stores and activates protein kinase C-dependent signaling (Abdul-Ghani et al., 1996; Dhami and Ferguson, 2006). Group 2 and group 3 mGluRs are both coupled to the Gi pathway and produce a decrease in intracellular levels of cAMP when activated (Niswender and Conn, 2010).

1.4 Role of mGluR5 in Neurodegenerative Disease

mGluR5 is primarily located on the post-synaptic terminal and is abundant throughout the striatum, hippocampus, cortex, caudate nucleus, and nucleus accumbens, which are areas involved in emotion, motivation and cognition (Simonyi et al., 2005). mGluR5 is known to be coupled to the $G_{q/11}$ pathway, where it activates PLC and stimulates calcium release from neuronal stores (Simonyi et al., 2005). Additionally, mGluR5 signaling has been found to be linked to many neurodegenerative diseases. The over activation of mGluR5 has been found to result in glutamate excitotoxicity, which refers to the injury and death of neurons due to the prolonged exposure to glutamate. Because mGluR5 are coupled to the IP_3/Ca^{2+} signal transduction pathway, this prolonged exposure in turn leads to release of Ca^{2+} from neuronal stores and an excessive influx of Ca^{2+} into the cell. The overload of Ca^{2+} results in an accumulation of toxic aggregates, delayed induction of autophagy, and neuronal cell death (Ferraguti et al., 2008; Conn and Pin, 1997; F. Ribeiro et al., 2010; Zeron et al., 2002; Dong et al., 2009). mGluR5 antagonists (MTEP and MPEP) have shown the ability to elicit neuroprotective effects (Lea et al., 2005). However, it has also been shown that selective mGluR5 agonists also have the ability to evoke neuroprotective effects (Chen et al., 2012; Doria et al., 2013; Loane et al., 2014). Because of these contradicting findings, mGluR5 is a very attractive and important area of medicine to study currently, as these pathways could lead to disease-altering therapeutics for several neurodegenerative diseases.

1.5 Regulation of Autophagy via mGluR5

Recently, it has been discovered using various Alzheimer's and Huntington's disease mouse models that two different mGluR5 signaling pathways, GSK3β/ZBTB16/ATG14 and mTOR/ULK1/ATG13, have the ability to regulate autophagy (Abd-Elrahman et al, 2017; Abd-Elrahman et al., 2018) (Figure 4). Specifically, mGluR5 activation functions by promoting inhibitory phosphorylation of GSK3β, which then increases levels of Zinc finger and BTB domain-containing protein 16 (ZBTB16). ZBTB16 then actively degrades the autophagy protein, ATG14, thus resulting in an accumulation of p62 and indicating the overall inhibition of autophagy (Zhang et al., 2015). As well, the activation of mGluR5 promotes the activation of mammalian target of rapamycin (mTOR) (Page et al., 2006), which phosphorylates Unc-51-like kinase (ULK1) at Ser_{757}. This results in decreased ULK1 kinase activity, and the inability to recruit the autophagy protein, ATG13 (Kim et al., 2011). A decrease in ATG13 protein levels results in an accumulation of p62 and a general inhibition of autophagy (Kim et al., 2011; Abd-Elrahman & Ferguson, 2019). With recent findings demonstrating the importance of mGluR5 and autophagy in neurodegenerative diseases, these mGuR5-regulated autophagy pathways are particularly interesting and attractive to investigate further.

1.6 mGluR5 Downstream Cell Signaling Partners

The most well-known mGluR5 signaling pathway is the $G_{q/11}$ coupled pathway, where the activation of mGluR5 leads to the activation of PLC, due to its coupling to the

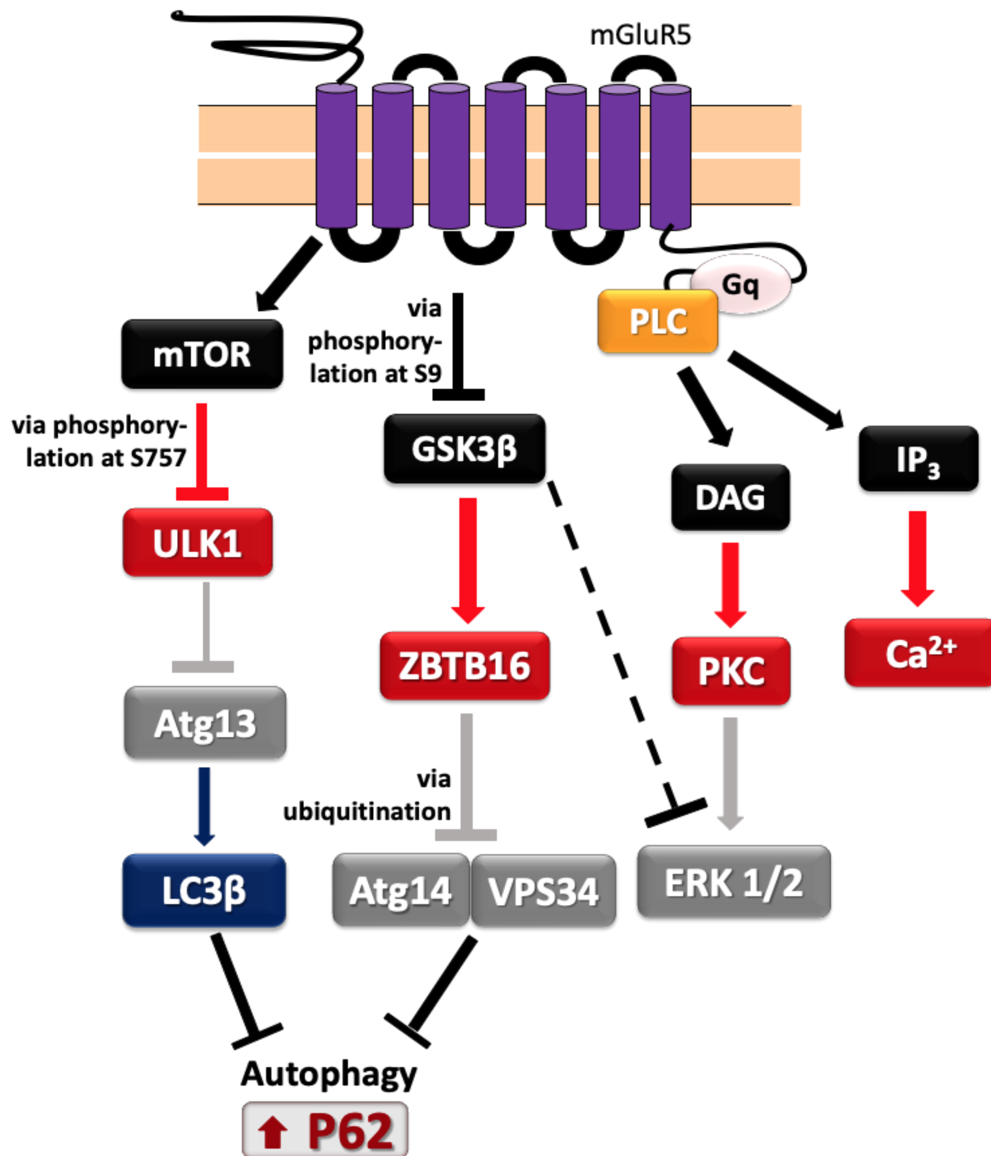


Figure 4. Various mGluR5 signalling cascades. Schematic drawing demonstrating various mGluR5 signalling cascades, including mTOR/ULK1/Atg13 pathway, GSK3β/ZBTB16/Atg14 pathway, DAG/PKC/ERK1/2 pathway, and PLC/IP_3/Ca^{2+} pathway.

$G_{q/11}$ proteins. PLC hydrolysis of IP_3 leads to the release of calcium from neuronal stores. PLC also initiates the formation of DAG, leading to the activation of the PKC and the phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (Menard and Quirion, 2012). ERK1/2 is a well-known cell survival marker, as it is activated in response to various stress stimuli (Strniskova et al., 2002; Kyosseva, 2004; Roux and Blenis, 2004). The activation of the PLC/PKC/ERK1/2 pathway has been shown to greatly impact important biological processes, including cell proliferation and survival (Olmo et al., 2016; Parrales et al., 2013). The activation of ERK1/2 has also been shown to activate p70S6K, which is important in synaptic plasticity and memory (Page et al., 2006; Menard and Quirion, 2012; Mukherjee and Manahan-Vaughan, 2013). Additionally, when ERK1/2 is activated in the CaMK-ERK1/2-p70S6K signaling cascade by group 1 mGluR triggered Ca^{2+} and CaM, this signaling pathway has been shown to lead to the upregulation of several long term depression (LTD)-related proteins and promote synaptic depression in the hippocampus (Sethna et al., 2016; Gallagher et al., 2004). LTD is important to allow for the encoding of new information and synaptic modification, an important aspect that is affected in several neurological disorders. Contrarily, activation of ERK1/2 plays a role in cell death processes upstream of capase-3 in a variety of cell types and animal models of brain injury (Zhuang and Schnellman, 2006). The phosphorylation of ERK1/2 leads to the activation of TNFα and Caspase-3, and the induction of apoptosis. (Zhuang & Schnellman, 2006). Overall, the activation of mGluR5 impacts several downstream cell signaling partners and cell processes, including autophagy, synaptic transmission and protein synthesis.

1.7 Role of OPTN in Neuronal Functions

OPTN is a cytosolic protein containing 577 amino acid residues and 16 exons (Ying and Yue, 2012). It contains many protein domains and binding sites, including a NEMO-like domain, a leucine zipper, an LC3-interacting region, multiple coiled-coil motifs, a ubiquitin-binding domain, and a zinc finger at the C-terminus (Figure 5) (Kachaner et al., 2012). OPTN has several other names, including FIP-2 (14.7K-interacting protein 2) (Li et al., 1998), NRP (NEMO-related protein) (Schwamborn et al., 2000), HIP7 (Huntingtin-interacting protein 7), HYPL (Huntingtin yeast partner L) (Faber et al., 1998), and TFIIIA-INTP (transcription factor IIIA-interacting protein) (Moreland et al., 2000). Studies have shown that OPTN plays a key role in many basic cell functions, including vesicle trafficking, maintenance of the Golgi apparatus, regulation of the NF-κB pathway, induction of autophagy and a role in cell survival. OPTN interacts with multiple proteins, including but not limited to: TBK1, Rab8, Huntingtin, LC3/GABARAP, Myosin VI, mGluR1/5, Transcription factor IIIA (TFIIIA), CYLD lysine 63 deubiquitinase (CYLD), Receptor-interacting serine/threonine-protein kinase 1 (RIP1), and Tax1/2 (Ying and Yue, 2012). The protein has been found to be expressed in many regions of ocular tissue (retina, cornea, iris, etc.), as well as, non-ocular tissue, including the brain, heart, skeletal muscle, kidney, liver and pancreas (Li et al., 1998; Rezaie and Sarfarazi, 2005; Rezaie et al., 2005).

OPTN was first found to be associated with normal tension glaucoma (NTG), a subtype of open-angle glaucoma (POAG), which is one of the leading causes of irreversible bilateral blindness (Rezaie et al., 2002). The mutants of OPTN associated with normal tension glaucoma are generally found to be single copy missense

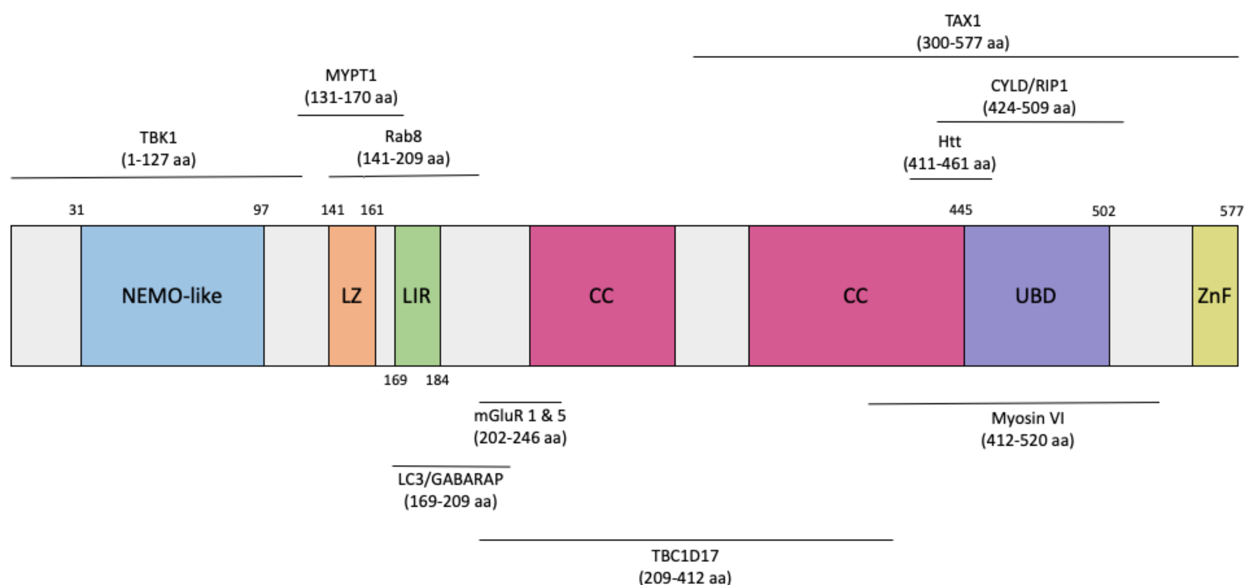


Figure 5. OPTN protein domains and binding sites. NEMO-like domain – NFκB essential modulator domain; LZ – leucine zipper; LIR – LC3-interacting region; CC – coiled-coil motif; UBD – ubiquitin binding domain; ZnF – Zinc finger; aa – amino acid

mutations, such as, E50K, M98K, and R545Q (Bansal et al., 2015; Rezaie et al., 2002). These missense mutations have been identified as being the cause of NTG in 16.7% of families. Mutations involving OPTN have also been linked to amyotrophic lateral sclerosis (ALS), a progressive disorder characterized by the degeneration of motor neurons of the primary cortex, brainstem and spinal cord, leading to the paralysis of voluntary muscle movement (Leigh, 2007). The mutants of OPTN associated with ALS range from missense mutations to nonsense mutations to deletions (Bansal et al., 2015). It has been shown that some patients with ALS display a homozygous Q398X nonsense mutation, resulting in premature stop codon and the deletion of the coiled coil 2 domain and the ubiquitin binding domain (Maruyama et al., 2010) while other ALS patients revealed a heterozygous E478G missense mutation, leading to the reduced binding of ubiquitin (Maruyama et al., 2010). As well, some patients with ALS displayed deletions of full exons, including a homozygous deletion of exon 5, a heterozygous deletion of exon 3,4 and 5, and a heterozygous deletion of exons 1,2,3 and 4, all resulting in null alleles (Maruyama et al., 2010; Iida et al., 2012). OPTN also interacts with HTT, linking HTT to Group I mGluR signaling. When HTT and mHTT are co-expressed with OPTN, only mHTT expression results in the increase of OPTN binding to mGluR1a, leading to an increased uncoupling of mGluR1a to PLC and an impairment of mGluR signaling (Anborgh et al., 2005). This suggests that mHTT may play a role in Huntington's disease by further antagonizing mGluR1/5 signaling through the activation of OPTN. As well, it has been shown that OPTN may contribute to the formation of toxic aggregates in Alzheimer's and Parkinson's disease. Immunohistochemical examination of the amyloid plaques and neurofibrillary tangles from several Alzheimer's disease

patients demonstrated that both the plaques and tangles were OPTN-positive. Similarly, OPTN immunoreactivity of Lewy bodies from numerous Parkinson's disease patients exhibited a strong positive reaction to anti-OPTN antibody (Osawa et al., 2011). Overall, in many studies, a mutation or alteration (overexpression or knockdown) of OPTN has been associated with the development of neurodegenerative diseases (Ying and Yue, 2012).

OPTN is also known as an autophagy receptor, specifically it has the ability to transport ubiquitinated debris to the autophagosome in order to be degraded through autophagic clearance (Figure 6). Ubiquitinated misfolded proteins and bacteria bind to OPTN via its ubiquitin binding domain. OPTN then transports the cargo to the phagophore, where it binds to LC3-II via its LC3-interacting region. The phagophore matures into an autophagosome, which then binds to the lysosome and carries out the degradation process (Randow and Youle, 2014). It has been found that it is necessary for TANK-binding kinase 1 (TBK1) to bind and phosphorylate OPTN on Ser_{177}, in order to enhance the binding affinity to both the target cargo and LC3, and ultimately enhance autophagic clearance (Korac et al., 2013; Heo et al., 2015). Additionally, when TBK1 is silenced, there is an impairment of autophagic clearance, suggesting further its importance in the phosphorylation of OPTN and autophagy process (Wild et al., 2011). Furthermore, the phosphorylation of OPTN at Ser_{177} is necessary for OPTN to facilitate the recruitment of the Atg12-5-16L1 complex to the phagophore, leading to the maturation of the phagophore into the autophagosome, as well as, allows OPTN to potentiate LC3-II production (Bansal et al., 2018). Mutations and alterations involving OPTN have been linked to impaired autophagic clearance (Ying and Yue, 2016), which

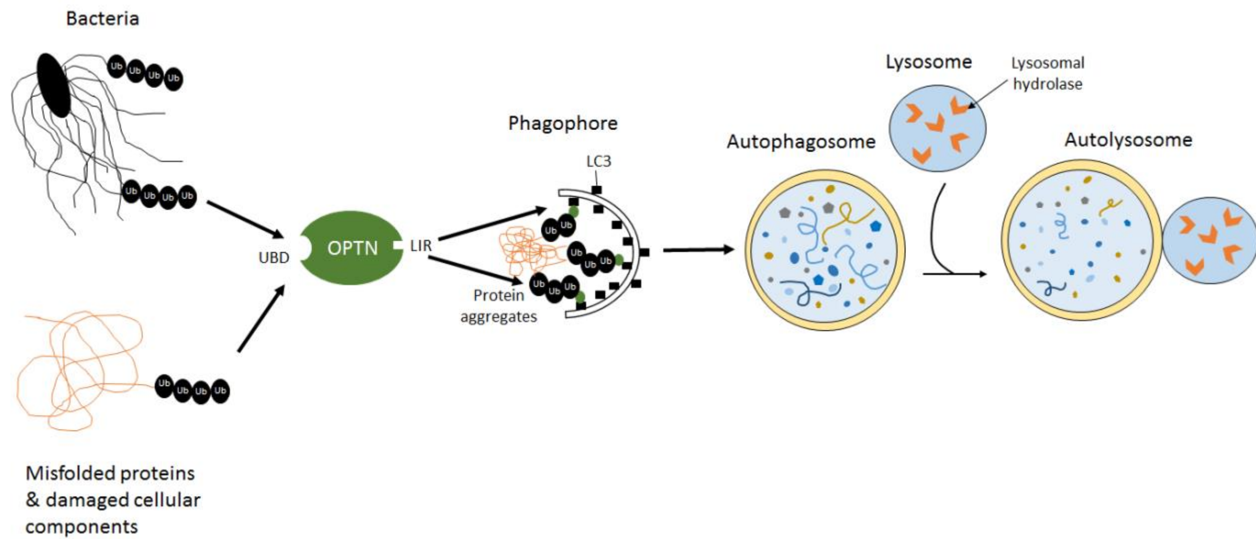


Figure 6. OPTN as an autophagy receptor. Schematic drawing representing the mechanism of how OPTN functions as an autophagy receptor.

can then result in the development of several neurodegenerative diseases (Ying and Yue, 2012).

1.8 OPTN Interaction with Group 1 mGluRs

Recently, OPTN has been identified as a potential contender involved in the altering of Group I mGluR-dependent signaling (Anborgh et al., 2005). We have previously shown that OPTN interacts with Group 1 mGluR protein to inhibit the coupling of mGluR to phospholipase C (PLC) and block the IP_3 signaling (Anborgh et al., 2005). mGluR5 has the ability to regulate autophagy through the GSK3β/ZBTB16/ATG14 and mTOR/ULK1/ATG13 pathways (Abd-Elrahman et al, 2017; Abd-Elrahman et al., 2018). Because OPTN is an autophagy receptor and has recently been shown to bind to group 1 mGluR complex to inhibit IP_3 signalling (Anborgh et al., 2005), it is important to further study the role of OPTN in group 1 mGluR signalling and determine how OPTN may alter mGluR-regulation of autophagy through the mTOR/ULK1/ATG13 and GSK3β/ZBTB16/ATG14 autophagy pathways.

1.9 Hypotheses and Objectives

1.9.1 Rationale and Hypotheses

mGluR5 is a major area of study currently in the development of therapies for several neurodegenerative diseases, including Huntington's and Alzheimer's disease. It is shown in various studies that over activation of group 1 mGluRs has the ability to elicit neurotoxic effects in Huntington's disease (reviewed in Sepers and Raymond, 2014), as well as, evoke neuroprotective mechanisms, such as autophagic signaling

pathways (Baskys et al., 2005; Bruno et al., 2001; Ribeiro et al., 2010). In addition, genetic deletion or chronic pharmacological inhibition of mGlur5 by the negative allosteric modulator, CTEP, rescues cognitive function and reduces Aβ oligomer aggregation in multiple mouse models of Alzheimer's disease (Abd-Elrahman et al., 2018). OPTN's ability to interact with mGluR5 makes it of particular interest in determining its role in autophagy signaling.

This thesis aims to determine the role of OPTN in mGluR5-dependent autophagy signaling. We hypothesize that silencing OPTN via two independent guides in ST*Hdh*$_{Q7/Q7}$ cells and in C57BL/6 mice will alter mGluR5-dependent autophagy signaling.

1.9.2 Objectives

1.9.2.1 Investigate the impact of silencing OPTN by CRISPR/Cas9 using two different guides on mGluR5-dependent regulation of autophagy.

1.9.2.2 Investigate the impact of knocking out OPTN in C57BL/6 mice on mGluR5-dependent regulation of autophagy.

2. Materials and Methods

2.1 Reagents

(S)-3,5-DHPG (Tocris) (0805/10) was purchased from Cedarlane Labs (Burlington, Ontario). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody was purchased from Bio-Rad (1662408EDU). Anti-vinculin antibody (129002) was purchased from Abcam (Cambridge, Massachusetts). Anti-SQSTM1/p62 antibody (56416) was purchased from Abcam (Cambridge, Massachusetts). Anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody (9101S) was purchased from Cell Signaling Technology (Danvers, Massachusetts). Anti-ERK1 + ERK 2 antibody - Loading Control (94484) was purchased from Abcam (Cambridge, Massachusetts). Phospho-GSK-3β (Ser9) (5B3) Rabbit (9323S) was purchased from New England Biolabs (Whitby, Ontario). GSK-3β (3D10) Mouse (9832S) was purchased from New England Biolabs (Whitby, Ontario). Anti-Plzf antibody (39354) was purchased from Abcam (Cambridge, Massachusetts). Recombinant Anti-VPS34 antibody (124905) was purchased from Abcam (Cambridge, Massachusetts). Phospho-ULK1 (Ser757) (D7O6U) Rabbit (14202S) was purchased from New England Biolabs (Whitby, Ontario). Reagents used for western blotting were purchased from Bio-Rad Laboratories (Hercules, California) and all other biochemical reagents were purchased from Sigma-Aldrich (St. Louis, Missouri).

2.2 Cell Lines

The ST*Hdh*$_{Q7/Q7}$ cell line was received from Dr. Ray Truant of the University of McMaster, who had purchased the cell line from Coriell Institute for Medical Research

(CH00097). The ST*Hdh*$_{Q7/Q7}$ cells are a striatal derived cell line from a knock in transgenic mouse, containing homozygous Huntingtin (HTT) loci with a humanized Exon 1 comprising 7 polyglutamine repeats. The ST*Hdh*$_{Q7/Q7}$ cells also expresses mGluR5, signifying it does not need to be transfected and is a more applicable cell line. OPTN was knocked out in two different guides using CRISPR/Cas9, which was performed by Vanessa Kissner and Stella MacDonald. Wild-type ST*Hdh*$_{Q7/Q7}$ and OPTN KO ST*Hdh*$_{Q7/Q7}$ were starved in HBSS for 1 hour and then treated with 10μM of (S)-3,5-DHPG (mGluR1/5 agonist) for either 5, 15, or 30 minutes to examine the effects of mGluR5 activation on autophagy signalling pathways. To stop the reaction, the cells were washed twice with ice-cold HBSS and placed on ice.

2.3 Animals

Animal care was in accordance with the University of Ottawa Institutional Animal Care Committee and the Canadian Council of Animal Care guidelines. Animals were housed in an animal care facility on a 12-hour light/12-hour dark cycle with food and water provided ad libitum. C57BL/6 heterozygous OPTN knockout mice were purchased from Dr. Henry Tseng and CMV-Cre mice were acquired from Dr. Mona Nemer. C57BL/6 heterozygous OPTN knockout mice and CMV-Cre mice were bred using cre/lox recombination technology to globally knock out OPTN. After wild-type and OPTN KO male animals were aged to 6-7 months of age, they were sacrificed by exsanguination, and brains were collected and dissected. The striatum and hippocampus regions were coronally sliced into 300µm slices and recovered in ACSF (127mM NaCl, 2mM KCl, 10mM glucose, 1.2 mM KH_2KO_4, 26 mM NaH_2CO_3, 1mM

$MgSO_4$, 1 mM $CaCl_2$, pH 7.4). Samples were gassed with 95% O_2/5% CO_2 and habituated for 90 minutes at 37℃ in 2ml tubes. The samples were transferred to 1.5ml tubes, gassed again and habituated for another 30 minutes at 37℃. Some of the tissue was then treated with CTEP (mGluR5 selective negative allosteric modulator) for 30 minutes and then all samples were treated with (S)-3,5-DHPG for 15 minutes. Treatment was halted by aspirating CTEP/DHPG and placing tube in liquid nitrogen.

2.4 Immunoblotting

ST*Hdh*$_{Q7/Q7}$ cells were lysed in 150µl of ice-cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10µg/ml leupeptin, and 2.5µg/ml aprotinin) and phosphatase inhibitors (10mM NaF and 500µM Na3VO4). Hippocampal tissue was lysed in 175µl of ice-cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10µg/ml leupeptin, and 2.5µg/ml aprotinin) and phosphatase inhibitors (10mM NaF and 500µM Na3VO4). Cell samples were centrifuged at 14,800 rpm at 4℃ for 10 minutes and brain samples were centrifuged twice at 14,800 rpm at 4℃ for 10 minutes each time. The supernatant in all samples was collected and total protein levels were quantified using Bradford Protein Assay (Bio-Rad). Lysates were diluted to 1µg/µl in a mix of lysis buffer and β-mercaptoethanol containing 3x loading buffer and boiled for 10 minutes at 90°C. Aliquots containing a total of 35µg of proteins were resolved by electrophoresis on either a 7.5% or 14% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Blots were blocked in Tris-buffered saline (pH 7.6) containing 0.05% of Tween 20 (TBST) and 5% non-fat dry milk for 1 hour at

room temperature. Following this, blots were incubated overnight at 4°C with primary antibodies diluted (1:1000) in TBST containing 1% non-fat dry milk. Membranes were washed 3 times in TBST and incubated with secondary antibodies (anti-rabbit/mouse) diluted (1:5000) in TBST containing 1% non-fat dry milk for 1 hour. Membranes were washed 3 more times in TBST, and bands were detected and quantified using a Bio-Rad chemiluminescence system.

2.5 Statistical Analysis

Means ± SEM for each independent experiment are shown in the various figure legends. Volumes were quantified using ImageLab and GraphPad Prism software was used to analyze the data for statistical significance. The statistical test used to analyze the data was a two-way ANOVA test and a P value less than 0.05 was considered statistically significant.

3. Results:

3.1 Successful generation of OPTN knockout cell lines

CRISPR Cas9 guides were designed using the Optimized CRISPR Design Tool from the Zhang Lab (http://crispr.mit.edu/) to target the first and second exon of the OTPN gene in *Mus musculus.* The first and second exons were chosen because the first exon is present in isoform 1 of OPTN and the second exon is common to isoform 1 and 2 of the gene. The nucleotide region between 9408-9573bp was used to search for guides in the first exon and the region between 18016-18126bp was used to search for guides in the second exon. The two guides that were selected were chosen based on having minimal overlap, no off-target sites and high-quality score (sequences listed in Figure 7). To anneal the top and bottom strands, top and bottom oligos (10μM) per guide were ligated with nuclease free water and T4 DNA ligase buffer (1X) by heating together on a dry heat block at 95ºC for 3 minutes. The heat block was then removed, allowing the mixture to cool at room temperature until it reached 50ºC. Each annealing product (1:20 dilution) was then added separately to a mixture of pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid (20 ng/μL), NEBuffer 2.1 (1X), Rapid T4 DNA ligase (1:40 dilution), Adenosine triphosphate (0.5mM), BbsI restriction enzyme (1:20 dilution), and nuclease free water. Each reaction was placed in a thermocycler with the following parameters: 12 cycles (37ºC for 5 minutes, 21ºC 5 minutes) and finishing with a 21ºC hold. D5H-α competent E. coli cells were transformed with the ligation product of each guide and plated on a pre-warmed ampicillin-containing agar-plate overnight at 37ºC, as the pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid is resistant to ampicillin. In order to screen colonies for the successful uptake of the plasmid, polymerase chain reaction

A

Sequence searched for guides in first exon (isoform 1, not 2 or 3): 9408-9573

atgtcccatcaacctctgagctgcctgactgagaaggggacagcccttgtgagaccccaggaaatggaccctccaatatggttcaccccagcctggacacattcaccctgaggagctgctgcagcaaatgaaggaactcctggt

Guide 1:
Original Sequence:
Top: 5’ GCTGGGGTGAACCATATTGG 3’
Bottom: 3’ CGACCCCACTTGGTATAACC 5’

Add overhangs:
5’ CACC GCTGGGGTGAACCATATTGG 3’
3’ CGACCCCACTTGGTATAACC CAAA 5’

Correct orientation for primer ordering:
Guide 1 Top: 5’ CACC GCTGGGGTGAACCATATTGG 3’
Guide 1 Bottom: 5’ AAAC CGACCCCACTTGGTATAACC 3’

B

Sequence searched for guides in second exon (common to isoform 1 and 2, not 3): 18016-18126

Atgcgccttcgggctgaaaaggcagacctgctgggcatcgtctcagaactgcagctcaaactcaactccggcggctcctcggaagactccttcgttgagatcaggatgacc

Guide 2:

Original Sequence:
Top: 5’ GGTCATCCTGATCTCAACGA 3’
Bottom: 3’ CCAGTAGGACTAGAGTTGCT 5’

Add overhangs:
5’ CACC GGTCATCCTGATCTCAACGA 3’
3’ CCAGTAGGACTAGAGTTGCT CAAA 5’

Correct orientation for primer ordering:
Guide 2 Top: 5’ CACC GGTCATCCTGATCTCAACGA 3’
Guide 2 Bottom: 5’ AAAC TCGTTGAGATCAGGATGACC 3’

Figure 7. Design of guide RNAs for CRISPR/Cas9 mediated silencing of OPTN gene.

Region in exon one (A) and exon two (B) used to design target gRNAs. Overhangs are shown in red text.

(PCR) was performed. Three colonies were selected per guide and resuspended separately in 10µL of nuclease free water. 1µL of this diluted colony was added to a mixture of: 2X phire green hot start II PCR master mix, forward primer 68 (TAAAATGGACTATCATATGC) (0.5µL/20µL), and the bottom strand of each guide (0.5µL/20µL). The thermocycler parameters for PCR colony screening were: 1 cycle of (98°C for 5 minutes), 27 cycles of (98°C for 10 seconds, 53°C for 15 seconds, and 72°C for 30 seconds), 1 cycle of (72°C for 5 minutes), and finishing with a 4°C hold. Agarose gel electrophoresis was then used to screen the PCR products compared to the negative control with no DNA per guide. Colonies that screened positive for the plasmid and guides were grown up in Lysogeny broth (LB media). DNA from the colonies were collected and purified and sent to the University of Ottawa StemCore Laboratories DNA Sequencing Facility. ST*Hdh*$_{Q7/Q7}$ cells were split into 10cm dishes at 40-50% confluency to reach a confluency of 50-70% 18 hours after. Transfection of the guides occurred 18 hours later, where the plasmids containing each set of guides and GFP were co-transfected into the ST*Hdh*$_{Q7/Q7}$ cells using PEI (4:1). Cells were incubated for 24 hours and visually examined for GFP. Following confirmation, cells were rinsed with 1X phosphate buffer saline (PBS), trypsinized, and quenched using DMEM + 10% FBS. Cells were then centrifuged for 2 minutes at 1.2K g, the media was aspirated, and the cells were resuspended in PBS containing 0.2% BSA. 96 well plates with DMEM + 20% FBS in each well were prepared for each guide. Using the Flow Cytometry Core Facility at the University of Ottawa, fluorescence activated cell sorting was performed in order to seed a single GFP expressing cell into each well. The 96 well plates were placed in an incubator at 37°C to grow for approximately 3 weeks. Once a colony reached 90-100%

confluency, it was trypsinized and maintained on two 24 well plates. One of the 24 well plates was marked for experiments and the other was marked for screening. This process was repeated to transfer the colonies to 12 well plates, and eventually 6 well plates. Colonies marked for screening were then lysed with 1% RIPA containing protease inhibitors (1 mM AEBSF, 10μg/ml leupeptin, and 2.5μg/ml aprotinin) and phosphatase inhibitors (10mM NaF and 500μM Na3VO4), and western blotting was performed to screen for knockouts. Successful knockouts were then sent for DNA sequencing at the Ottawa Hospital Research Institute DNA Sequencing Facility to further confirm that a successful knockout was generated. The western blot screening and sequencing of the two colonies generated from two different guides that were used for further experiments are listed in (Figure 8).

3.2 mGluR5 activation in ST*Hdh*$_{Q7/Q7}$ cells increases phosphorylation of ERK1/2 in WT cells but has no effect on OPTN knockouts

We subsequently tested what the effect of knocking out OPTN expression using CRISPR/Cas9 in a ST*Hdh*$_{Q7/Q7}$ cell line using two different guide RNAs would have upon the phosphorylation of status of cell signaling molecules that were activated downstream of endogenously expressed mGluR5. Initial studies examined the effect of treating ST*Hdh*$_{Q7/Q7}$ wild-type and ST*Hdh*$_{Q7/Q7}$ OPTN knockout cells with 10μM DHPG for 5 minutes on ERK1/2 phosphorylation. We found that ERK1/2 phosphorylation was significantly increased by 278% +/- 76.7% in response to DHPG treatment of wild-type ST*Hdh*$_{Q7/Q7}$ cells, whereas ERK1/2 phosphorylation was not induced in response to DHPG treatment in either G1 or G2 ST*Hdh*$_{Q7/Q7}$ OPTN knockout cell lines (Figure 10).

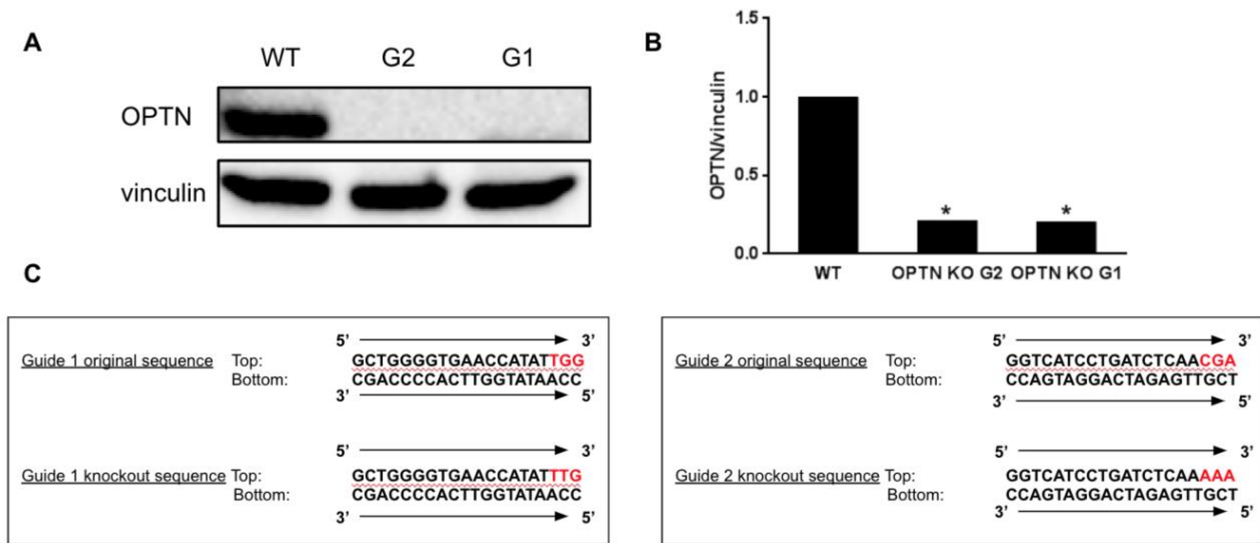


Figure 8. Successful knockout of OPTN in *STHdh$_{Q7/Q7}$* *cells using two gRNAS.* *Representative* western blot (A) and densiometric quantification (B) of OPTN and corresponding vinculin for guide 1 and guide 2 knockout colonies. (C) Original sequencing results and corresponding knockout sequencing results for guide 1 and guide 2. Red text shows mutated region of original and knockout sequence.

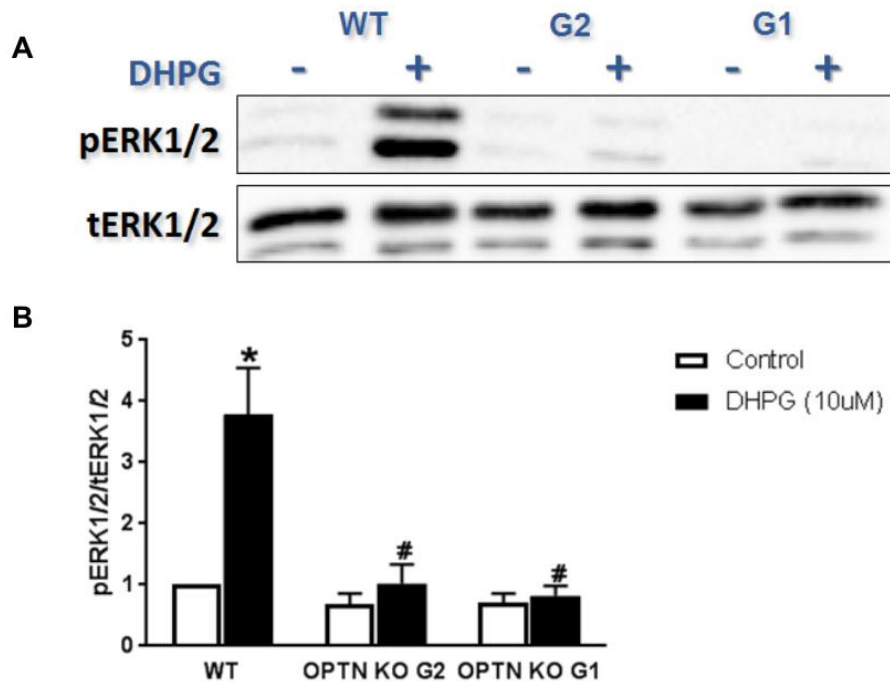


Figure 9. mGluR5 activation increases phosphorylation of ERK1/2 in WT ST*Hdh*$_{Q7/Q7}$cells but has no effect on OPTN knockouts. Representative blots (A) and densiometric quantification (B) of pERK1/2 (n=6) expression in OPTN knockout cells for guide one (G1) and guide two (G2) compared to wild-type (WT) cells treated with DHPG (10 μM) or HBSS (NT). Data represents mean $\pm$ SEM of 6 independent experiments. Significantly different (p<0.05) results when compared to non-treated wild-type cells and to treated wild-type cells are depicted by * and #, respectively.

3.3 Loss of OPTN in ST*Hdh*$_{Q7/Q7}$ cells activates autophagy by GSK3β/ZBTB16/ATG14 pathway

We previously demonstrated that activation of mGluR5 inhibited autophagy by stimulating inhibitory (Ser$_{9)}$ phosphorylation of GSK3β, thereby inactivating the GSK3β/ZTBTB-Cullin3-Roc1 E3 ubiquitin ligase/ATG14 autophagy pathway that resulted in increased p62 protein expression (Abd-Elrahman et al., 2017). However, the precise mechanism by which GSK3β phosphorylation was modulated was yet to be determined. Therefore, we tested whether OPTN knockout in ST*Hdh*$_{Q7/Q7}$ cells altered the extent of GSK3β phosphorylation, as well as protein expression of ZBTB16, and VPS34, an important protein involved in the class III phosphatidylinositol 3-kinase complex with ATG14 and Beclin-1 to promote autophagosome formation. We found that phosphorylation of GSK3β at Ser$_9$ was significantly decreased in the ST*Hdh*$_{Q7/Q7}$ OPTN knockout cells and mGluR5 agonism did not alter this decrease (Figure 10). Furthermore, we found that ZBTB16 protein expression levels were significantly reduced in the ST*Hdh*$_{Q7/Q7}$ OPTN knockout guides, and activation of mGluR5 did not correct this reduction (Figure 11). We also discovered VPS34 protein levels were significantly increased in the ST*Hdh*$_{Q7/Q7}$ OPTN knockout cells, and DHPG treatment did not alter this increase in protein expression (Figure 12). Together, these data suggested OPTN plays a role in the inhibition of autophagy via the GSK3β/ZBTB16/Atg14 pathway.

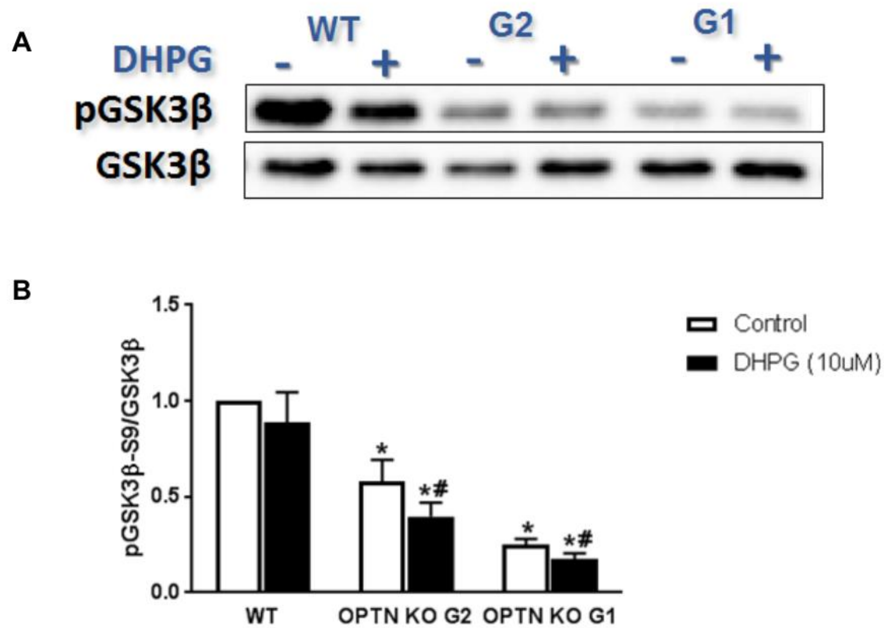


Figure 10. OPTN knockout ST*Hdh*Q7/Q7 cells exhibit a decrease in GSK3β-S9 phosphorylation. Representative blots (A) and densiometric quantification (B) of pGSK3β (n=6) expression in OPTN knockout cells for G1 and G2 compared to WT cells treated with DHPG (10 μM) or HBSS (NT). Data represents mean $\pm$ SEM of 6 independent experiments. Significantly different (p<0.05) results when compared to non-treated wild-type cells and to treated wild-type cells are depicted by * and #, respectively.

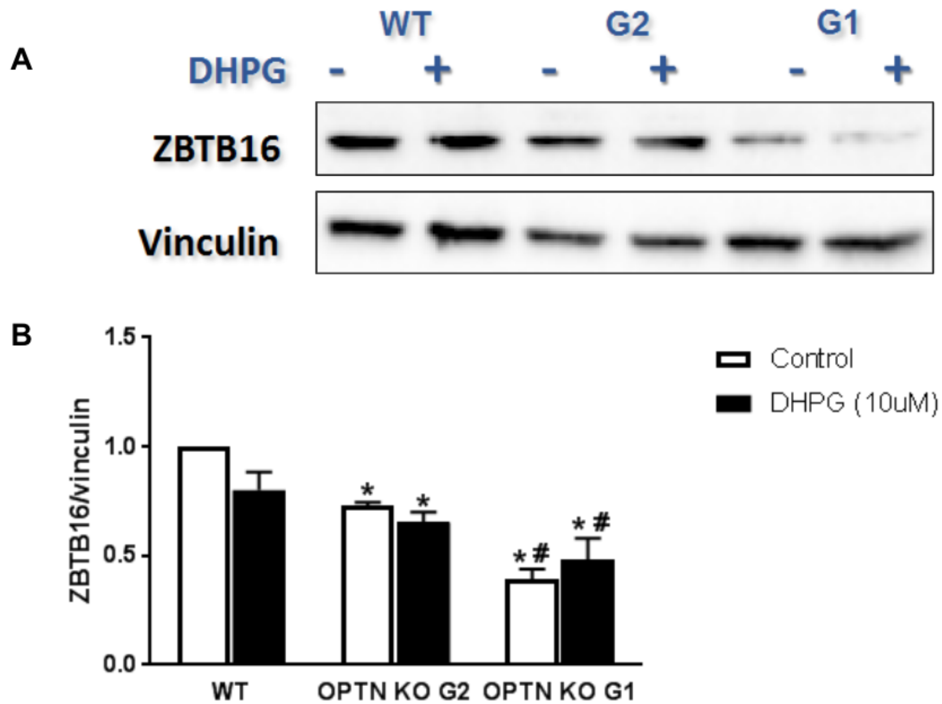


Figure 11. OPTN knockout ST*Hdh*$_{Q7/Q7}$ cells exhibit a decrease in ZBTB16 protein level expression. Representative blots (A) and densiometric quantification (B) of ZBTB16 (n=6) expression in OPTN knockout cells for G1 and G2 compared to WT cells treated with DHPG (10 μM) or HBSS (NT). Data represents mean $\pm$ SEM of 6 independent experiments. Significantly different (p<0.05) results when compared to non-treated wild-type cells and to treated wild-type cells are depicted by * and #, respectively.

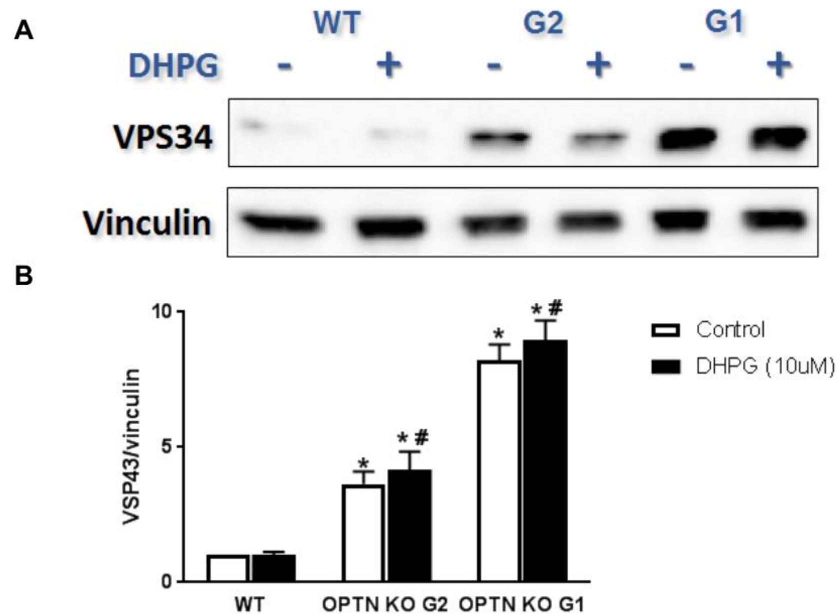


Figure 12. OPTN knockout ST*Hdh*$_{Q7/Q7}$ cells exhibit an increase in VPS34 protein level expression. Representative blots (A) and densiometric quantification (B) of VPS34 (n=6) expression in OPTN knockout cells for G1 and G2 compared to WT cells treated with DHPG (10 μM) or HBSS (NT). Data represents mean $\pm$ SEM of 6 independent experiments. Significantly different (p<0.05) results when compared to non-treated wild-type cells and to treated wild-type cells are depicted by * and #, respectively.

3.4 Knocking out OPTN in ST*Hdh*$_{Q7/Q7}$ cells inhibits autophagy by mTOR/ULK1 pathway

We previously demonstrated that chronic antagonism of mGluR5 corrected the abnormal activation of mTOR in a Huntington's disease mouse model, and thereby prevented the inhibitory phosphorylation of ULK1 at Ser$_{757}$, leading to the activation of autophagy (Abd-Elrahman et al., 2018; Abd-Elrahman & Ferguson, 2019). However, when mGluR5 was activated, this pathway lead to inhibition of autophagy and lack of autophagosome degradation, which resulted in an accumulation of LC3β and p62 protein levels (Abd-Elrahman & Ferguson, 2019; Kim et al., 2011). The precise mechanism by which this pathway was regulated remained to be investigated. Therefore, we tested whether knocking out OPTN in ST*Hdh*$_{Q7/Q7}$ cells altered ULK1 phosphorylation, along with LC3β and p62 protein levels. We found that the inhibitory phosphorylation of ULK1 at Ser$_{757}$ was significantly increased in the ST*Hdh*$_{Q7/Q7}$ OPTN knockout cells and mGluR5 activation did not alter this increased phosphorylation (Figure 13). Additionally, we found both LC3β and p62 protein expression levels were significantly increased in the ST*Hdh*$_{Q7/Q7}$ OPTN knockout cells and mGluR5 agonism did not affect either of these increases, respectively (Figure 14 & Figure 15). Together, these data suggested OPTN plays a role in the initiation of autophagy via the mTOR/ULK1 pathway.

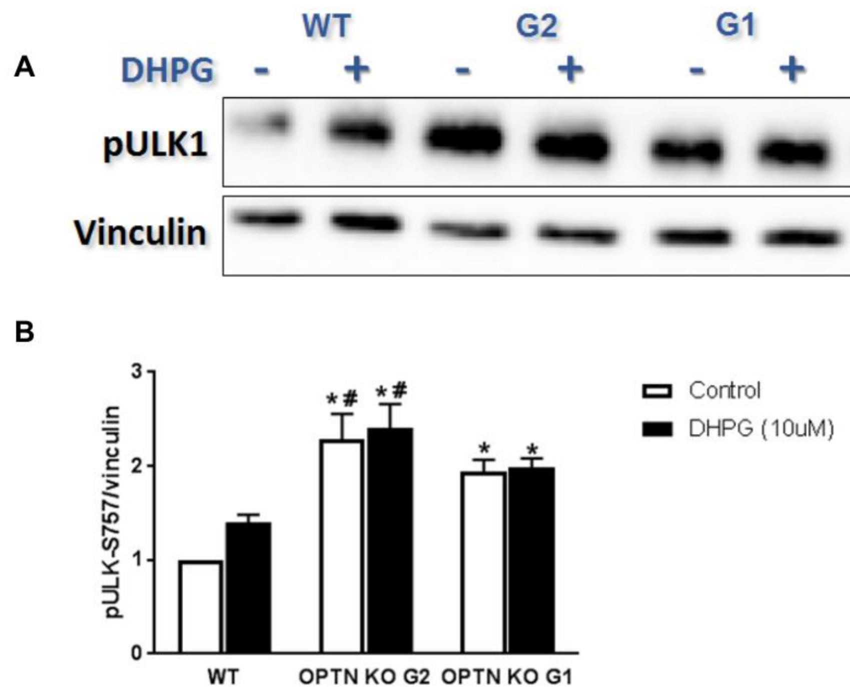


Figure 13. OPTN knockout ST*Hdh*$_{Q7/Q7}$ cells display an increase in ULK1-S757 phosphorylation. Representative blots (A) and densiometric quantification (B) of pULK1 (n=5) expression in OPTN knockout cells for G1 and G2 compared to WT cells treated with DHPG (10 μM) or HBSS (NT). Data represents mean $\pm$ SEM of 5 independent experiments. Significantly different (p<0.05) results when compared to non-treated wild-type cells and to treated wild-type cell are depicted by * and #, respectively.

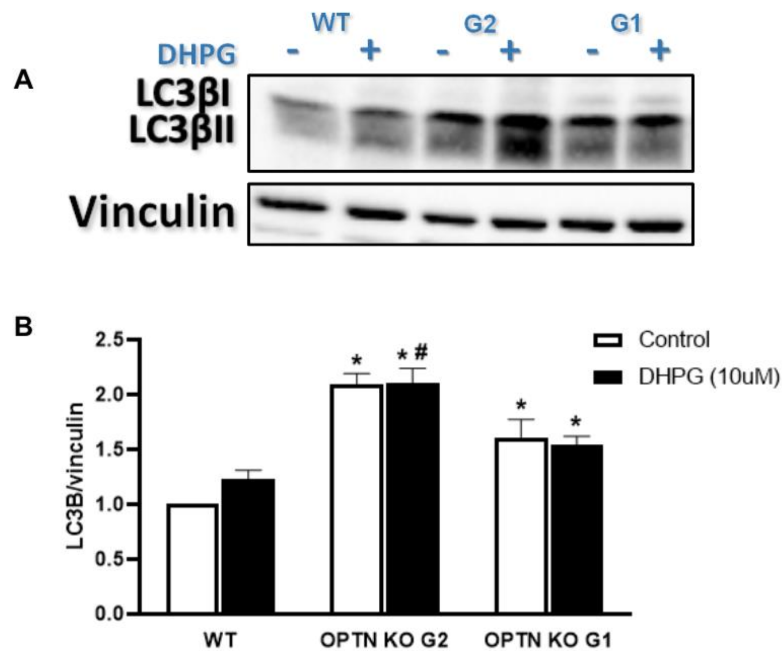


Figure 14. OPTN knockout ST*Hdh*$_{Q7/Q7}$ cells display an increase in LC3β expression. Representative blots (A) and densiometric quantification (B) of LCβI/II (n=5) expression in OPTN knockout cells for G1 and G2 compared to WT cells treated with DHPG (10 μM) or HBSS (NT). Data represents mean $\pm$ SEM of 5 independent experiments. Significantly different (p<0.05) results when compared to non-treated wild-type cells and to treated wild-type cell are depicted by * and #, respectively.

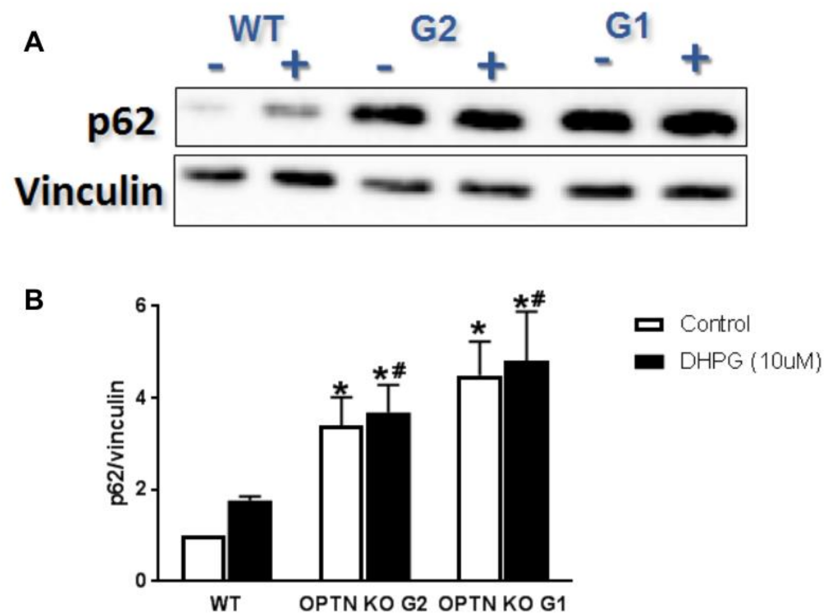


Figure 15. OPTN knockout ST*Hdh*$_{Q7/Q7}$ cells display an accumulation of p62. Representative blots (A) and densiometric quantification (B) of p62 (n=5) expression in OPTN knockout cells for G1 and G2 compared to WT cells treated with DHPG (10 μM) or HBSS (NT). Data represents mean $\pm$ SEM of 5 independent experiments. Significantly different (p<0.05) results when compared to non-treated wild-type cells and to treated wild-type cell are depicted by * and #, respectively.

3.5 Transfection of OPTN into OPTN KO cells rescues phosphorylation of ERK1/2

Lastly, we had begun to test whether the re-expression of OPTN back into each CRISPR/Cas9 ST*Hdh*$_{Q7/Q7}$ OPTN knockout cell line re-established wild-type ERK1/2 phosphorylation levels in the absence and presence of DHPG treatment. We then planned to measure the same autophagy and cell survivor markers using western blot analysis to observe if alterations in the autophagy pathways can be rescued. Due to the recent COVID-19 pandemic and the closure of the laboratory, we were not able to finish the experiment and were only able to obtain n value of 2 for pERK1/2/tERK1/2 blots. However, from the 2 blots, it is evident that when both ST*Hdh*$_{Q7/Q7}$ OPTN knockout guides were transfected with OPTN-YFP, there was a noticeable rescue of the phosphorylation of ERK1/2 following activation of mGluR5 (Figure 16). Although the OPTN-YFP transfected wild-type cells and knockout guides already display a high level of phosphorylation of ERK1/2, as can be seen in the non-treated cells, it was promising to see the rescue effects in the OPTN-YFP transfected cells. These results helped validate the previous results that showed that knocking out OPTN in the ST*Hdh*$_{Q7/Q7}$ cells abolished the canonical phosphorylation of ERK1/2.

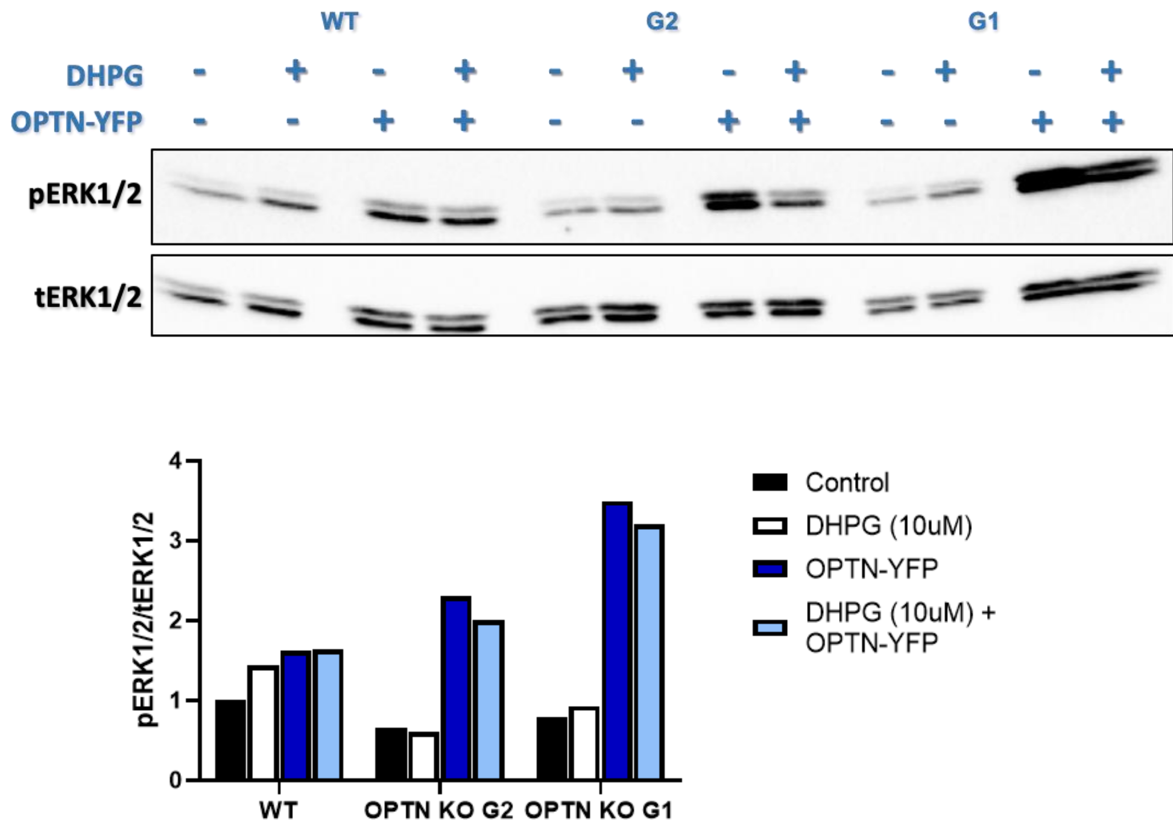


Figure 16. Transfection of OPTN-YFP into OPTN KO cells rescues phosphorylation of ERK1/2. Western blot (A) and densiometric quantification (B) of pERK1/2 (n=2) expression in OPTN knockout cells for guide one (G1) and guide two (G2) compared to wild-type (WT) cells treated with DHPG (10 μM) or HBSS (NT) that are either non-transfected or transfected with OPTN-YFP. Data represents mean in 2 independent experiments.

3.6 Activation of mGluR5 in the hippocampus increases phosphorylation of ERK1/2 in WT but has no effect on OPTN knockouts

The observed alterations in the phosphorylation and protein expression status of proteins in the mTOR/ULK1 and GSK3β/ZBTB16/ATG14 autophagy pathways in the ST*Hdh*$_{Q7/Q7}$ OPTN knockout cells prompted us to further investigate these alterations in cell signaling in OPTN knockout mice. OPTN knockout was confirmed by immunoblotting for OPTN expression in wild-type and OPTN knockout mice (Figure 17). Subsequently, we examined the effect of treating wild-type mouse hippocampus and OPTN knockout mouse hippocampal slices with 50μM DHPG or 50μM DHPG + 10μM CTEP for 15 minutes on ERK1/2 phosphorylation. We found that ERK1/2 phosphorylation in wild-type mouse hippocampus was significantly increased by 73% +/- 29.1% in response to DHPG treatment, which was antagonized by CTEP pre-treatment (Figure 18). However, ERK1/2, similar to what was observed for CRISPR/Cas9 ST*Hdh*$_{Q7/Q7}$ OPTN knockout cells (Figure 9), ERK1/2 phosphorylation was abolished in the OPTN knockout mouse hippocampal slices (Figure 18).

3.7 mGluR5 agonism in the hippocampus inhibits autophagy by the GSK3β/ZBTB16/ATG14 pathway in WT but has no effect in OPTN knockouts

We previously demonstrated that mGluR5 activation by β-amyloid inhibited autophagy by promoting inhibitory phosphorylation of GSK3β, increasing of ZBTB16 expression and actively degrading ATG14 (Abd-Elrahman et al., 2017). The ST*Hdh*$_{Q7/Q7}$

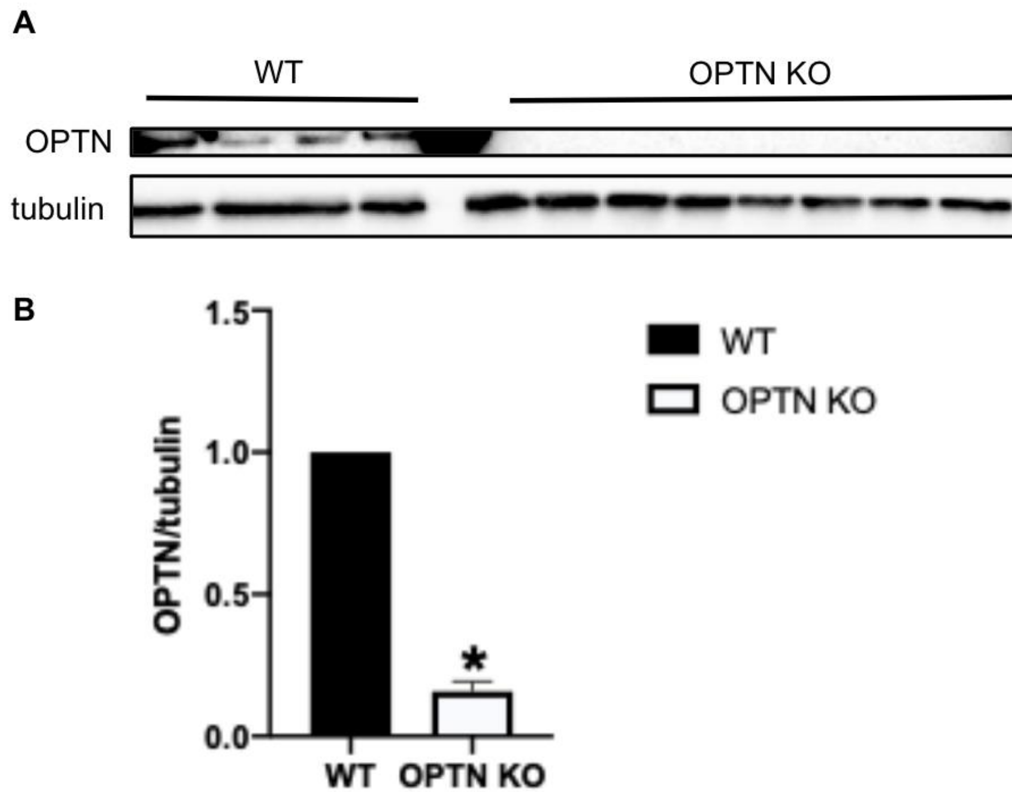


Figure 17. Successful knockout of OPTN in C57BL/6 mice using cre/lox recombination technology. *Representative* western blot (A) and densiometric quantification (B) of OPTN and corresponding vinculin for wild-type and OPTN KO animals.

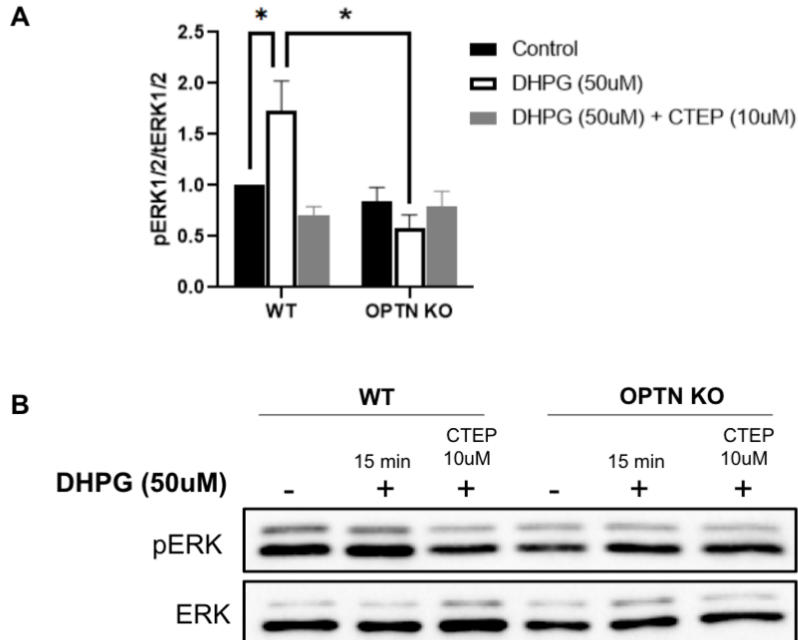


Figure 18. Activation of mGluR5 in the hippocampus increases phosphorylation of ERK1/2 in WT but has no effect on OPTN knockouts. Representative blots (B) and densiometric quantification (A) of pERK1/2 (n=4) expression in OPTN knockout mouse hippocampus compared to WT mouse hippocampus treated for 15 minutes with DHPG (50 μM), DHPG (50 μM) + CTEP (10 μM) or ACSF (NT). Data represents mean $\pm$ SEM of 4 independent experiments. Significantly different (p<0.05) results are depicted by *.

OPTN knockout cells displayed alterations in the GSK3β/ZBTB16/ATG14 pathway. Therefore, we tested whether OPTN knockout altered GSK3β phosphorylation, as well as, ZBTB16 and VPS34 protein levels. We found that mGluR5 agonism significantly increased the phosphorylation of GSK3β at Ser_9 in the wild-type mouse hippocampal slices, however GSK3β phosphorylation at Ser_9 was not induced in response to DHPG treatment in the OPTN knockout mouse hippocampus (Figure 19). Furthermore, we found that ZBTB16 expression was significantly increased in the wild-type mouse hippocampus in response to 50 μM DHPG treatment, an effect which would be antagonized by CTEP pre-treatment (Figure 20). However, agonist-stimulated increases in ZBTB16 expression was not detected in the OPTN knockout mouse hippocampal slices following mGluR5 activation with 50 μM DHPG (Figure 20). DHPG treatment had no effect on VPS34 protein expression in wild-type hippocampal slices and although there was a trend towards increased basal expression in OPTN knockout hippocampal slices, the data was not statically significantly different (Figure 21). Together, these findings demonstrated that mGluR5 activation inhibited autophagy via the GSK3β/ZBTB16/ATG14 pathway in the wild-type mouse hippocampus. However, in the OPTN knockout mouse hippocampus, mGluR5-mediated regulation of this pathway was lost.

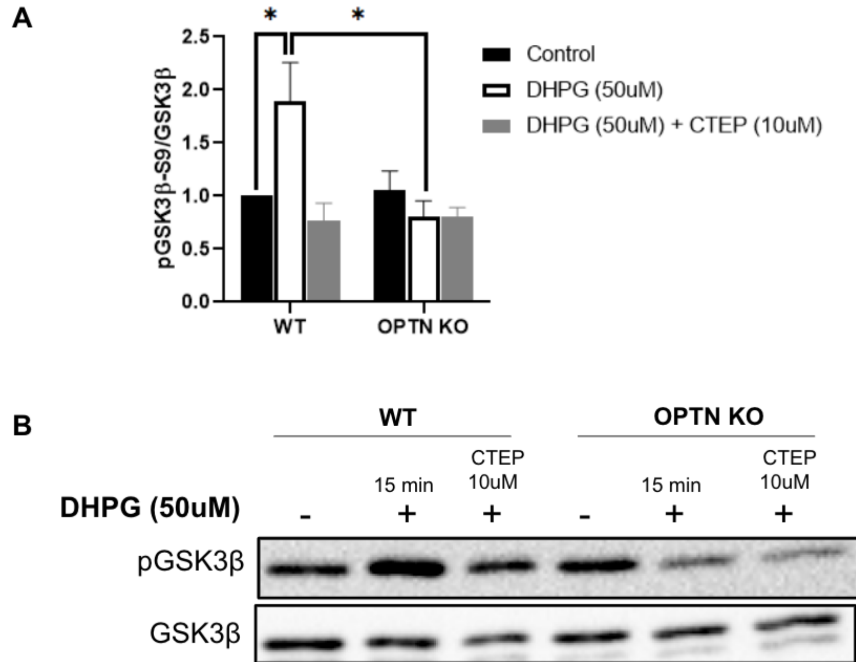


Figure 19. mGluR5 agonism in the hippocampus of increases phosphorylation of GSK3β-S9 in wild-type brain slices but has no effect in OPTN knockouts. Representative blots (B) and densiometric quantification (A) of pGSK3β (n=4) expression in OPTN knockout mouse hippocampus compared to WT mouse hippocampus treated for 15 minutes with DHPG (50 μM), DHPG (50 μM) + CTEP (10 μM) or ACSF (NT). Data represents mean $\pm$ SEM of 4 independent experiments. Significantly different (p<0.05) results are depicted by *.

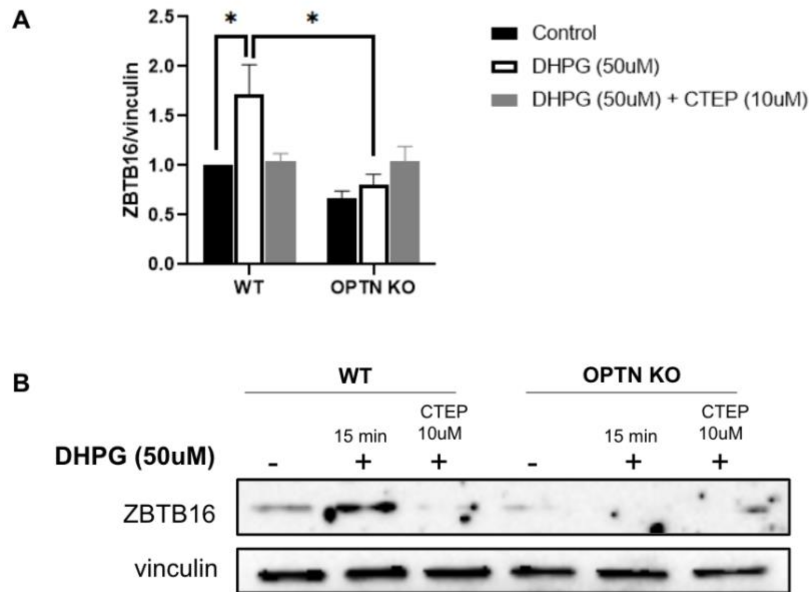


Figure 20. mGluR5 agonism in the hippocampus of increases ZBTB16 protein levels in wild-type brain slices but has no effect in OPTN knockouts. Representative blots (B) and densiometric quantification (A) of ZBTB16 (n=4) expression in OPTN knockout mouse hippocampus compared to WT mouse hippocampus treated for 15 minutes with DHPG (50 μM), DHPG (50 μM) + CTEP (10 μM) or ACSF (NT). Data represents mean $\pm$ SEM of 4 independent experiments. Significantly different (p<0.05) results are depicted by *.

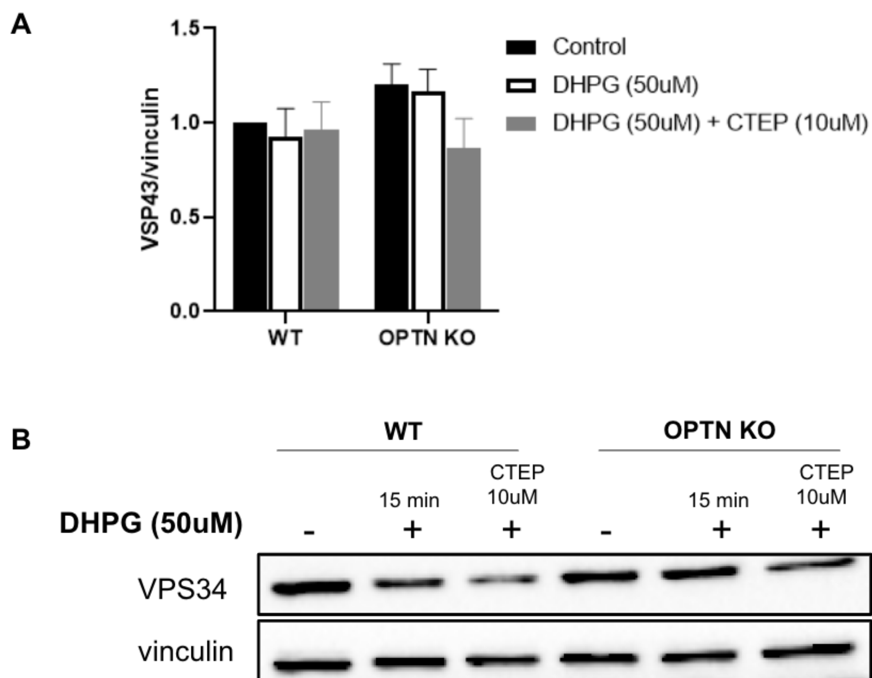


Figure 21. mGluR5 agonism had no effect on VPS34 protein expression in wild-type hippocampal slices or OPTN knockout hippocampal slices. Representative blots (B) and densiometric quantification (A) of VPS34 (n=4) expression in OPTN knockout mouse hippocampus compared to WT mouse hippocampus treated for 15 minutes with DHPG (50 μM), DHPG (50 μM) + CTEP (10 μM) or ACSF (NT). Data represents mean $\pm$ SEM. Significantly different (p<0.05) results are depicted by *.

3.8 Loss of OPTN inhibits autophagy by the mTOR/ULK1 pathway in hippocampus, however recovered with DHPG treatment

We previously demonstrated that the mGluR5-dependent activation of mTOR and the inhibitory phosphorylation of ULK1 at Ser_{757} lead to the inhibition of autophagy (Abd-Elrahman et al., 2018; Abd-Elrahman & Ferguson, 2019). The ST*Hdh*$_{Q7/Q7}$ OPTN knockout cells displayed alterations in the mTOR/ULK1 pathway, as well as in several well-known autophagy markers. However, it still remained unknown whether this pathway is modulated in an OPTN-dependent manner in intact mouse brain. Therefore, we examined whether OPTN knockout in mouse hippocampus affected the phosphorylation of ULK1, as well as p62 protein levels. We found that ULK1 phosphorylation at Ser_{757} was increased in the OPTN knockout mouse hippocampal slices when compared with wild-type tissue and that mGluR5 activation reduced phosphorylation of ULK1 to wild-type levels (Figure 22). However, CTEP treatment did not antagonize the effects of DHPG treatment. Finally, we found that p62 protein levels were significantly increased in the OPTN knockout mouse hippocampal slices in the absence of drug treatment and that treatment of slices with 50 μM DHPG reduced expression to wild-type levels, but that CTEP treatment did not antagonize the effects of DHPG on p62 expression (Figure 23).

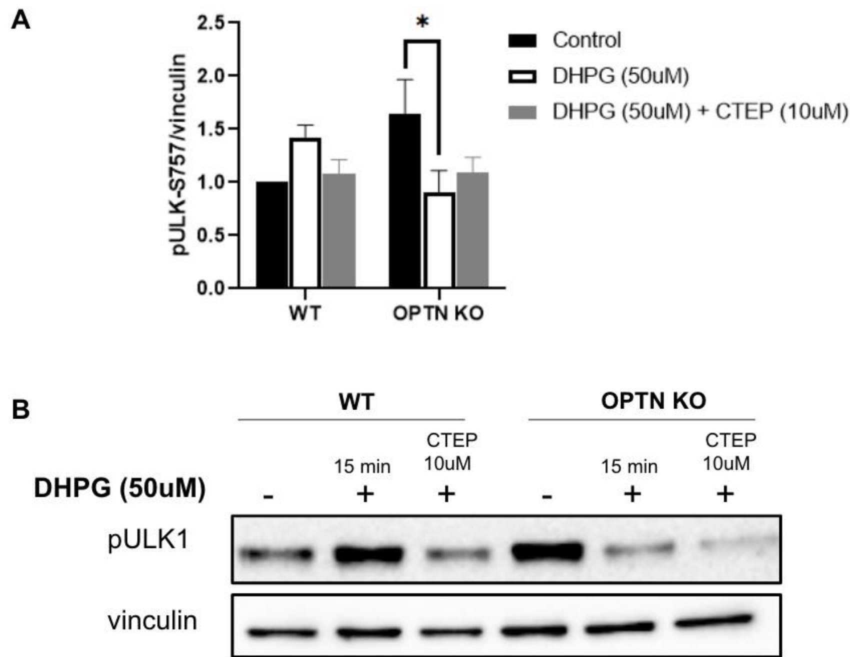


Figure 22. Loss of OPTN increases phosphorylation of ULK1-S757 in hippocampus, however recovered by mGluR5 activation. Representative blots (B) and densiometric quantification (A) of pULK1 (n=4) expression in OPTN knockout mouse hippocampus compared to WT mouse hippocampus treated for 15 minutes with DHPG (50 μM), DHPG (50 μM) + CTEP (10 μM) or ACSF (NT). Data represents mean $\pm$ SEM of 4 independent experiments. Significantly different (p<0.05) results are depicted by *.

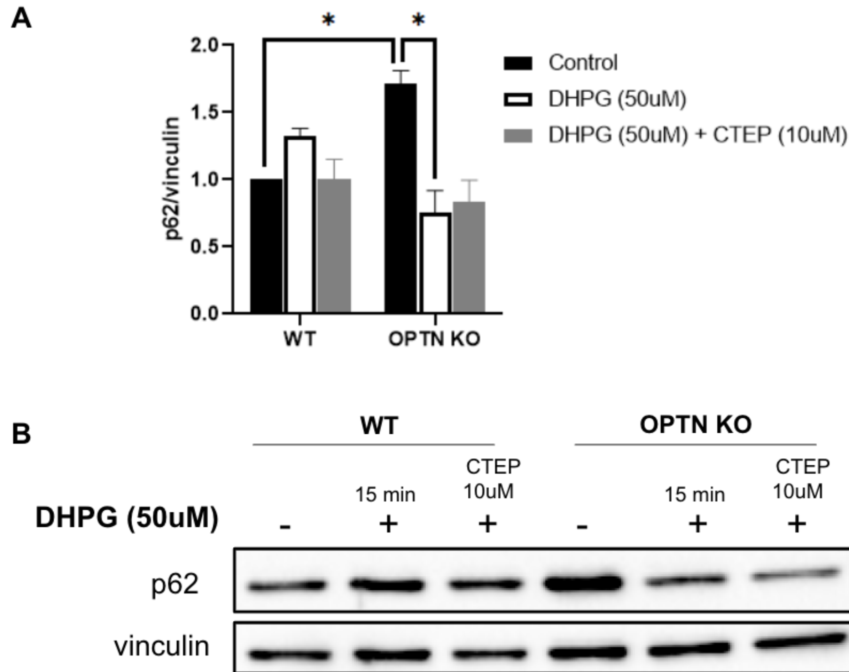


Figure 23. Loss of OPTN increases p62 protein levels in hippocampus, however recovered by mGluR5 activation. Representative blots (B) and densiometric quantification (A) of p62 (n=4) expression in OPTN knockout mouse hippocampus compared to WT mouse hippocampus treated for 15 minutes with DHPG (50 μM), DHPG (50 μM) + CTEP (10 μM) or ACSF (NT). Data represents mean $\pm$ SEM of 4 independent experiments. Significantly different (p<0.05) results are depicted by *.

4. Discussion

Neurodegenerative diseases are known as the progressive degeneration and death of nerve cells, which results in problems involving movement and/or mental functioning. Examples of neurodegenerative diseases include, Alzheimer's disease, Huntington's disease and Parkinson's disease. A common characteristic found in all of these diseases is the accumulation of a toxic aggregate, which ultimately leads to neuron degeneration and death. In order to clear the various toxic aggregates associated with neurodegenerative diseases, we propose inducing autophagy as an effective mechanism. Although it is difficult to pharmacologically target autophagy, it has recently been reported that GPCR signaling has the ability to regulate autophagy through two novel pathways (Zhang et al., 2015; Zhu et al., 2018). The first pathway, the mGluR5-mediated inhibition of autophagy as the consequence of increased GSK3β phosphorylation results in an increase in ZBTB16 protein levels, an important component of the ZBTB16-Cullin3-Roc1 E3 ubiquitin ligase complex. ZBTB16 actively promotes the degradation of the autophagy protein, ATG14, resulting in deficiency of autophagosome degradation and an accumulation of p62 protein aggregates (Abd-Elrahman et al., 2017). Autophagy is also targeted through another canonical pathway, as activation of mGluR5 promoted mTOR activity (Abd-Elrahman & Ferguson, 2019; Zhu et al., 2018). When activated, mTOR phosphorylated ULK1 at Ser_{757}, which is an important kinase involved in promoting autophagosome formation. When phosphorylated, $ULK1_{757}$ kinase activity is suppressed, resulting in insufficient autophagosome formation and inhibition of autophagy (Abd-Elrahman et al., 2018; Satyavarapu et al., 2018). Recent results show that OPTN, an autophagy receptor and

protein linked to several neurodegenerative diseases, might be involved in altering mGluR5-mediated autophagic signaling (Anborgh et al., 2005). We investigated here, whether OPTN knockout impacts mGluR5-dependent autophagy signaling pathways.

When assessing the effects of knocking out OPTN in the ST*Hdh*$_{Q7/Q7}$ cells, we find that GSK3β phosphorylation at Ser$_9$ is significantly decreased in the OPTN knockout groups, resulting in lower levels of ZBTB16 expression and increased VPS34 protein levels. VPS34 is a protein that is a part of the PI3K CIII complex, containing ATG14 (Su et al., 2017; Yoon, 2015). Although we have experienced difficulties with ATG14 antibodies, it is clear that lower levels of ZBTB16 correlates with increased activity of the PI3K CIII complex, which contains VPS34. It is notable that the two knockout guides represent different mutations and present slightly different phenotypes. Guide 1 expressed less ZBTB16 protein levels than guide 2, which is associated with higher VPS34 protein levels in guide 1 compared to guide 2. Overall, this suggests that silencing OPTN in the ST*Hdh*$_{Q7/Q7}$ cells results in suppression of mGluR5 activity and initiation of autophagy through the GSK3β/ZBTB16/ATG14 pathway. However, we also find that the inhibitory phosphorylation of ULK1 at Ser$_{757}$ is significantly increased in the OPTN knockout ST*Hdh*$_{Q7/Q7}$ cells, blocking autophagy-initiated autophagosome biogenesis and accumulating LC3β and p62 protein aggregates. This contradicts the results of the OPTN knockout ST*Hdh*$_{Q7/Q7}$ cells via the GSK3β/ZBTB16/ATG14 pathway, as it instead suggests that silencing OPTN in the ST*Hdh*$_{Q7/Q7}$ cells further activates mGluR5 and inhibits autophagy via the mTOR/ULK1 pathway. Although these two pathways display contradicting findings, we believe knocking out OPTN in ST*Hdh*$_{Q7/Q7}$ cells inhibits autophagy, as OPTN knockout ST*Hdh*$_{Q7/Q7}$ cells displayed an

increase in both p62 and LC3β, well-known autophagy markers, which indicated an overall inhibition of autophagy. However, these opposing results demonstrate the possibility that OPTN interacts in a different manner with the GSK3β/ZBTB16/ATG14 pathway than the mTOR/ULK1 pathway, providing support for further investigation in the contribution of OPTN to pathological mGluR5 signaling. Overall, these data suggest OPTN plays a critical role in autophagy signaling, which agrees with previous reports showing disease-causing mutations in OPTN resulted in the presentation of autophagic defects (Ryan and Tumbarello, 2018). However, it still remains unknown if the alterations found in the GSK3β/ZBTB16/ATG14 and mTOR/ULK1 pathways caused by knocking out OPTN in the ST*Hdh*$_{Q7/Q7}$ cells are mGluR5-dependent, as exposure to DHPG treatment did not alter the significant changes. In order to validate our findings in the OPTN knockout ST*Hdh*$_{Q7/Q7}$ cells and further investigate what the effect of knocking out OPTN expression might have upon the activation status of cell signaling molecules downstream of endogenously expressed mGluR5, we have also examined the effect of knocking out OPTN in C57BL/6 mice in combination with DHPG treatment.

When assessing the effects of globally knocking out OPTN in the C57BL/6 mice, the current study provides further evidence for the pivotal role of mGluR5 in mediating autophagy and a novel function of OPTN in mGluR5-dependent regulation of autophagy. We evaluated the effect of globally knocking out OPTN in mouse hippocampus, as atrophy of the hippocampus is a major symptom in Alzheimer's disease patients (Josephs et al., 2017). When mGluR5 is activated in the wild-type mouse hippocampus, we find a significant increase in inhibitory phosphorylation of GSK3β at Ser$_9$, accompanied by a significant increase in ZBTB16 protein levels

compared to the wild-type non-treated group, which is consistent with previous reports (Abd-Elrahman et al., 2017). However, the loss of OPTN eliminates the effects of DHPG treatment as there was no significant changes in GSK3β phosphorylation and ZBTB16 protein levels between the treated and non-treated OPTN knockout mouse hippocampus groups. Similar results are observed in ULK_{757} phosphorylation and p62 protein levels following activation of mGluR5, as both appear to show an increase, although not significantly significant, in the wild-type mouse hippocampus following exposure to DHPG treatment. Additionally, we detect a significant elevation in $ULK1_{757}$ phosphorylation and p62 expression in the non-treated OPTN knockout mouse hippocampus group, followed by an abolishment of these increases after exposure to DHPG treatment. However, there are no significant changes observed in VPS34 expression levels. This may occur because VPS34 is further downstream of GSK3β and ZBTB16, indicating that in order to induce changes in protein levels, we may have needed to expose the tissue to a higher dose of DHPG or expose it for a longer period of time. Together, these data suggest a novel finding that OPTN plays a role in the GSK3β/ZBTB16/ATG14 and mTOR/ULK1 autophagy pathways by facilitating the activation of mGluR5 and inhibition of autophagy.

The GSK3β/ZBTB16/ATG14 and mTOR/ULK1 pathways are activated by a mGluR5-homer interaction, leading to the activation of PI3K enhancer protein (PIKE). Stimulation of PIKE activates PI3K, resulting in the downstream activation of Akt (Gross et al., 2012; Hou and Klann, 2004). Akt has the ability to activate mTOR through

direct phosphorylation and inhibition of tuberous sclerosis complex 2 or through an indirect mechanism regulating cellular energy and inhibition of AMPK (Memmott and Dennis, 2009; Hahn-Windgassen et al., 2005). Akt also has the ability to phosphorylate GSK3β at Ser_9, resulting in its deactivation (Jaworski et al., 2019; Liu et al., 2005) (Figure 24). Because our results suggest a novel finding that OPTN is involved in both the GSK3β/ZBTB16/ATG14 and mTOR/ULK1 pathways to facilitate the activation of mGluR5 and inhibition of autophagy, we expect that OPTN participates in activating these autophagy pathways by interacting with mGluR5 itself or cell signaling molecules immediately downstream of mGluR5, including but not limited to, Homer, PIKE, PI3K, or Akt. This theory is based on the knowledge that the PIKE/PI3K/Akt pathway is upstream and activates both the GSK3β/ZBTB16/ATG14 and mTOR/ULK1 pathways. Overall, this novel discovery is important in regard to neurodegenerative diseases, as the absence of OPTN could prevent mGluR5 activation and allow for the initiation of autophagy to degrade toxic aggregates associated with neurodegenerative diseases.

Accompanying the inhibition of autophagy is the canonical abolishment of the phosphorylation of ERK1/2 following mGluR5 activation. In our study, we have found that ERK1/2 phosphorylation in ST*Hdh*$_{Q7/Q7}$ wild-type cells significantly increased with treatment of DHPG, which agrees with the findings reported in a Huntington's disease mouse model (Ribeiro et al., 2010). However, knocking out OPTN in the ST*Hdh*$_{Q7/Q7}$ cells abolished the canonical phosphorylation of ERK1/2 following mGluR5 activation. Knocking out OPTN in C57BL/6 mice results in similar outcomes, as ERK1/2 phosphorylation significantly increases in the wild-type mouse hippocampus following exposure to DHPG treatment, however ERK1/2 phosphorylation is not induced in

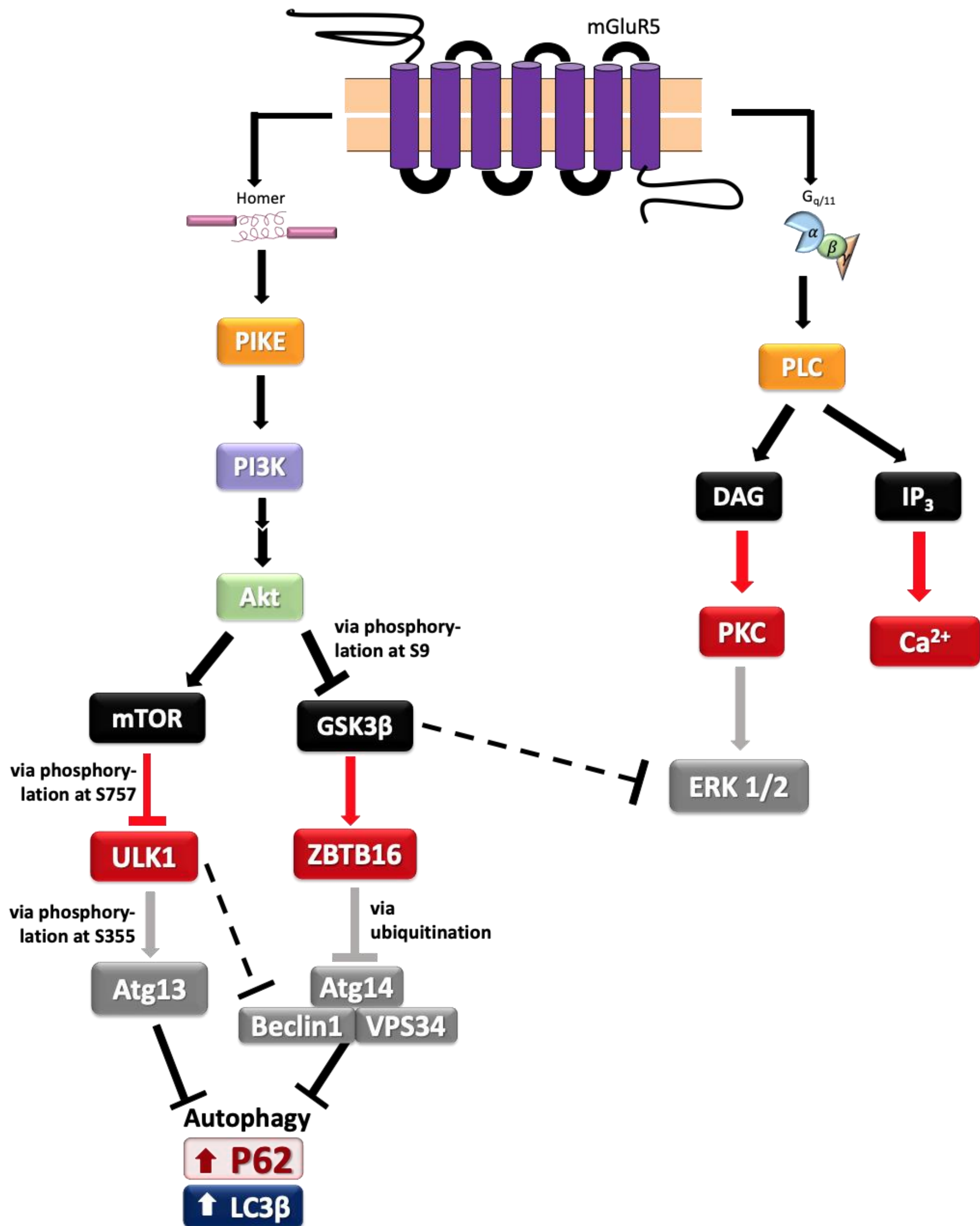


Figure 24. mGluR5 signalling cascades. Schematic drawing demonstrating various mGluR5 signalling cascades, including the PI3K pathway leading to mTOR/ULK1/Atg13 and GSK3*β*/ZBTB16/Atg14 pathways, as well as the $G_{q/11}$ coupled pathway leading to the DAG/PKC/ERK1/2 and IP_3/Ca^{2+} pathway.

response to treatment with DHPG in the OPTN knockout mouse hippocampus group. This novel finding provides further evidence for the role of OPTN and its effects on mGluR5-dependent phosphorylation of ERK1/2. It is an important area to further investigate in order to determine what the effects would be on cell signaling pathways downstream of ERK1/2 when OPTN expression is silenced. Phosphorylation of ERK1/2 has been shown to activate TNFα and Caspase-3, leading to the induction cell death processes (Zhuang and Schnellman, 2006). However, activation of ERK1/2 by Group 1 mGluR triggered Ca^{2+} has also been shown to induce LTD, by promoting the upregulation of several LTD-related proteins, including Arc (Sethna et al., 2016). As well, this pathway activates p70S6K, which is an important protein involved in synaptic plasticity and memory (Page et al., 2006; Menard and Quirion, 2012; Mukherjee and Manahan-Vaughan, 2013). It is important to further investigate how these cell signaling processes downstream of ERK1/2 are impacted by silencing OPTN expression and abolishing ERK1/2 phosphorylation, as they are all impacted and correlated with neurodegenerative diseases.

It would be interesting to further investigate why silencing OPTN leads to the abolishment of ERK1/2 phosphorylation. Some report that ERK1/2 is phosphorylated by PKC (Tsao et al., 2013; Monick et al., 2000; Clark et al., 2004) while others report that ERK1/2 is phosphorylated by the MAPK/ERK kinase 1/2 (MEK) downstream of β-arrestin (Luttrell et al., 2001; Roberts and Der, 2007). In the current study, we suspect that ERK1/2 is being phosphorylated by PKC because our results are mGluR5-dependent and mGluR5 regulates PKC activity through the PLC-DAG-PKC signalling cascade (Vanzulli and Butt, 2015). It is possible that OPTN indirectly activates PLC or

PKC, and thus without its presence, PKC is unable to phosphorylate ERK1/2. Another possibility of why the loss of OPTN results in the abolishment of ERK1/2 phosphorylation is a phosphatase being activated when OPTN is removed. It is known that both calcineurin, a phosphatase, and calcineurin inhibitor protein (CAIN), a phosphatase inhibitor, both form a complex with mGluR5 (Ferreira et al., 2009; Alagarsamy et al., 2005; Dale et al., 2001). We suggest that when OPTN expression is lost, that CAIN may also be lost from the receptor complex as well, which will facilitate the ability of calcineurin to dephosphorylate signaling proteins, such as ERK1/2 (Figure 25). Overall, this is an important area to further research to determine why ERK1/2 phosphorylation is abolished and the downstream effects of this canonical abolishment.

Although the OPTN knockout mouse hippocampus group displayed an overall suppression of mGluR5 activity and inhibition of autophagy when exposed to DHPG treatment, the non-treated OPTN knockout group displayed differences in the activation status of cell signaling molecules between the GSK3β/ZBTB16/ATG14 and mTOR/ULK1 pathways. While the non-treated OPTN knockout mouse hippocampus did not present with significantly higher levels of GSK3β phosphorylation or ZBTB16 compared to the non-treated wild-type group, the non-treated OPTN knockout mouse hippocampus did present with significantly elevated levels of $ULK1_{757}$ phosphorylation and p62 expression. This observation suggests autophagy is inhibited via the mTOR/ULK1 pathway when OPTN is silenced, however knocking out OPTN does not inhibit autophagy via the GSK3β/ZBTB16/Atg14 pathway. As mentioned previously, this suggests that OPTN may play a different role in each of these pathways. Additionally, the OPTN knockout mouse hippocampus group exhibited increased levels of

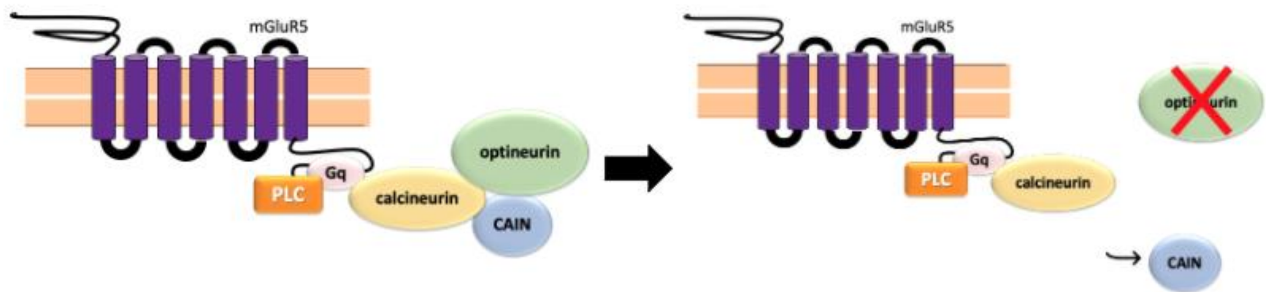


Figure 25. mGluR5-OPTN-calcineurin-CAIN complex. Schematic drawing demonstrating the possibility of CAIN being removed from the mGluR5 complex when OPTN is knocked out.

ULK_{757} phosphorylation and p62 protein levels when compared to wild-type tissue and exposure to DHPG treatment significantly reduced phosphorylation of ULK1 and p62 expression to wild-type levels. However, CTEP treatment did not antagonize the effects of DHPG on ULK1 phosphorylation or p62 expression, respectively. This suggests that the decrease in $pULK_{757}$ and p62 protein levels may not be mGluR5-dependent, as it is known that CTEP is selective for mGluR5. Because DHPG is not selective for mGluR5 and also targets mGluR1, it is possible DHPG is mediating these responses through mGluR1. The effects of DHPG on mGluR1 in the ST*Hdh*$_{Q7/Q7}$ cells was not a concern, as the ST*Hdh*$_{Q7/Q7}$ cell line is a mouse, striatal derived cell line. It is known that mGluR1 is not expressed in the striatum, and therefore is not expressed in the ST*Hdh*$_{Q7/Q7}$ cell line. However, mGluR1 is endogenously expressed in the hippocampus, and therefore could be an alternative target for DHPG in mediating these responses that cannot be reversed by CTEP treatment.

4.1 Planned Unfinished Research

From these results, there were many unanswered questions that should be further investigated. Due to the recent pandemic and lab closures, there were a few experiments we had planned to finish for my thesis. However, I was unable to complete these studies. The striking results seen in the abolishment of ERK1/2 phosphorylation when knocking out OPTN in both the ST*Hdh*$_{Q7/Q7}$ cells, as well as, in the hippocampus brain slices was intriguing to me. My plan was to investigate whether there are any changes in PKC activity, as this was likely the protein responsible for phosphorylating ERK1/2 (Vanzulli and Butt, 2015). It was previously known that PKCα, PKCβI, PKCβII,

and PKCγ were all activated in response to group 1 mGluR activation, as they exhibit a repetitive translocation between the cytosol and the plasma membrane (Babwah et al., 2003; Uchino et al., 2004). I had planned to perform a live imaging experiment on the confocal microscope to determine if whether this PKC translocation and oscillatory patterning could be observed in the OPTN KO cells in response to DHPG treatment.

For the planned experiment, I would first transfect both the wild-type and OPTN KO ST*Hdh*$_{Q7/Q7}$ cells with plasmid encoding GFP-PKCβII. Then I would treat with DHPG while performing live imaging to observe if there are any differences in redistribution of PKC from the cytosol to the plasma membrane between the wild-type cells and the OPTN knockout groups. If I did not observe PKC plasma membrane translocation in OPTN KO cells, this would help to better understand why ERK1/2 phosphorylation is abolished when OPTN is knocked out and complete the DAG-PKC-ERK1/2 pathway.

While further investigating the reason behind the abolishment of the ERK1/2 phosphorylation in the OPTN KO ST*Hdh*$_{Q7/Q7}$ cells and in the OPTN KO hippocampus brain slices, I hypothesized that calcineurin was being activated in this absence of OPTN expression. This I presumed to be the consequence of a loss of CAIN within the mGluR5 scaffolded signaling complex. In order to execute this theory, my plan was to first perform a Co-IP experiment to confirm that mGlur5, calcineurin, CAIN and OPTN form a complex in the wild-type cells. When this was verified, I was then planning to use a calcineurin activity kit to measure the difference in activity between the wild-type and OPTN KO ST*Hdh*$_{Q7/Q7}$ cells. If calcineurin was more active in the OPTN KO cells, it would be evident that CAIN was removed from the complex, allowing calcineurin to be activated and dephosphorylate ERK1/2 and other proteins.

Lastly, I had started to perform a rescue experiment, however as I stated in the results chapter, I was only able to obtain an n value of 2. In this rescue experiment, I had planned to transfect OPTN-YFP into the ST*Hdh*$_{Q7/Q7}$ wild-type and OPTN KO cells. I would then treat the cells with DHPG and measure the same autophagy and cell survival markers (GSK3β, ZBTB16, VPS34, ULK1, LC3β, p62, ERK1/2) using immunoblots to observe if alterations previously seen in these pathways can be rescued. This would validate the results previously observed, where silencing OPTN disrupted these mGluR5-dependent autophagy pathways.

4.2 Further Research

The effects observed by the lack of phosphorylation of ERK1/2 due to knocking out OPTN in both the ST*Hdh*$_{Q7/Q7}$ cells and the hippocampal slices was extremely striking. It has previously been reported that ERK1/2 phosphorylation can be stimulated by mGluR5 agonism (Ribeiro et al., 2010), which is consistent with the results we have observed in the ST*Hdh*$_{Q7/Q7}$ wild-type cells, as well as, in the wild-type hippocampus brain slices. However, it is extremely evident from our results that OPTN is necessary for this phosphorylation of ERK1/2 following mGluR5 stimulation. As mentioned previously, the phosphorylation of ERK1/2 is involved in several cellular and molecular mechanisms, including cell growth mechanisms and cell death mechanisms. In order to determine the downstream effects of the abolishment of ERK1/2 phosphorylation, several experiments can be performed. The same experimental procedure can be performed, where both the ST*Hdh*$_{Q7/Q7}$ wild-type and OPTN KO cells are exposed to DHPG treatment, or the wild-type and OPTN KO hippocampal brain slices are treated

with DHPG. The cells and brain slices are then lysed, quantified and specific proteins are detected by immunoblot. In order to determine the effects knocking out OPTN and the abolishment of ERK1/2 phosphorylation on cell death processes and apoptosis, it would be important to blot for TNFα and Caspase-3. With a decrease in phosphorylation of ERK1/2, it is expected that there would also be a decrease in both TNFα and Caspase-3, resulting in an overall inhibition of apoptosis (Zhuang & Schnellman, 2006). To validate these results, it would also be important to blot for Brain-derived Neurotropic Factor (BDNF), as it is known that an increase in BDNF correlates with an inhibition of apoptosis (Petersen et al., 2001). Without the activation of ERK1/2, cell death processes and apoptosis will be inhibited, which might be beneficial for cell survival. Another important cell process to investigate is long term depression and synaptic plasticity, as ERK1/2 plays an important role in the CaMK-ERK1/2-p70S6K signaling cascade, which is activated by group 1 mGluR triggered Ca^{2+} and CaM activity. This signaling cascade leads to the upregulation of several LTD proteins and overall synaptic depression. It is important to blot for p70S6K, as this is an important protein involved in memory and synaptic plasticity (Page et al., 2006; Menard and Quirion, 2012; Mukherjee and Manahan-Vaughan, 2013), as well as, Activity-regulated cytoskeleton-associated protein (Arc), as this is a well-known LTD protein that is upregulated following the activation of ERK1/2 by group 1 mGluRs, and leads to synaptic modification (Sethna et al., 2016; Gallagher et al., 2004). Blotting for all of these cell death related proteins and synaptic modification proteins would be a promising start to determine the effects of the abolishment of ERK1/2 phosphorylation on the cell and tissue function.

To further determine the effects of knocking out OPTN and its role in mGluR5 regulation of autophagy, the next step would be to cross an OPTN KO mouse model with a Huntington's or Alzheimer's disease mouse model. Our results suggest that OPTN is necessary to facilitate the activation of mGluR5, leading to the inhibition of autophagy. It would be interesting to determine if the absence of OPTN decreased the activation of mGluR5 in the Huntington's/Alzheimer's disease mouse model, leading to the activation of autophagy and clearance of toxic aggregates. This would be an important discovery in advancing mGluR5-targetted therapeutics for neurodegenerative diseases.

5. Conclusion

In summary, our results show a novel discovery that OPTN plays a role in the inhibition of autophagy through the GSK3β/ZBTB16/ATG14 and mTOR/ULK1 autophagy pathways by facilitating the activation of mGluR5 in the hippocampus. Furthermore, it demonstrates that OPTN is an important protein involved in the canonical phosphorylation of ERK1/2 following mGluR5 activation. Together, all these discoveries are important for further advancing mGluR5-targetted therapeutics for several neurodegenerative diseases, including Alzheimer's, Huntington's and Parkinson's disease.

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