INVOLVEMENT OF THE SIGMA-1 RECEPTOR IN NEURONAL CELL DEATH AND ALZHEIMER'S DISEASE

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ABSTRACT

Dysfunction in the Sigma-1 receptor (Sig-1R) is implicated in many neurodegenerative diseases such as Alzheimer's Disease (AD). Recently, agonists of the Sig-1R have been found to be neuroprotective in AD and provide significant improvements in symptoms. The hallmarks of AD are aggregation of amyloid-β (Aβ) plaques and development of neurofibrillary tau tangles in the brain, which are thought to be correlated with progressive neuronal cell death in AD. Aβ leads to increased endoplasmic reticulum (ER) stress, decreased autophagy, and increased apoptosis, all of which may be contributing to the neuronal cell death that is seen in AD. The Sig-1R is known to reduce ER stress, increase autophagy, and decrease apoptosis. However, as of yet there is little research on the ability of the Sig-1R to specifically reduce Aβ toxicity through these pathways. Therefore, through the use of *in vitro* and *ex vivo* models, this study examined the pathways through which activation of the Sig-1R may exert its protective effects against Aβ toxicity. Here, it is shown that activation of the Sig-1R reduces neuronal cell apoptosis *in vitro*, and reduces tissue death in the CA3 region of the hippocampus *ex vivo*. Furthermore, this reduction in cell and tissue death may be a result of reduction of ER stress and a return towards baseline levels of autophagy. Together, this research provides insight as to how the Sig-1R may be an important therapeutic target in AD through protection against apoptosis and tissue death.

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LIST OF ABBREVIATIONS

AD – Alzheimer's Disease
ALS – Amyotrophic lateral sclerosis
Aβ - Amyloid-β 1-42
AβR – Amyloid-β reverse/42-1
ATF4 – Activating transcription factor 4
ATF6 – Activating transcription factor 6
ATG5 – Autophagy-related gene 5
ATG12 – Autophagy-related gene 12
ATG16 – Autophagy-related gene 16
BiP – Binding immunoglobulin protein
CHOP – C/EBP homologous protein
CNS – Central nervous system
DAPK – Death-associated protein kinase
DHEA – Dehydroepiandrosterone
DG – Dentate gyrus
DMT – N,N-dimethyltryptamine
ER – Endoplasmic reticulum
ERAD – Endoplasmic reticulum associated degradation
eIF2α – Eukaryotic initiation factor 2α
FTD – Frontotemporal dementia
GADD34 – Growth arrest and DNA damage-inducible protein
GPCR – G protein-coupled receptors
HD – Huntington's Disease
IP3Rs – Inositol 1,4,5-triphosphate receptors
IRE1 – Inositol-requiring protein 1
JNK – c-Jun N-terminal kinase
KD – Knock-down
KO – Knock-out
Kv – Voltage-gated K^+ channels
MAM – Mitochondrial-associated membrane
mRNA – Messenger ribonucleic acid
Nav – Voltage-gated sodium channel
NFT – Neurofibrillary tau tangles
NMDAR – N-methyl-D-aspartate receptor
OHSC – Organotypic hippocampal slice culture
PBS – Phosphate buffered saline
PD – Parkinson's Disease
PERK – PKR-like endoplasmic reticulum kinase
PFA – Paraformaldehyde

PTZ – (+)-Pentazocine
qPCR – Quantitative polymerase chain reaction
Sig-1R – Sigma-1 receptor
UPR – Unfolded protein response
Vps34 – Vacuolar protein sorting 34
WT - Wildtype

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1. INTRODUCTION

1.1 The Sigma-1 Receptor

1.1.1 Relevance

Since the discovery of the sigma-1 receptor (Sig-1R) in 1976 by Gilbert and Martin, there has been increasing interest in understanding the role of this receptor in a wide range of pathophysiological conditions. Dysfunction in the Sig-1R has been shown to be associated with neurological disorders such as Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), Parkinson's disease (PD), Multiple Sclerosis, stroke, and brain injury (Lisak et al., 2020; Ryskamp et al., 2019). In addition, activation of the Sig-1R through a variety of agonists leads to signalling functions that are generally protective and anti-apoptotic, thus promoting cell survival (Hayashi and Su, 2007). Recent research suggests that the Sig-1R may be a promising therapeutic target for neurodegenerative diseases since activation of the Sig-1R has anti-amnesic, synaptogenic, and neuroprotective functions (reviewed in Ryskamp et al., 2019). Sig-1R agonists have been shown to reduce symptoms and slow disease progression in mouse models of ALS, FTD, PD, HD, and AD (Francardo et al., 2014; Hyrskyluoto et al., 2013; Mancuso et al., 2012; Ryskamp et al., 2019a). Furthermore, exciting novel research shows that patients with AD have overall improvements in their functional abilities during ongoing clinical trials in Phase 3 using ANAVEX ®2-73, a selective Sig-1R agonist (Hampel et al., 2018). Despite its clear relevance to neurological disorders and potential as a

therapeutic target, there is still relatively little known about the neuroprotective effects of Sig-1Rs and the mechanism underlying these effects.

1.1.2 Background

The Sig-1R is a small transmembrane protein that has a wide range of cellular functions, many of which are not fully understood. Originally, the Sig-1R was mislabelled as a type of opioid receptor based on its high affinity for SKF10,047 and other benzomorphans (Gilbert and Martin, 1976). In later studies, it was determined that while (-)-isomers were the primary agonists of opioid receptors, they had a lower affinity for Sig-1Rs, and that (+)-isomers were the preferential agonists of Sig-1Rs (Young and Khazan, 1984). The Sig-1R was found to have a high affinity for a variety of (+)-benzomorphans including (+)-cyclazocine, dextrallorphan, and (+)-pentazocine, and the binding site of these drugs had low affinity for other opiates and opioid peptides (Su, 1982). In addition, the binding of these (+)-benzomorphans to the Sig-1R was unaffected by the opioid receptor antagonist naloxone (Su, 1982). This led to the conclusion that the Sig-1R was its own class of receptor rather than a sub-type of opioid receptor.

The endogenous ligand of the Sig-1R, if it exists, is still undetermined. There is some evidence to show that N,N-dimethyltryptamine (DMT), a naturally occurring hallucinogen, binds to the Sig-1R and acts as an agonist in Sig-1R-mediated Na^+ channel modulation thereby suggesting that this is a potential endogenous ligand for the Sig-1R (Fontanilla et al., 2009; Szabo et al., 2016). Additionally, the Sig-1R has been found to bind progesterone and testosterone which act as antagonists, as well as a neuroactive steroid dehydroepiandrosterone which may act as an agonist (DHEA; Chen et al., 2006; Ganapathy

et al., 1999). Since its discovery, many aspects of the structure and function of Sig-1Rs have been elucidated, however there is still much to learn about this protein.

1.1.3 Structure and location

The Sig-1R was first cloned in 1996 from guinea pig, and this provided the first insight on its structure (Hanner et al., 1996). It is a small, 28 kDa transmembrane protein, which has no similarity to any other mammalian protein. The Sig-1R was predicted to have one transmembrane domain based on hydrophobicity analysis that indicated a highly hydrophobic region at the amino-terminus likely to be a transmembrane helix (Hanner et al., 1996). This receptor forms as a triangular trimer with a transmembrane domain in each corner, and each subunit has a β-barrel flanked by two α-helices (Schmidt et al., 2016; Smith and Su, 2017). The β-barrel is likely the site of binding for both agonists and antagonists whereby the ligand binds to one of the three subunits (Smith and Su, 2017). The structure of the Sig-1R changes when it is bound to agonists and antagonists. The antagonists haloperidol and NE-100 fit into the region of the β-barrel close to the membrane (Schmidt et al., 2018). The agonist (+)-pentazocine was found to bind into the same region of the β-barrel, however there was a slight difference in the positions of the α-helices in comparison to the antagonist binding conformation (Schmidt et al., 2018). These small differences in binding between agonists and antagonists could be what leads to the differences in their effects.

At the sub-cellular level, the Sig-1R is an endoplasmic reticulum (ER) transmembrane protein. Membrane fractionation experiments revealed that the Sig-1R resides primarily at the mitochondrial-associated ER membrane (MAM; Hayashi and Su, 2007). However, it has also been found to localize to the plasma and nuclear membrane

particularly when it is activated by agonists (Hayashi and Su, 2007; Tsai et al., 2015). The Sig-1R has additionally been found to interact with a variety of proteins, including chaperones, transcription factors, ligand-gated and voltage-gated ion channels, and G protein-coupled receptors (GPCRs; Su et al., 2016). Accordingly, the Sig-1R may be involved in a range of physiological processes including neurotransmission, Ca^{2+} dynamics, and overall cell survival (Smith and Su, 2017).

The Sig-1R does not appear to have its own signalling pathway. Upon ligand-binding the Sig-1R translocates between different organelles and operates by involving itself in different protein-protein interactions to modulate the activity of other ion channels and proteins (Su et al., 2010). Agonists tend to lead to translocation of the Sig-1R, for example (+)-pentazocine, a Sig-1R ligand, was found to cause Sig-1Rs to translocate away from the MAM (Hayashi and Su, 2007). The Sig-1R also translocates in response to stressors. During cell stress triggered by glucose deprivation or depletion of ER Ca^{2+}, the Sig-1R becomes highly mobile at the ER membrane and relocates from the MAM to plasmalemmal ER cisternae and nuclear envelopes (Hayashi and Su, 2007).

1.1.4 Functions of the Sig-1R

Sig-1Rs have a variety of known functions in the cell. It resides primarily at the MAM, where it may be involved in IP3R-dependent Ca^{2+} flux, lipid dynamics, stability of the MAM, and the unfolded protein response (UPR; Figure 1; Hayashi and Su, 2007; Ryskamp et al., 2019b; Su et al., 2016). Under resting conditions, the Sig-1R has reduced chaperone activity, and forms a complex with binding immunoglobulin protein (BiP; Hayashi and Su, 2007). Agonists of the Sig-1R, as well as conditions of ER stress or dysregulated Ca^{2+}, trigger Sig-

1Rs to dissociate from BiP and relocate to other positions in the ER and plasma membranes (Hayashi and Su, 2007). Once active, the Sig-1R interacts with a variety of other ER and plasma membrane proteins such as IP3Rs, ion channels, GPCRs, and kinases (Su et al., 2016). At baseline, Sig-1Rs appear to have no effect on Ca^{2+} dynamics at the MAM. However, when ER Ca^{2+} is depleted, the association of Sig-1Rs with IP3Rs seems to increase the uptake of Ca^{2+} from the ER into the mitochondrion, which can facilitate the production of ATP (Figure 1; Gregianin et al., 2016; Hayashi and Su, 2007). Complete KO of the Sig-1R was associated with increased formation of reactive oxygen species, which may be a result of dysregulation of Ca^{2+} flux into the mitochondrion (Wang et al., 2015).

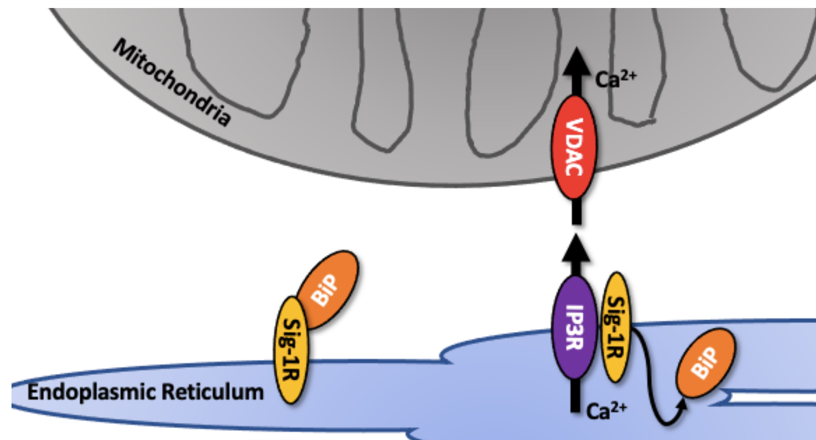


Figure 1. Location of the Sigma-1 Receptor (Sig-1R) at the endoplasmic reticulum (ER) mitochondrial associated membrane. In a resting state Sig-1Rs are found closely associated with binding immunoglobulin protein (BiP). Under conditions of ER stress or reduced mitochondrial Ca^{2+} Sig-1Rs dissociate with BiP and bind closely to Inositol 1,4,5-triphosphate receptors (IP3R) which allow Ca^{2+} flux from the ER into the cytoplasm adjacent to the mitochondria. Voltage-dependent anion channels (VDAC) then allow uptake of cytosolic Ca^{2+} into the mitochondria.

Particularly in the case of neuronal cells, Sig-1Rs are important contributors to neuronal excitability as they interact with many ligand-gated and voltage-gated ion channels. Sig-1R agonists have been shown to modulate function of K^{+}, Ca^{2+}, Na^{+}, Cl^{-}, and SK

channels, as well as N-methyl-D-aspartate receptors (NMDAR; Maurice and Su, 2009). For example, Sig-1R agonists lead to the dissociation of the Sig-1R from voltage-gated Na^+ (Nav) channels and suppress their activity (Johannessen et al., 2009). Sig-1Rs are also implicated in the function of voltage-gated K^+ channels (Kv; Abraham et al., 2019). Activation of the Sig-1R with cocaine leads to its increased interaction with Kv1.2, which is then trafficked to the plasma membrane where it decreases excitability of dopaminergic neuronal cells (Delint-Ramirez et al., 2020). In the case of NMDARs, the Sig-1R is thought to facilitate the signalling and neurotransmission in the hippocampus potentially by inhibiting SK channels or by promoting the expression of NMDARs and increasing their trafficking to the plasma membrane (Martina et al., 2007; Pabba et al., 2014).

Sig-1Rs also have potential indirect effects of transcription of certain genes. Sig-1Rs may regulate transcription through interactions with the ER protein inositol-requiring protein 1 (IRE1). Sig-1R promotes dimerization of IRE1, eventually leading to splicing and activation of x-box binding protein 1 (XBP1) and upregulation of a multitude of ER chaperone proteins (Mori et al., 2013). Furthermore, the Sig-1R has been implicated in cocaine-induced transcriptional regulation through its interaction with emerin, a nuclear envelope protein, and through its involvement in recruiting chromatin-remodelling factors such as histone deacetylases (Tsai et al., 2015). Knock-down (KD) Sig-1R hippocampal neuronal cell cultures showed altered transcription of genes involved in ubiquitination, oxidative stress, sterol biosynthesis, and modelling of actin (Tsai et al., 2012). Therefore implying a role of the Sig-1R in transcription of these genes.

The functions of the Sig-1R are broad and still not fully understood. In addition to its role in Ca^{2+} flux through IP3Rs, regulation of ion channels, and potential transcriptional

activity, the Sig-1R is also known to play a role in the ER, autophagy, and in apoptosis (Su et al., 2016). Its roles in these pathways are of particular interest as they may be involved in cell survival. However, more research is required to gain a better understanding of this protein.

1.1.5 Sig-1R involvement ER stress

The ER is a eukaryotic cellular organelle involved in protein production and folding, synthesis of lipids, and Ca^{2+} storage. When the cell experiences certain stresses such as increased protein secretion or accumulation of misfolded or mutated proteins, there is a conflict between the demand for protein production and the ability of the ER to properly fold proteins. This leads to a cellular state called ER stress. In order to detect and resolve ER stress the cell employs a group of signalling pathways collectively termed the unfolded protein response (UPR; Figure 2; reviewed in Lin et al., 2008). The UPR is mediated by three signalling branches initiated by three different proteins: IRE1, PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). Under physiological conditions these three proteins are bound to BiP. When ER stress occurs BiP is released and the UPR is activated (Kopp et al., 2019). Activation of the UPR through these three branches can promote cell survival, or lead to cell death under prolonged conditions of ER stress (Lin et al., 2008).

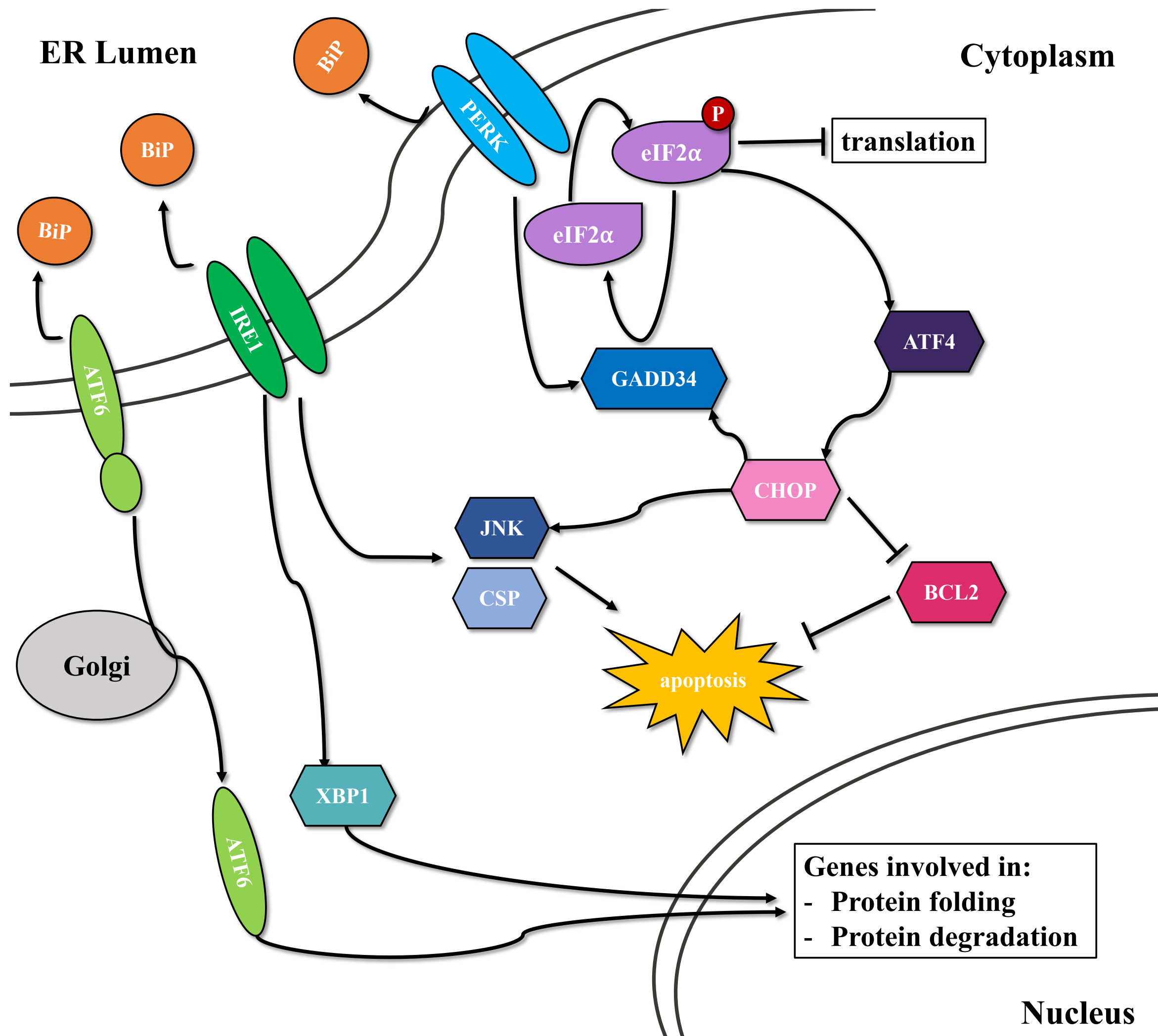


Figure 2. Unfolded protein response (UPR) pathways. The UPR is mediated by ATF6, IRE1, and PERK, which are found associated to BiP in resting conditions. Under conditions of endoplasmic reticulum (ER) stress ATF6 is cleaved in the Golgi apparatus and this cleaved ATF6 is translocated to the nucleus where it upregulates genes involved in protein folding and degradation. The IRE1 pathway is initiated when IRE1 dimerizes in response to ER stress and leads to increased expression of a spliced version of XBP1, which also acts to upregulate many of the same genes as ATF6. The IRE1 pathway also leads to upregulation of JNK and caspases (CSP) which both lead to apoptosis. The PERK pathway is activated when PERK dimerizes in response to ER stress and through its kinase activity it phosphorylates eIF2α leading to the inhibition of translation. Phosphorylated eIF2α also leads to upregulation of ATF4 and CHOP. CHOP primes cells for apoptosis by leading to increased JNK and by inhibiting the function of BCL2 which is generally anti-apoptotic. PERK activation also leads to upregulation of GADD34 which acts in a negative feedback loop and dephosphorylates eIF2α.

The IRE1 pathway is initiated when IRE1 dimerizes within the ER membrane in response to ER stress (Lin et al., 2008). Once dimerized, IRE1 acts from the ER membrane and splices XBP1 mRNA in the cytosol, and this spliced version of XBP1 acts as a transcription factor that upregulates proteins involved in ER protein-folding and degradation of misfolded proteins, thus improving the ability of the ER to properly fold proteins and removing the ER stressor (Calfon et al., 2002; Yoshida et al., 2001). IRE1 also has pro-apoptotic functions when activated. IRE1 activates c-Jun N-terminal kinase (JNK) which has been shown to trigger cell death (Urano et al., 2000). In addition, IRE1 activates caspases, which are a class of proteins involved in relaying the cell death signal or directly triggering apoptosis (Szegezdi et al., 2003).

ATF6 resides in the ER and has a stress-sensing domain in the ER lumen (Lin et al., 2008). In response to ER stress, ATF6 is trafficked from the ER to the Golgi, where its transmembrane domain is cleaved before it is sent to the nucleus (Ye et al., 2000). Once in the nucleus, it acts as a transcription factor to upregulate UPR target genes involved in protein-folding and degradation of misfolded proteins like those activated by XBP1 in the IRE1 pathway (Haze et al., 1999).

Induction of the PERK pathway begins when the ER-luminal domain of PERK senses ER stress and PERK dimerizes, activating its kinase function (Lin et al., 2008). PERK phosphorylates eukaryotic initiation factor 2α (eIF2α), a cofactor involved in assembly of the 80S subunit of the ribosome (Zhang et al., 1999). When eIF2α is phosphorylated it can no longer complete this role thus preventing ribosomal assembly, directly leading to reduced protein translation (Wek et al., 2006). In order to avoid complete shutdown of protein production, growth arrest and DNA damage-inducible protein (GADD34) is also activated

by the PERK pathway and it promotes dephosphorylation of eIF2α, therefore restoring the function of eIF2α in assembly of the ribosome (Chou and Roizman, 1994). Conversely, for certain mRNAs, activation of PERK increases their translation. Among these mRNAs is *atf4,* encoding activating transcription factor 4 (ATF4), which enhances transcription of ER chaperones leading to a reduction in ER stress (Harding et al., 2000a). ATF4 is also involved in pro-apoptotic signalling by upregulating C/EBP homologous protein (CHOP; Harding et al., 2000b). CHOP may increase apoptosis by increasing GADD34 expression and increasing oxidative stress (Marciniak et al., 2004; Zinszner et al., 1998).

The Sig-1R is thought to be involved in ER stress. Under periods of prolonged ER stress, Sig-1Rs are redistributed to other areas of the cell where it is thought that they may have generally protective effects (Su et al., 2016). The Sig-1R also has a direct involvement in the IRE1 pathway. The chaperone function of Sig-1Rs with IRE1 allows proper transmission of ER stress signals to the nucleus, and results in overall upregulation of anti-stress and anti-oxidant proteins (Mori et al., 2013).

The IRE1 and PERK branches of the UPR are especially interesting as they seem to be involved in the switch between pro-survival and pro-apoptotic signalling of the UPR. Recent studies have implicated the Sig-1R in promoting cell survival under ER stress by mediating CHOP, JNK, and caspase signalling (Zhao et al., 2019). When ER stress is induced by a variety of cell stressors (e.g. tunicamycin or thapsigargin), expression of the Sig-1R is drastically increased and this increase in expression is thought to occur through transcriptional upregulation, which is induced immediately in the acute phase of ER stress (Mitsuda et al., 2011). Furthermore, it was found that expression of the Sig-1R correlates with ATF4 expression, but not expression of ATF6 or XBP1, indicating that ATF4 is likely

a regulator of Sig-1R expression under ER stress (Mitsuda et al., 2011). In addition, ATF4 is found to bind directly to the 5' upstream region of the Sig-1R gene further implicating ATF4 in upregulation of the Sig-1R (Mitsuda et al., 2011). As ATF4 is a downstream element of the PERK pathway, it seems that the PERK pathway is the most involved in Sig-1R regulation during ER stress, and as such it is a good pathway for investigating Sig-1R function.

1.1.6 Sig-1R involvement in autophagy

In addition to the UPR, when ER stress occurs a second signalling mechanism is employed: ER-associated degradation (ERAD). ERAD, like the UPR, has multiple branches. ERAD can occur through ubiquitin-proteosome dependent ERAD or autophagy-lysosome dependent ERAD (reviewed in Song et al., 2018). Autophagy is a mechanism by which the cell attempts to reduce stress and conserve energy through lysis of aggregated, unfolded, and misfolded proteins, or organelles (Mizushima, 2007). Autophagy can be initiated by a variety of cellular stresses with ER stress among these. This form of autophagy has been termed ER stress-mediated autophagy. The UPR is directly involved in regulating ER stress-mediated autophagy.

As previously discussed, when ER stress occurs, IRE1 dimerizes and is phosphorylated, eventually leading to downstream activation of JNK (Figure 3; Gardner and Walter, 2011; Lin et al., 2008). JNK promotes autophagy by phosphorylating BCL2, releasing it from the BCL2/Beclin-1 complex, thereby allowing Beclin-1 to form a complex with vacuolar protein sorting 34 (Vps34). This allows for the production of isolation membranes, the precursor to autophagosomes which are organelles involved in autophagy

(Pattingre et al., 2005; Song et al., 2018). Furthermore, XBP-1 is involved in triggering autophagy through activation of Beclin-1 (Gardner and Walter, 2011; Zalckvar et al., 2009).

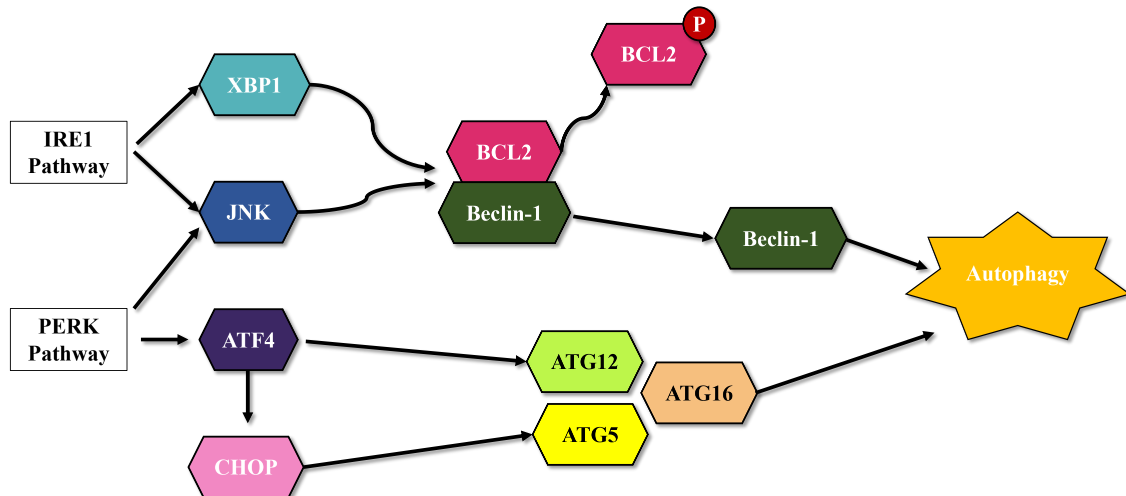


Figure 3. Pathways involved in autophagy. Autophagy can be triggered by activation of the PERK and IRE1 branches of the unfolded protein response (UPR). The IRE1 pathway leads to upregulation of spliced XBP1 and JNK. JNK promotes phosphorylation of BCL2 which releases it from its complex with Beclin-1. XBP1 is also involved in activating Beclin-1 and leads to increased autophagy. The PERK pathway also leads to upregulation of JNK and upregulation of ATF4. ATF4 transcriptionally upregulates ATG12 and CHOP. CHOP then upregulates ATG5. ATG12 and ATG5 form a complex with ATG16 and eventually lead to increased autophagy.

The PERK branch of the UPR is also involved in initiating ER stress-mediated autophagy. ATF4, when upregulated through activation of PERK, transcriptionally upregulates autophagy-related gene 12 (*atg12*) and transcriptionally induces autophagy-related gene 5 (*atg5*) through activation of CHOP (B'chir et al., 2013). ATG5 and ATG12 form a complex with autophagy related protein 16 (ATG16), and this complex is involved in formation of the autophagosome (B'chir et al., 2013; Kang et al., 2017).

Another important relationship between the ER and autophagy is through Ca^{2+} dynamics. The ER plays an important role in intracellular Ca^{2+} homeostasis, and during ER stress the ER releases Ca^{2+} into the cytoplasm through IP3Rs (Borodkina et al., 2016; Høyer-Hansen et al., 2007). This Ca^{2+} release through IP3Rs leads to activation of death-associated protein kinase (DAPK) which is involved in phosphorylation of Beclin-1 leading to its dissociation from BCL2, thereby inducing autophagy (Simon et al., 2016; Zalckvar et al., 2009). When ER Ca^{2+} is dysregulated or upon activation with a ligand, Sig-1Rs are found to prolong Ca^{2+} signalling to the mitochondria through IP3Rs (Hayashi and Su, 2007). Therefore, this is a potential mechanism through which the Sig-1R may be involved in increasing autophagy.

In general, autophagy is considered to be protective since it allows for degradation of cytoplasmic components and excess damaged proteins in order to conserve energy. Nevertheless, like in the case of the UPR, prolonged autophagy can lead to cell death (Mariño et al., 2014). The adaptive aspects of autophagy are strongly dependent on autophagic flux, which corresponds to the rate at which material is degraded through autophagy (Lumkwana et al., 2017). The association between Sig-1Rs and autophagy is a relatively new topic of study. Recently, it was shown that a mutant form of the Sig-1R led to an accumulation of autophagic components and reduced autophagy (Christ et al., 2019). Agonists of the Sig-1R are thought to increase autophagic flux (Christ et al., 2019). Correspondingly, in Sig-1R knock-out (KO) cells there was a partial blockage of the clearance of autophagosomes, preventing the process of autophagy (Yang et al., 2019). Autophagy is a process implicated in cell survival and apoptosis, and proteins implicated in regulation of autophagy, such as the Sig-1R may be important in promoting the anti-apoptotic functions of autophagy.

Therefore, elucidating the role of the Sig-1R in autophagy may be a significant step towards uncovering the protective roles of the Sig-1R.

1.1.7 Sig-1R involvement in apoptosis

Apoptosis can be triggered by a variety of cellular events including ER stress. When ER stress is prolonged and cannot be resolved, apoptosis can be triggered by components downstream in the ER stress pathways (Schröder and Kaufman, 2005). Activation of the UPR through PERK, IRE1, and ATF6 does not directly cause apoptosis. Rather, it is through secondary effectors and the downstream components like CHOP, JNK, and BCL2 that lead to apoptosis (Szegezdi et al., 2006).

Each of the branches of the UPR have differing but overlapping effects on the process of apoptosis (Figure 2). The PERK pathway generally acts as an anti-apoptotic signalling pathway through activation of ATF4, which induces genes involved in amino acid metabolism, redox reactions, stress response, and protein secretion (Harding et al., 2003). However, ATF4 also increases the expression of CHOP and GADD34, which are known pro-apoptotic proteins (Szegezdi et al., 2006). The use of CHOP KO mice allows for elucidation of the role of CHOP in ER stress-induced apoptosis. Mouse embryonic fibroblasts made from CHOP KO mice showed a partial resistance to apoptosis triggered by ER stress (Zinszner et al., 1998). The pro-apoptotic effects of CHOP may occur through *gadd34,* which is a target gene of CHOP, and expression of GADD34 has been shown to enhance or initiate apoptosis (Adler et al., 1999).

IRE1 activation may also play an important role in ER stress-induced apoptosis in a multitude of ways. One of these pathways is through JNK which is activated by a cascade of

events triggered by IRE1 activation (Nishitoh et al., 1998; Szegezdi et al., 2006). JNK phosphorylates BCL2 which suppresses its natural anti-apoptotic function (Davis, 2000). Overexpression of BCL2 blocks ER stress-induced apoptosis by preventing release of cytochrome *c,* a protein that is important in initiating apoptosis (Häcki et al., 2000). However, similar to PERK, IRE1 activation also results in anti-apoptotic signalling including increased expression of ER chaperone proteins that improve the ability of the ER to properly fold proteins (Szegezdi et al., 2006). The interplay between the pro-apoptotic and anti-apoptotic signalling pathways by IRE1 and PERK are complex and highly regulated, and ultimately lead to either cell death or cell survival in situations of ER stress. The final stages of ER stress-induced apoptosis all occur by the cumulative activation of caspases downstream of the aforementioned pathways such as regulation of BCL2 and JNK (Szegezdi et al., 2006). Processing of several caspases has been observed in ER stress, and overall caspase activation is what ultimately leads to the regulated break down of the cell.

Multiple studies have demonstrated a protective role of the Sig-1R against apoptosis. In a human lens cell line (FHL124) silencing of the Sig-1R with siRNA led to an increase in activated caspases, indicating that these cells were more primed for apoptosis than WT cells (Wang and Duncan, 2006). Similarly, in MIN6 cells, silencing of the Sig-1R led to an increase in CHOP expression and a higher rate of apoptosis following induction of ER stress (Ke et al., 2021). In general, agonists of the Sig-1R have been shown to protect cells against apoptosis. SKF10,047, a Sig-1R agonist, was found to reduce apoptosis in prostate cancer cells, possibly by preventing cleavage of caspases (Das et al., 2016). (+)-Pentazocine, has also been shown to reduce apoptosis in microglia following oxidative stress (Heiss et al.,

2016). The mechanism behind the protective action of the Sig-1R against apoptosis, and the pathways through which it may exert these effects have yet to be investigated.

The wide range of functions and diverse protein interactions of the Sig-1R have led many researchers to conclude that it may be involved in human diseases (Su et al., 2016). In particular, the Sig-1R may have important implications in diseases of the central nervous system (CNS). Its role in protecting against ER stress, oxidative stress, and apoptosis in general has been extensively studied in relation to a variety of neurodegenerative diseases. However, research is still needed to elucidate its potential neuroprotective effects and the mechanism behind these.

1.1.8 Sig-1R involvement in neurodegenerative diseases

The Sig-1R is expressed in many tissues throughout the body including the pancreas, heart, liver, lungs, and brain. In the brain, Sig-1Rs are found in high concentration in the brainstem motor nuclei, cranial nerves, cerebellum, basal ganglia, and hippocampus, suggesting they may play a role in motor function, learning, and memory (Matsumoto et al., 2006). The role of the Sig-1R in the nervous system has been extensively studied as it has been found to be implicated in mood disorders, schizophrenia, and many neurodegenerative diseases (Nguyen et al., 2015).

Agonists of the Sig-1R have been shown to be neuroprotective in multiple neurological disorders. In experimental stroke models, activation of the Sig-1R with an agonist reduced infarct volume and improved functional recovery, and these effects were blocked by simultaneously administering a Sig-1R antagonist (Ruscher and Wieloch, 2015; Shen et al., 2008; Takahashi et al., 1997). In models of ALS and PD, treatment with Sig-1R

agonists reduced the progressive loss of motor neurons and dopaminergic neurons respectively (Francardo et al., 2014; Mancuso et al., 2012). In addition, activation of Sig-1Rs with an agonist led to a reduction in neuroinflammation, a contributor to disease pathogenesis in PD (Francardo et al., 2014). Sig-1Rs are also found to be downregulated in the brains of early-stage PD, indicating that their loss may play a role in the disease progression (Mishina et al., 2005). One of the most promising applications of Sig-1R agonists is for treatment of patients with AD. In the last 10 years, clinical trials have focused on using Sig-1R agonists in this context (Lisak et al., 2020).

1.2 Alzheimer's Disease

1.2.1 Hallmarks

AD and other related dementias are the most common neurodegenerative diseases worldwide, currently affecting more than 44 million people. The clinical characterization of AD is a gradual decline of many cognitive functions eventually resulting in incapacitation of patients who require complete care in the later stages of the disease. There is currently no cure for AD and only a few treatments that modestly reduce symptoms (Citron, 2004; Yiannopoulou and Papageorgiou, 2020). Acetylcholinesterase inhibitors and NMDAR antagonists are currently the most used treatments for improving cognitive function in patients, however, they do not have any effect on slowing disease progression (Citron, 2004; Yiannopoulou and Papageorgiou, 2020).

There are several common neurological characteristics of AD patients. They all show gradual neuronal cell and synapse loss particularly in the hippocampus, cerebral cortex, and

basal ganglia, and show accumulation of two hallmark lesions, amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs) which are composed of hyperphosphorylated tau protein (Blessed et al., 1968). Aβ peptides are cleaved from amyloid precursor protein (APP) and are thought to be the main cause of neurotoxicity in AD (Selkoe, 1991). Correspondingly, NFTs are also likely contributors to neurodegeneration. In patients with FTD, the second most-common form of dementia, there are signs of NFTs without any Aβ accumulation (Cairns et al., 2007). Progression of Aβ plaque accumulation and NFT formation are correlated with disease progression in AD, and loss of synapses is one of the earliest events associated with cognitive decline (Braak and Braak, 1995; Selkoe, 2002). Aβ is usually secreted as an Aβ1-40 or Aβ1-42 amino acid peptide, and synthetic versions of both peptides are often used for *in vitro* experiments (Perez et al., 1999). Hardy and Higgins (1992), were the first to propose the Aβ cascade hypothesis which states that Aβ is the primary toxic species in AD. Since this hypothesis was proposed, there have been many studies which show that Aβ aggregations are toxic to neurons, and when accumulation of Aβ decreases, toxicity is diminished (Figure 4; Gilbert, 2013).

Figure 4. Amyloid cascade hypothesis. The amyloid cascade hypothesis suggests that amyloid-β is the primary toxic species that leads to the development of AD. Cleavage of amyloid-precursor protein (APP) is the first step which leads to formation of amyloid plaques composed of Aβ1-40 and Aβ1-42. These proteins were thought to lead to increased neurofibrillary tau tangles (NFTs), then eventual neuronal cell death and the development of Alzheimer's Disease (AD). Figure adapted from Karran et al. (2011) *Nature Reviews Drug Discovery*.

In addition to accumulation of Aβ plaques and NFTs, another feature of AD is cerebral inflammation. Evidence of an inflammatory response is seen in the brains of AD patients (Heneka et al., 2015). Microglia are phagocytes that are activated during the immune response, they have been shown to become activated and are observed around Aβ plaques (Serrano-Pozo et al., 2011; Wake et al., 2009). Their numbers also increase in response to damaged neurons attributed to accumulation of NFTs (Serrano-Pozo et al., 2011). In addition, reactive astrocytes are also found in close proximity to Aβ plaques, healthy astrocytes generally function in the neuroprotection of damaged neurons, however reactive astrocytes are thought to be neurotoxic (Steele and Robinson, 2012).

Early symptoms of AD include disorientation, loss of context, and impairment of memory (Small et al., 2011). Neuronal cell loss and loss of synapses are the features that most correlate with these cognitive deficits (Gómez-Isla et al., 1997). Loss of synapses often precedes neuronal cell loss, and this synaptic loss is thought to primarily be driven by deposition of Aβ plaques and NFTs (Overk and Masliah, 2014). There may also be a compensatory mechanism involved however, since remaining synapses tend to be larger and more robust (Serrano-Pozo et al., 2011). One of the regions of the brain that sees the most severe neuronal cell loss and synaptic changes is the hippocampus (Llorens-Martín et al., 2014). The hippocampus is a brain structure that plays a particularly important role in memory. The hippocampus is divided into different regions including the dentate gyrus (DG), CA1, CA2, CA3, and CA4 (Figure 5). Granule cells in the dentate gyrus project mossy fibres to the pyramidal cells of the CA3 which then connects to the CA1 through the schaffer-collateral pathway. AD results in subtle synaptic changes throughout the hippocampus, in

addition to neuronal cell death that are both correlated with the memory deficits seen (Setti et al., 2017).

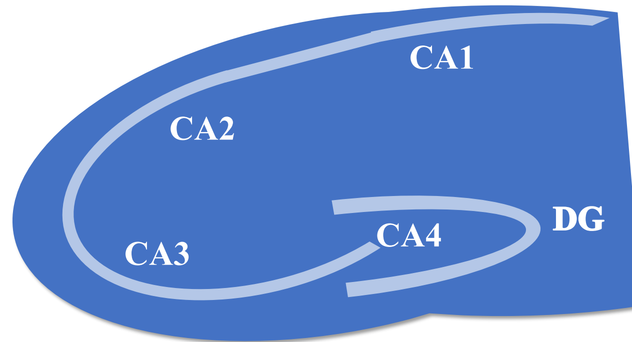


Figure 5. Regions of the hippocampus. The hippocampus is divided into five regions, the dentate gyrus (DG), CA1, CA2, CA3 and CA4.

AD can either be sporadic or familial. The vast majority of cases (>95%) are sporadic with no known underlying genetic causes. Age is the most significant risk factor in sporadic AD, but there is also increased risk with the presence of cardiovascular disease (de Oliveira et al., 2014; Philbert et al., 2021; Roher et al., 2003). Familial AD is less common and there are genetic mutations associated. Risk of developing sporadic and familial AD can be increased by certain environmental risk factors including diet (e.g. high meat and fat ingestion, low vegetable ingestion), physical activity (high physical activity reduces risk), sleep (chronic loss of sleep associated with higher risk), and education level (higher education level is correlated with lower incidence of AD) (reviewed in Brewer, 2018). In familial AD, mutations have been identified in the genes encoding APP, presenilin-1 (PS1), and presenilin-2 (PS2; reviewed in Bertram and Tanzi, 2005). APP, as named, is the precursor to Aβ, and is cleaved by γ-secretase to become Aβ1-42, which is the full-length Aβ protein that is most present in Aβ plaques (Knowles et al., 2014). The APP mutation seen

in familial AD causes an increase in the cleavage of APP into Aβ1-42 (Citron et al., 1992). PS1 and PS2 encode components of γ-secretase and the mutations in these two proteins that are seen in familial AD also lead to an increase in cleavage of APP into Aβ1-42 (Veugelen et al., 2016). Other than the differences in origin of Aβ accumulation and age of onset between familial versus sporadic AD the two diseases are essentially phenotypically identical in terms of how they progress.

1.2.2 ER stress, autophagy, and apoptosis in AD

Evidence of ER stress is seen in post-mortem brain samples of AD patients, animal models, and *in vitro* models (Ho et al., 2012; Katayama et al., 2001). Some of this evidence includes enhanced ER chaperone expression and immunohistochemical reactivity for markers of UPR activity (Unterberger et al., 2006). In addition, phosphorylation of PERK and eIF2α are increased in the neurons of patients with AD compared to healthy controls (Hoozemans et al., 2005). NFTs and Aβ have both been implicated in the occurrence of ER stress, and reciprocally ER stress may also regulate the pathways that produce Aβ (Jung et al., 2015). There appears to be a link between Aβ and tau phosphorylation, whereby *in vitro* studies have found that Aβ induces ER stress which correlates with tau phosphorylation (Resende et al., 2008). Activation of the UPR is thought to lead to tau phosphorylation, and neurons with activated PERK protein are expressed in AD brains (Hoozemans et al., 2009). Many studies have shown that PERK and eIF2α phosphorylation is increased in cells treated with Aβ providing evidence for ER stress, and this has been seen in cell lines, primary neuronal cultures, and organotypic slices (Katayama et al., 2004). Treatment of cells with Aβ leads to the activation of caspase-12 which is a known marker of ER stress and correlates with

apoptosis (Costa et al., 2012a). However, toxicity of Aβ is dependent on whether it is in the soluble oligomer or insoluble fibril form. Recent research has shown that soluble Aβ oligomers, as opposed to the Aβ fibrils that compose plaques are the toxic Aβ species (Tamagno et al., 2006). These oligomers may have the ability to enter cells and cause ER stress (Oddo et al., 2007).

Though it is widely accepted that ER stress is present in neurons of patients with AD, less is known about how ER stress contributes to pathogenesis. Studies analyzing the implication of PERK in AD models demonstrate multiple roles. PERK activation occurs relatively early in the development of AD and before the formation of NFTs, however activation PERK increases following the formation of NFTs (Hoozemans et al., 2009). PERK activation may play a role in neuronal plasticity and cognition. Protein translation controlled by eIF2α phosphorylation is known to negatively regulate synaptic plasticity as well as learning and memory, which may contribute to some of the symptoms seen in AD (Costa-Mattioli et al., 2009). Furthermore, a common feature of an AD brain is neuronal cell loss. It has been suggested that ER stress-induced apoptosis is involved in the neuronal cell loss that occurs in AD (Katayama et al., 2004). The induction of ER stress by Aβ oligomers induces CHOP expression, a downstream marker of the PERK pathway, which leads to neuronal degeneration (Figure 2; Katayama et al., 2004). However, the implications of PERK activation are somewhat controversial as certain studies have found PERK to be neuroprotective against the effects of Aβ (Lee et al., 2010). In different models of AD, inhibition of the PERK pathway has been found to be both protective and deleterious (Devi and Ohno, 2014; Radford et al., 2015; Yang et al., 2015). In general the UPR is an adaptive response that promotes cell survival, though it can promote cell death when it is prolonged

(Schröder and Kaufman, 2005). The question of whether ER stress in response to Aβ is beneficial or detrimental is still under investigation, but could be an important piece in determining effective treatments for AD.

There is also extensive evidence of abnormal autophagy in the AD brain (Nixon et al., 2005). In general, a certain amount of autophagy is neuroprotective since it allows for clearance of misfolded proteins and conservation of energy (Nixon and Yang, 2011). The disruption of proper autophagic processes can be detrimental in protein misfolding disorders. Autophagic vacuoles are shown to accumulate particularly in regions where there are Aβ plaques and synapse loss (Nixon et al., 2005). This indicates that in these regions of the brain there is an abundance of misfolded/undigested proteins. In healthy brains, autophagic vacuoles are uncommon, and accumulation of these tend to only occur during excessive autophagy or if there is an inability of the cell to clear autophagic vacuoles indicating an overall reduction in autophagic flux (Mizushima et al., 2008). In the case of AD there seems to be dysfunction in the process of clearance of autophagic vacuoles by lysosomes, which leads to the accumulation of autophagic vacuoles (Nixon and Yang, 2011). Restoring the proper function of the autophagic pathway could be an important step in protecting neuronal cells from degeneration.

In AD, apoptosis plays a pivotal role in neuronal cell degeneration (Obulesu and Lakshmi, 2014). There are significantly higher levels of caspases in neurons of AD brains compared to healthy controls, indicating increased levels of apoptosis (Sun et al., 2011). Since neuronal cell loss is a feature of AD, understanding how and why apoptosis occurs in AD may provide more options for treatments. There is some evidence showing that Aβ could be involved in neuronal apoptosis. Aβ has been shown to induce neuronal apoptosis in AD,

however the exact mechanism is still unknown (Simon et al., 2011). It is likely a combination of pathways that eventually lead to apoptosis, and among these could be ER stress-induced apoptosis. A pathway for reducing apoptosis may be important in the context of AD in order to spare neuronal cells from death. Since the Sig-1R shows overall anti-apoptotic effects, this may be a potential avenue to be studied for the purpose of preventing neuronal cell death in AD.

1.2.3 Sig-1R as a therapeutic target for AD

Considering the overlap between Sig-1R function in ER stress, autophagy, and apoptosis, and the progression of neurodegeneration in AD, it is clear that the Sig-1R is a potential target for remedying the dysfunction seen in these pathways due to AD. An early study involved testing several Sig-1R agonists ((+)-pentazocine, PRE-084, and cutamesine) on mice injected with the Aβ fragment Aβ25-35. Aβ25-35 toxicity was induced only 1 week post-injection. The animals were then injected with the Sig-1R agonists, and underwent behaviour testing. All the Sig-1R agonists reduced the impairments observed in this behaviour testing due to Aβ25-35 peptide, and this effect was blocked with the co-treatment of haloperidol, a Sig-1R antagonist (Maurice et al., 1998). Further *in vivo* studies demonstrated that Sig-1R agonists PRE-084, ANAVEX2-73, and ANAVEX1-41 all had neuroprotective effects in pharmacological models of AD (Maurice et al., 2001; Villard et al., 2009, 2011). Markers of oxidative stress, neuroinflammation, apoptosis, and cell loss were improved following treatment with these Sig-1R agonists. Furthermore, these treatments were shown to decrease tau hyperphosphorylation, indicating that Sig-1R agonists

could not only improve symptoms, but also reduce the extent of the hallmark protein aggregates.

As previously mentioned, ANAVEX2-73 is currently undergoing a phase 2b/3 trial with 450 patients enrolled (www.anavex.com). It has been shown to decrease the phosphorylation effects of Aβ on tau protein (Lahmy et al., 2013). This agonist binds both Sig-1R and muscarinic receptors, and pre-clinical studies showed that ANAVEX2-73 in combination with donepezil (an acetylcholinesterase inhibitor) had a neuroprotective effect (Maurice, 2016). This combination drug is now undergoing a clinical trial, and so far has proven to reduce memory loss and neuronal loss by 80% compared to either drug on its own (Maurice, 2016).

Though there is significant evidence that Sig-1R activation protects against behavioural deficits and apoptosis *in vivo* and *in vitro* models of AD, there is still little known about the cellular mechanisms underlying these effects. As clinical trials using Sig-1R agonists as AD treatments progress, it is important that these mechanisms are better understood. Overall, the Sig-1R may be a promising novel therapeutic target for AD based on its involvement in the UPR, oxidative stress, Ca^{2+} -signalling, and apoptosis. Investigating the role Sig-1Rs may play in reducing ER stress, autophagy, and apoptosis induced by Aβ will provide important information for current and future clinical studies investigating the Sig-1R as a treatment option for AD.

1.3 Hypothesis and Objectives

HYPOTHESIS

Aβ induces ER stress, autophagy, and apoptosis *in vitro* and *ex vivo* and this effect can be rescued by activation of the Sig-1R *via* treatment with (+)-pentazocine, a Sig-1R agonist.

OBJECTIVES

1. Determine whether Aβ leads to increased ER stress, autophagy, and apoptosis *in vitro* and whether these can be alleviated by activation of the Sig-1R with (+)-pentazocine.

2. Determine whether Aβ leads to increased ER stress, autophagy, and tissue death *ex vivo* and whether these can be alleviated by activation of the Sig-1R with (+)-pentazocine.

2. MATERIALS AND METHODS

2.1 Study models

2.1.1 In vitro model

In vitro experiments were performed using *SH-SY5Y* cells, a human derived neuron-like cell line. These cells were subcloned from the *SK-N-SH* cell line, which was isolated from a patient with neuroblastoma (Biedler et al., 1973). *SH-SY5Y* cells were used in this study due to their neuronal characteristics. These cells were grown in Dulbecco's modified Eagle medium / Ham's F12 50:50 mix (DMEM:F12 Wisent Inc.), containing 10% filtered fetal bovine serum, 100 U/mL penicillin/streptomycin, and 2mM L-glutamine in a humidified 37°C, 5% CO_2 incubator. Cells are passaged when they reach ~80% confluency, approximately twice weekly. Cells were briefly washed with phosphate buffered saline (PBS; 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 137 mM NaCl, and 2.68 mM KCl; pH 7.2) then separated from culture dishes by trypsination (0.05% trypsin; Gibco/Invitrogen).

2.1.2 Ex vivo model

All experiments involving animals were approved by uOttawa Animal Care and Veterinary Services. *Ex vivo* experiments were performed using organotypic hippocampal slice cultures (OHSCs) produced from WT and KO B6.129-Sigmar1tm1Lmon mice generated in the uOttawa transgenic core facility. Slices were cultured in Neurobasal A medium (Thermofisher), containing 1X B27 (Thermofisher), 10% Horse Serum (Gibco), 10mM HEPES (Thermofisher), 25mM D-glucose (Thermofisher), 100 U/mL penicillin/

streptomycin (Invitrogen), and 2mM L-glutamine (Thermofisher) in a humidified 34ºC, 5% CO_2 incubator. Culture media is changed the day following plating, then every 2-3 days after that. Slices were cultured for 10 days before doing any pharmacological treatments. Organotypic slices generally last several weeks, allowing for long-term pharmacological treatments. They also maintain structural and synaptic organization of the tissue, thus providing a model more comparable to an *in vivo* organism than single-cell colonies.

2.1.3 Organotypic preparation

Mice aged P7-P9 were sacrificed by rapid decapitation and the brain was placed immediately in ice-cold choline cutting solution containing 110mM choline Cl, 12mM Na^+-ascorabte, 7mM $MgSO_4 \cdot 7H_2O$, 2.5mM KCl, 1.25 NaH_2PO_4, 26 mM NaH_2CO_3, 0.5 mM $CaCl_2$, and 10mM glucose (bubbled with 95/5% carbogen for 30 min. and frozen in -80°C). Under sterile conditions, the hippocampi from both hemispheres are quickly dissected in icy choline cutting solution and sliced into 300μm slices using an MX-TS Tissue Slicer (Automate Scientific). The intact full hippocampi slices were then transferred to a pre-incubated 6-well plate and placed on top of biopore membranes on 0.4μm culture inserts (Millipore). Each culture insert contained 3-5 slices and 1mL of the neurobasal culture media was added per well underneath the insert.

2.2 Drugs and solutions

2.2.1 Aβ preparation

Aβ1-42 (Aβ) and Aβ42-1 (Aβ R) aggregated soluble oligomers were used for treatments. These proteins were supplied in solid form (beta-amyloid peptide 1-42 ab120301; beta-amyloid peptide 42-1 ab120481; Abcam). 1mg of each peptide was dissolved in 1mL of 1,1,1,3,3,3-hexafluoro-2-propanol (Millipore Sigma) and incubated at room temperature with gentle rocking for 24 hours, this allows for the formation of oligomers but not fibrils. After 24 hours the 1,1,1,3,3,3-hexafluoro-2-propanol was allowed to evaporate, and the leftover solid was dissolved in 110.74μL of DMSO to achieve a concentration of 2mM Aβ. This solution was aliquoted in 10μL quantities and stored at -80°C until used. Aliquots were thawed when needed and 90μL of DPBS was added (Thermo Fisher Scientific). The aliquots were then incubated at 37°C for 24 hours before being added to culture media.

2.2.2 (+)-Pentazocine preparation

5mg of (+)-pentazocine (Sigma-Aldrich) was dissolved in 350 μL of warmed 0.1N HCl and mixed thoroughly. 1.4mL of PBS was added to the solution then is aliquoted and stored at -20°C for up to one month.

2.3 Treatments

2.3.1 SH-SY5Y treatments

SH-SY5Y cells underwent the treatments outlined in Table 1. Both WT Sig-1R and KD Sig-1R cells were used in each treatment. Cells were plated in 6-well culture dishes at an approximate cell density of 0.2 x 10^6 cells /mL. 24 hours following plating, siRNA for the human *Sig-1R* (Dharmacon; J-017475-06-0002) was transfected using DharmaFECT 1 transfection reagent following manufacturer's instructions (Dharmacon). 24 hours following siRNA transfection, KD Sig-1R and WT Sig-1R cells were treated with combinations of Aβ, Aβ R, and (+)-pentazocine as outlined in Table 1. Aβ and Aβ R were added directly to the cell culture media at a concentration of 100nM. (+)-Pentazocine was added simultaneously at a concentration of 9μM. Cells were then placed back into the incubator at 37°C and incubated for 24 hours before being collected for experiments.

Table 1. Treatments of *SH-SY5Y* cells and organotypic hippocampal slice cultures (OHSCs). (+)-Pentazocine (PTZ), Aβ1-42 (Aβ), and Aβ42-1 (Aβ R) are added to culture media of cells and OHSCs for 24 hours at specified concentrations before collection for western blot, qPCR, flow cytometry, and imaging experiments.

Study Model	**Genotype**	**Control**	**Aβ**	**Aβ R**
SH-SY5Y	WT Sig-1R	± PTZ (9μM)	Aβ (100nM) ± PTZ (9μM)	Aβ R (100nM) ± PTZ (9μM)
SH-SY5Y	KD Sig-1R	± PTZ (9μM)	Aβ (100nM) ± PTZ (9μM)	Aβ R (100nM) ± PTZ (9μM)
OHSCs	WT Sig-1R	± PTZ (9μM)	Aβ (10μM) ± PTZ (9μM)	Aβ R (10μM) ± PTZ (9μM)
OHSCs	KO Sig-1R	± PTZ (9μM)	Aβ (10μM) ± PTZ (9μM)	Aβ R (10μM) ± PTZ (9μM)

2.3.2 Organotypic hippocampal slice cultures (OHSC) treatments

Organotypic hippocampal slice cultures (OHSC) underwent the treatments outlined in Table 1 after 10 days *in vitro*. Both WT Sig-1R and KO Sig-1R cultures were used in each treatment. Aβ and Aβ R were added directly to the culture media at a concentration of 10μM, and (+)-pentazocine was added at the same time at a concentration of 9μM. OHSCs were then placed back in the incubator at 34°C and incubated for 24 hours before being collected for experiments.

2.4 Western blotting

Following treatments for 24 hours, cells and OHSCs were lysed on ice with 200μL of radioimmunoprecipitation buffer (RIPA: 150 mM NaCl, 50 mM Tris, 0.5% Na deoxycholate, 0.1% NP-40, 5 mM Na pyrophosphate, 2 mM β-glycerophosphate, 1×EDTA-free protease inhibitor (Fisher Scientific), pH 7.5). Samples were sonicated for 15 seconds at 20%, and centrifuged at 14,000 rcf for 10 min. The supernatant was collected and total protein concentration was quantified by DC protein assay. Samples were then diluted to a concentration of 1.5mg/mL and stored at -80°C until use.

Protein was resolved on a Tris-glycine SDS-PAGE (20μg of protein per lane), then transferred onto a nitrocellulose membrane. After transfer, blots were blocked in 5% skim milk/ Tris-buffered saline (TBS; 50mM Tris-Cl, 150mM NaCl, pH 7.5) for 1 hour at room temperature with gentle rocking. The membranes were incubated in primary antibody overnight in 5% skim milk/ TBS - Tween 20 solution (TBS-T; 50mM Tris-Cl, 150mM NaCl, 0.05% Tween 20 (Fisher Scientific), pH 7.5). The primary antibodies used were β-actin HRP-

conjugated antibody (1:10,000, GenScript; Table 2), and Sigma-1R antibody (1:1000, Atlas Antibodies; Table 2). After incubation with primary antibodies, the membranes were washed with TBS-T and incubated in secondary HRP-conjugated rabbit antibody (1:15000; Jackson ImmunoResearch) for 1 hour at room temperature. Membranes were washed again, then developed with Luminata Forte and imaged using LI-COR Odyssey Fc (LI-COR, Lincoln, Nebraska USA). Sig-1R band intensities were normalized to β-actin using ImageJ.

Table 2. Antibodies used for western blots, flow cytometry, and imaging of *SH-SY5Y* cells and organotypic hippocampal slice cultures (OHSCs).

Experiment (Study Model)	**Antibody/stain name**	**Company (Cat. No.)**	**Dilution**
Western Blot (*SH-SY5Y* / OHSCs)	β-actin	GenScript (A00702-100)	1:10,000
Western Blot (*SH-SY5Y* / OHSCs)	Sig-1R	Atlas Antibodies (HPA018002)	1:1000
Flow Cytometry/Imaging (*SH-SY5Y*)	Annexin-V	Thermofisher (A13199)	1:200
Flow Cytometry/Imaging (*SH-SY5Y*)	PI	Thermofisher (V13242)	0.1μg/mL
Imaging (*SH-SY5Y*)	Hoechst	Thermofisher (62249)	1:1000
Imaging (OHSCs)	PI	Thermofisher (V13242)	2μg/mL
Imaging (OHSCs)	DAPI	Sigma Aldrich (D9542)	2ng/mL

2.5 Quantitative polymerase chain reaction (qPCR)

Following treatments for 24 hours, cells and OHSCs were collected with 1mL of TRIzol (Thermofisher) and RNA was isolated according to manufacturer's instructions. RNA concentration was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Burlington, ON, Canada). Reverse transcription was performed on 2μg of RNA per reaction using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA samples were stored at 4°C until use.

The rotor-gene SYBR Green PCR kit (Qiagen, 204074) was used for qPCR experiments. cDNA samples were run in triplicates on a Rotor-Gene Q real-time PCR system (Qiagen) using the following thermocycler program: 5 min. at 95°C, 40 cycles of 95°C for 5 seconds followed by 60°C for 10 seconds, then a melting cycle from 60°C to 95°C with a 1°C temperature rise each step with a 90 second pre-melt conditioning on the first step and 5 seconds for each step after. Primers used are outlined in Table 3. Each sample was run with β-actin as the housekeeping gene for normalization. Results of qPCR were fitted to standard curves for each gene before analysis.

Table 3. Primer sequences for qPCR.

Gene	Species	Sequence (5'-3')	
		Forward	Reverse
β-actin	human	GCGGGAAATCGTGCGTGACATT	GATGGAGTTGAAGGTAGTTTCGTG
β-actin	mouse	CGCAGCCACTGTCGAGTC	GTCATCCATGGCGAACTGGT
CHOP	human	ACCTGAGGAGAGAGTGTTCAA	GGCTGGAACAAGCTCCATGT
CHOP	mouse	CCTGAGGAGAGAGTGTTCCAG	ACACCGTCTCCAAGGTGAAAG
GADD	mouse	TCTAAAAGCTCGGAAGGTACAC	GGCTTCGATCTCGTGCAAAC
ATG5	human	GCAACTCTGGATGGGATTGC	TCCTAGTGTGTGCAACTGTCC
ATG5	mouse	TCACAGTACATTTCAAGAGTTTTCC	ACTGGTCAAATCTGTCATTCTGC
ATG12	mouse	TGGCCTCGGAACAGTTGTTA	TTCCCACAGCACCGAAATGT

2.6 Flow Cytometry

Following treatments for 24 hours, floating and adherent *SH-SY5Y* cells were collected by trypsination and resuspended in 1mL of Annexin-V binding buffer (Thermofisher). Propidium iodide (PI; 0.2ug/mL; Thermofisher) and FITC Annexin-V conjugate (1:200;

Thermofisher) are both added to each cell sample. Annexin-V is a commonly used apoptosis stain, however it also stains dead cells. To account for this PI, a dead cell stain, is used in combination. Therefore the cells that are positively stained for Annexin-V and negatively stained for PI are deemed to be apoptotic. To allow for proper gating for analysis, unstained, PI only, and Annexin V only samples were prepared as well. Cells were incubated at room temperature for 15 min. 5μL of 123count eBeads (Thermofisher) were added to each sample before samples were brought to BD LSR Fortessa Flow Cytometer (BD Biosciences) at the Flow Cytometry facility in uOttawa. Fluorescence emission is measured at 530nm and >575nm. The percentage of apoptotic cells was then determined by identifying the cells stained

2.7 Imaging

2.7.1 SH-SY5Y live cell imaging

For imaging experiments, cells were plated on 8-well μ-slides (ibidi) before undergoing treatments. Following 24-hour treatments, cell media was replaced with Annexin-V binding buffer (Thermofisher). PI (0.2μg/mL; Thermofisher), FITC Annexin-V conjugate (1:200; Thermofisher), and Hoechst (20μM; Thermofisher) were added to cells. Cells were incubated with the stains for 15 min. at room temperature before imaging with a confocal fluorescent microscope at 40X magnification (Zeiss LSM 800) .

2.7.2 OHSCs imaging

Following 24-hour treatments of OHSCs, slices were stained with 2 μg/mL PI in serum-free Neurobasal A medium for 30 min. at 33.5ºC. OHSCs were then collected and fixed in 4% Paraformaldehyde (PFA) for 30 min. at 4ºC, then washed 3 times in PBS with DAPI (2ng/mL; Sigma-Aldrich) added for 20 min. in the second PBS wash. OHSCs were then mounted on slides with Fluoromount G (Southern Biotech) and coverslipped, then set to dry for at least 24 hours. OHSCs were imaged with a confocal fluorescent microscope at 20X magnification (Zeiss LSM 800).

2.8 Analysis and statistics

One-way analysis of variance (ANOVAs) were performed using the software "R" 3.5.3 (R Core Team, 2019) between control, Aβ, and Aβ + (+)-pentazocine groups for each experiment. When significant differences were detected ($p<0.05$), Tukey HSD with Bonferroni correction for multiple comparisons was done as a post-hoc analysis.

3. RESULTS

3.1 Expression of the Sig-1R does not change following 24 hour treatment with (+)-pentazocine or Aβ

Studies have shown that over an acute period of ER stress, expression of the Sig-1R is increased (Mitsuda et al., 2011). Tunicamycin induced ER stress over a period of 15-120 min. and led to upregulation of the Sig-1R, particularly through activation of the PERK pathway (Mitsuda et al., 2011). However, in the case of AD, disease progression and accumulation of Aβ in the brain occurs over many years. As such, it is important to study a longer treatment model. Furthermore, in the brains and post-mortem tissues of AD patients there is actually a decrease in expression of the Sig-1R (Mishina et al., 2008). For these reasons, the objective of this current study was to determine whether a longer treatment of Aβ would alter the expression of the Sig-1R in both an *in vitro* and *ex vivo* model. Following a 24-hour treatment with 100nM Aβ/Aβ R (*SH-SY5Y*) or 10μM Aβ/Aβ R (OHSCs), the protein expression of the Sig-1R was assessed by western blot.

A representative western blot showing Sig-1R expression following Aβ treatments is shown in Figure 6A and 6B. Knock-down of the Sig-1R in *SH-SY5Y* with siRNA for the human Sig-1R gene lead to a decrease of Sig-1R expression to approximately 30% of the WT levels. Treatment of WT/KD Sig-1R *SH-SY5Y* cells or WT Sig-1R OHSCs with Aβ or Aβ R resulted in no significant change in expression of the Sig-1R compared to the control group (Figure 6C,D).

In addition, certain studies have shown that some Sig-1R agonists increase its expression. For example, fluvoxamine, a potent Sig-1R agonist, has been shown to alleviate ER stress through upregulation of the Sig-1R after 12 and 24 hour treatments (Omi et al.,

2014). Hence, the expression of the Sig-1R in response to treatment with (+)-pentazocine with and without Aβ was assessed *in vitro* and *ex vivo*. Following a 24 hour treatment of *SH-SY5Y* cells and OHSCs with (+)-pentazocine alone, or Aβ and (+)-pentazocine, the protein expression of the Sig-1R was once again assessed by western blot.

A representative western blot showing Sig-1R expression following (+)-pentazocine treatments is shown in Figure 6A and 6B. Treatment of *SH-SY5Y* cells or OHSCs with (+)-pentazocine, or Aβ and (+)-pentazocine, resulted in no significant change in expression of the Sig-1R in comparison to the control group (Figure 6C,D).

These results indicate that when *SH-SY5Y* cells and OHSCs are treated with Aβ or (+)-pentazocine, there is no change in expression of the Sig-1R. Therefore, any changes observed from Aβ or (+)-pentazocine treatments are likely not due to changes in expression of the Sig-1R.

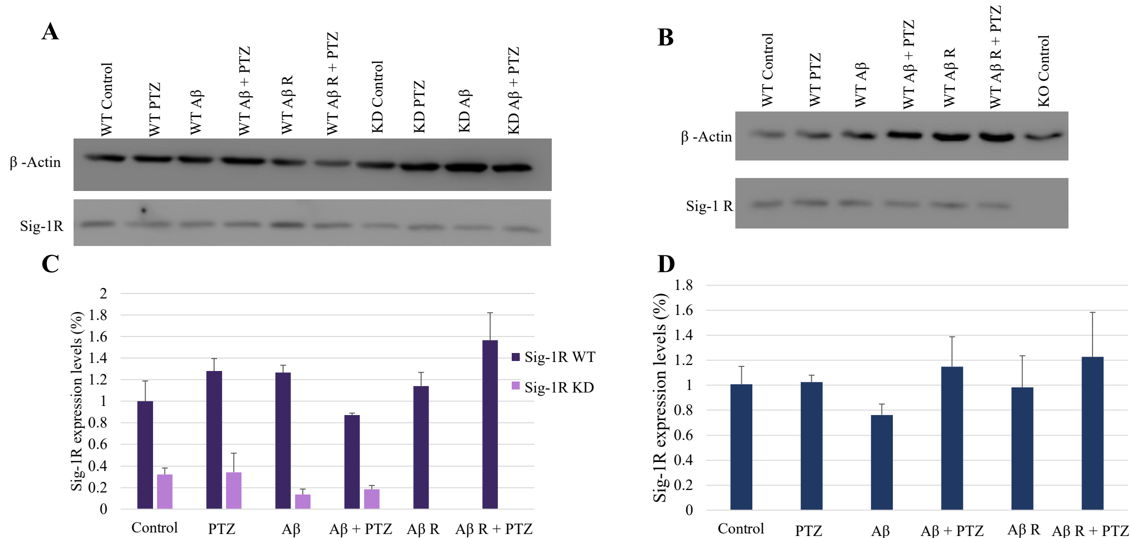


Figure 6. Sigma-1 receptor (Sig-1R) expression in response to amyloid-β 1-42 (Aβ) and (+)-pentazocine (PTZ). WT / KD Sig-1R *SH-SY5Y* cells and WT / KO Sig-1R organotypic hippocampal slice cultures (OHSCs) were treated with Aβ, Aβ 42-1(Aβ R) and PTZ for 24 hours.

(A) Representative western blots for β-actin and the Sig-1R in WT Sig-1R and KD Sig-1R cells.

(B) Representative western blots for β-actin and the Sig-1R in WT Sig-1R and KO Sig-1R OHSCs.

(C) Quantification of Sig-1R expression normalized to β-actin then to control in WT Sig-1R (dark purple) and KD Sig-1R (light purple) cells (n=4).

(D) Quantification of Sig-1R expression normalized to β-actin then to control in WT Sig-1R and KO Sig-1R OHSCs (n=4).

Error bars represent mean +SEM.

3.2 Activation of the Sig-1R reduces transcription of markers for ER stress and autophagy following Aβ treatment

3.2.1 Activation of the Sig-1R reduces transcription of ER stress markers

Activation of the Sig-1R reduces markers of ER stress in situations of acute ER stress induced by known ER stressors (Hyrskyluoto et al., 2013; Omi et al., 2014). In addition, it

is well-known that Aβ induces ER stress (Costa et al., 2012b; Hitomi et al., 2004; Resende et al., 2008). As of yet there is little research to show whether activation of the Sig-1R can reduce ER stress induced by Aβ specifically. This is an important distinction because ER stressors such as tunicamycin and thapsigargin do not perfectly mimic the effects of accumulation of unfolded proteins such as Aβ. For example, tunicamycin blocks a step of glycoprotein biosynthesis in the ER causing accumulation of unfolded glycoproteins (Oslowski and Urano, 2011). This is an inherently different mechanism from aggregation of Aβ1-42 peptides as a result of cleavage of APP.

In the context of Sig-1R activation as a potential treatment for AD, it is important to study the effects of Sig-1R activation in reducing ER stress induced by Aβ oligomers. It is therefore important to determine whether ER stress markers upregulated by Aβ oligomers could be downregulated following activation of the Sig-1R with (+)-pentazocine in *SH-SY5Y* cells and OHSCs. For those reasons, this study investigated whether transcripts levels of the ER stress markers *chop* (*SH-SY5Y* and OHSCs) and *gadd34* (OHSCs) were altered following Aβ, Aβ R and (+)-pentazocine treatments.

Treatment of WT/KD Sig-1R *SH-SY5Y* and WT/KO Sig-1R OHSCs with Aβ1-42 oligomers led to a significant upregulation of *chop,* and an upregulation of *gadd34* transcripts in OHSCs in comparison to the control group (Figure 7A,B; Figure 8A). Additionally, the mRNA transcript levels of *chop* and *gadd34* were significantly lower in WT Sig-1R *SH-SY5Y* and WT Sig-1R OHSCs treated with a combination of Aβ and (+)-pentazocine in comparison to the Aβ treatment alone (Figure 7A,B; Figure 8A). However, there was no significant difference in transcript levels of *chop* and *gadd34* in KD Sig-1R *SH-SY5Y* and

KO Sig-1R OHSCs treated with Aβ and (+)-pentazocine in comparison to the Aβ treatment alone (Figure 7A,B; Figure 8A).

These results indicate that ER stress occurs when WT/KD Sig-1R *SH-SY5Y* cells and WT/KO Sig-1R OHSCs are treated with Aβ oligomers. Furthermore, activation of the Sig-1R with (+)-pentazocine appears to protect WT Sig-1R *SH-SY5Y* cells and WT Sig-1R OHSCs from ER stress induced by Aβ. This result also suggests that activation of the Sig-1R may protect WT Sig-1R *SH-SY5Y* cells and WT Sig-1R OHSCs from ER stress-induced apoptosis based on lower expression of *chop,* which is also a marker for early apoptosis. Since there was no effect of (+)-pentazocine on KD Sig-1R *SH-SY5Y* groups and KO Sig-1R OHSCs, the reduction of ER stress markers seen in the WT groups treated with Aβ and (+)-pentazocine is likely due to activation of the Sig-1R.

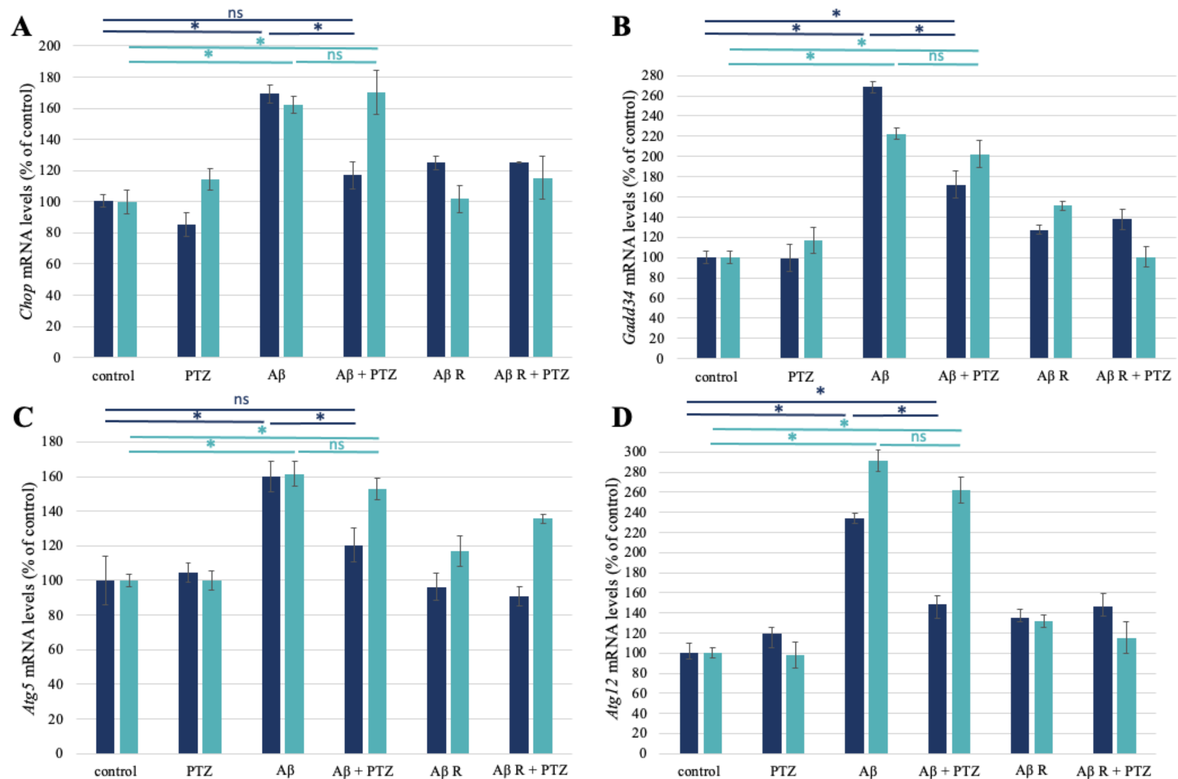


Figure 7. Endoplasmic reticulum stress and autophagy gene expression in organotypic hippocampal slice cultures (OHSCs) in response to amyloid-β 1-42 (Aβ) and (+)-pentazocine (PTZ). WT / KO Sig-1R organotypic hippocampal slice cultures (OHSCs) were treated with Aβ, Aβ 42-1(Aβ R) and PTZ for 24 hours.

(A) Quantification of *chop* mRNA levels normalized to β-actin then to control (n=5) in WT Sig-1R OHSCs (dark blue), and KO Sig-1R OHSCs (light blue).

(B) Quantification of *gadd34* mRNA levels normalized to β-actin then to control (n=4) in WT Sig-1R OHSCs (dark blue), and KO Sig-1R OHSCs (light blue).

(C) Quantification of *atg5* mRNA levels normalized to β-actin then to control (n=4) in WT Sig-1R OHSCs (dark blue), and KO Sig-1R OHSCs (light blue).

(D) Quantification of *atg12* mRNA levels normalized to β-actin then to control (n=4) in WT Sig-1R OHSCs (dark blue), and KO Sig-1R OHSCs (light blue).

Error bars represent mean ±SEM. *p<0.05 Tukey HSD test with Bonferroni correction

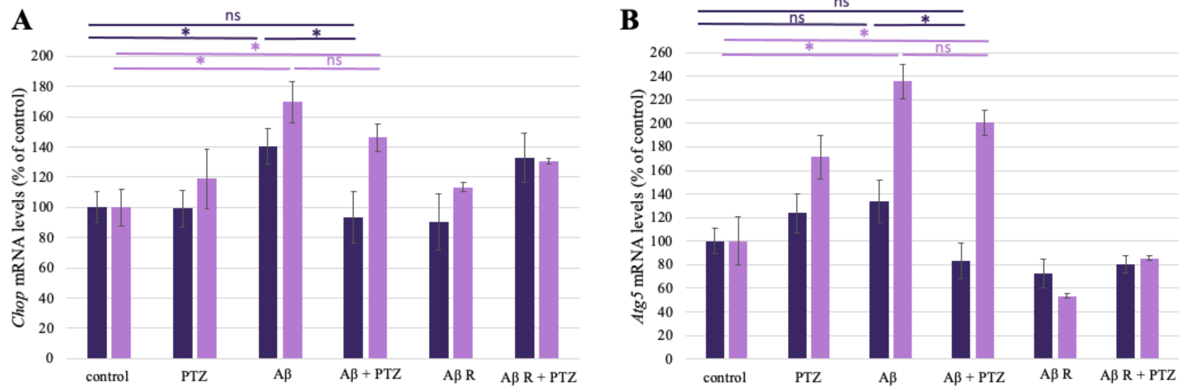


Figure 8. Endoplasmic reticulum (ER) stress and autophagy gene expression in *SH-SY5Y* cells in response to amyloid-β 1-42 (Aβ) and (+)-pentazocine (PTZ). WT / KD Sig-1R *SH-SY5Y* cells were treated with Aβ, Aβ 42-1(Aβ R) and PTZ for 24 hours.

(A) Quantification of *chop* mRNA levels normalized to β-actin then to control (n=5) in WT (dark purple), and KD (light purple) SH-SY5Y cells.

(B) Quantification of *atg5* mRNA levels normalized to β-actin then to control (n=4) in WT (dark purple), and KD (light purple) SH-SY5Y cells.

(C) Error bars represent mean ±SEM. *p<0.05 Tukey HSD test with Bonferroni correction.

3.2.2 Activation of the Sig-1R reduces transcription of autophagy markers

This study evaluated whether treatment of WT/KD Sig-1R *SH-SY5Y* and WT/KO Sig-1R OHSCs with Aβ oligomers ± (+)-pentazocine would lead to increases in transcript levels of autophagy markers. Following treatments outlined in Table 1, mRNA expression of autophagy markers was assessed using qPCR. The autophagy markers used were *atg5* (*SH-SY5Y* cells and OHSCs) and *atg12* (OHSCs). Treatment of KD Sig-1R *SH-SY5Y* cells and WT/KO Sig-1R OHSCs with Aβ oligomers led to a significant upregulation of *atg5*, and an upregulation of *atg12* in OHSCs compared to the control group (Figure 7C,D; Figure 8B). However, in WT Sig-1R *SH-SY5Y* cells there was no significant upregulation in *atg5* transcript levels (Figure 8B). The mRNA transcript levels of *atg5* and *atg12* were significantly lower in WT Sig-1R *SH-SY5Y* and WT Sig-1R OHSCs treated with a combination of Aβ and (+)-pentazocine in comparison to the Aβ treatment alone (Figure

7C,D; Figure 8B). There was no significant difference in transcript levels of *atg5* and *atg12* in KD Sig-1R *SH-SY5Y* and KO Sig-1R OHSCs treated with Aβ oligomers and (+)-pentazocine in comparison to the Aβ treatment alone (Figure 7C,D; Figure 8B).

These results indicate that autophagy may be triggered when WT/KD Sig-1R *SH-SY5Y* cells and WT/KO Sig-1R OHSCs are treated with Aβ oligomers. Furthermore, activation of the Sig-1R with (+)-pentazocine seems to reduce autophagy towards baseline levels in WT Sig-1R *SH-SY5Y* cells and WT Sig-1R OHSCs treated with Aβ. This result suggests that activation of the Sig-1R may protect WT Sig-1R *SH-SY5Y* cells and WT Sig-1R OHSCs from excessive stress-induced autophagy. Additionally, since there was no effect of (+)-pentazocine on the KD Sig-1R *SH-SY5Y* cells and KO Sig-1R OHSCs the reduction of autophagy markers seen in the WT groups treated with Aβ1-42 and (+)-pentazocine is likely mediated by the Sig-1R and not another mechanism of (+)-pentazocine activity.

3.3 Activation of the Sig-1R reduces apoptotic *SH-SY5Y* cells following Aβ treatment

One of the more troubling features of AD is the progressive neuronal cell death, which could exacerbate many of the overall cognitive dysfunctions seen in patients with this disease (Gómez-Isla et al., 1997; Llorens-Martín et al., 2014). Many theories hypothesize the potential causes for this neuronal cell death, one of them being the amyloid cascade hypothesis, which states that accumulation of Aβ plaques are the initiators of AD pathogenesis and lead to neurodegeneration (Figure 4; Hardy and Higgins, 1992). It has been well-established in cell culture models that Aβ leads to apoptosis and cell death (Gschwind

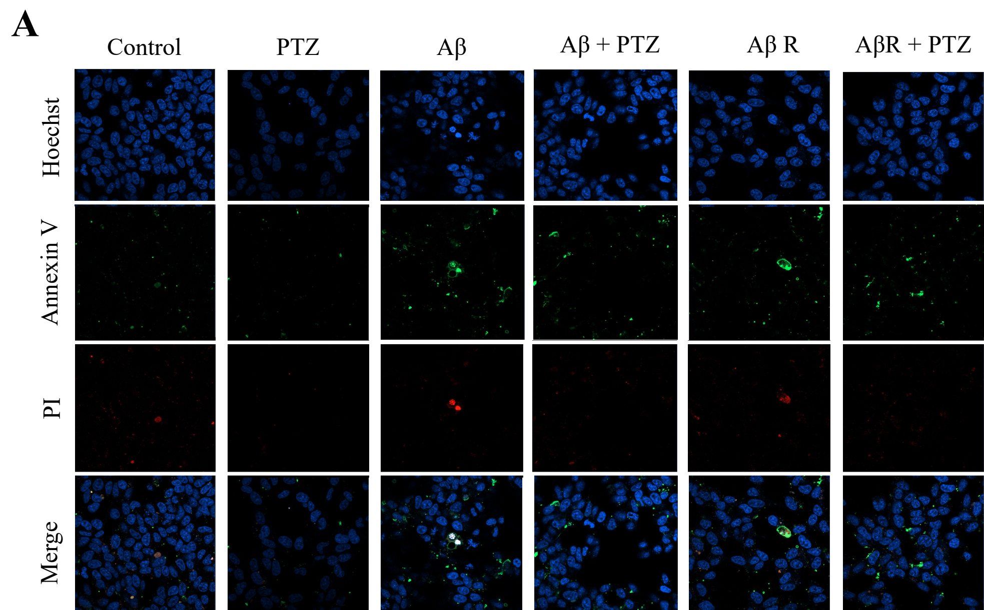
and Huber, 1995; Loo et al., 1993). Therefore, it is important to investigate potential mechanisms for reducing this cell death.

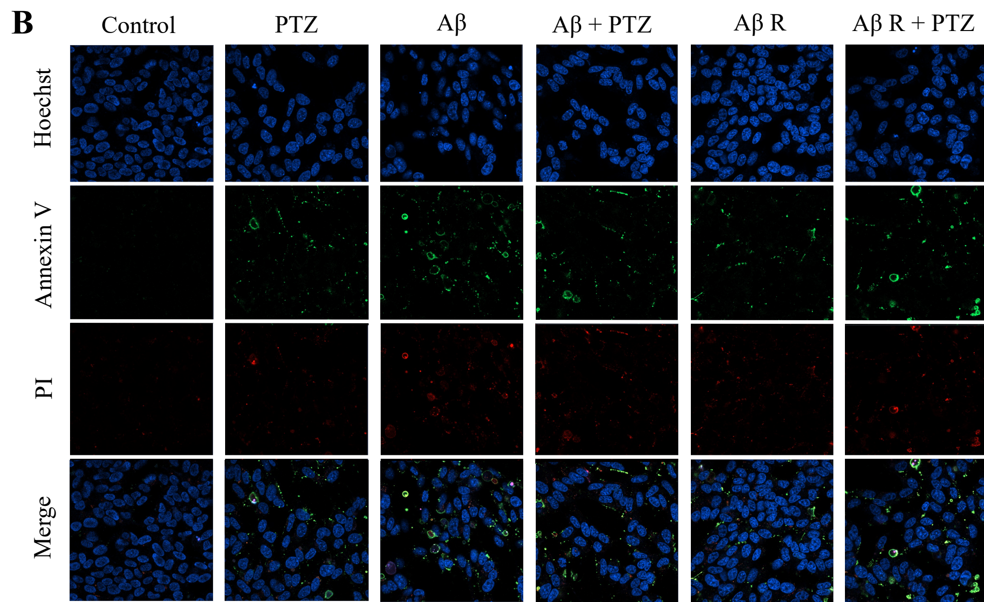
Activation of the Sig-1R with the agonist PRE-084 following a short term application of Aβ25-35 treatments was shown to improve neuronal cell survival (Marrazzo et al., 2005). The aim here was to confirm these results with our current study model. WT/KD Sig-1R *SH-SY5Y* cells were treated with Aβ and the percentage of apoptotic cells was assessed with or without (+)-pentazocine treatment. Annexin-V and PI were used to stain apoptotic cells, and flow cytometry was used as a method for counting cells that were positively stained.

Representative images of stained WT/KD Sig-1R *SH-SY5Y* cells are shown in Figure 9A,B. Treatment of WT/KD Sig-1R *SH-SY5Y* cells with Aβ oligomers led to a significant increase in apoptotic cells compared to the control group (Figure 9C,D). Additionally, the percentage of apoptotic cells were significantly lower in WT Sig-1R *SH-SY5Y* cells treated with a combination of Aβ and (+)-pentazocine in comparison to the Aβ treatment alone (Figure 9C,D). However, there was no significant difference in the percentage of apoptotic cells in KD Sig-1R *SH-SY5Y* and KO Sig-1R OHSCs treated with Aβ and (+)-pentazocine compared to Aβ treatment alone (Figure 9C,D). The results from flow cytometry were confirmed through imaging analysis (Figure A-1).

These results indicate that apoptosis occurs in WT and KD Sig-1R *SH-SY5Y* cells treated with Aβ. Furthermore, activation of the Sig-1R with (+)-pentazocine reduces this apoptosis in WT Sig-1R *SH-SY5Y* cells treated with Aβ. This result suggests that activation of the Sig-1R may protect WT Sig-1R *SH-SY5Y* cells from apoptosis induced by Aβ. Since there was no effect of (+)-pentazocine on the KD Sig-1R *SH-SY5Y* cells, the reduction of

apoptotic cells seen in the WT cells treated with Aβ and (+)-pentazocine confirmed that (+)-pentazocine is likely acting through activation of the Sig-1R.


A
Control
PTZ
Aβ
Aβ + PTZ
Aβ R
AβR + PTZ
Hoechst
Annexin V
PI
Merge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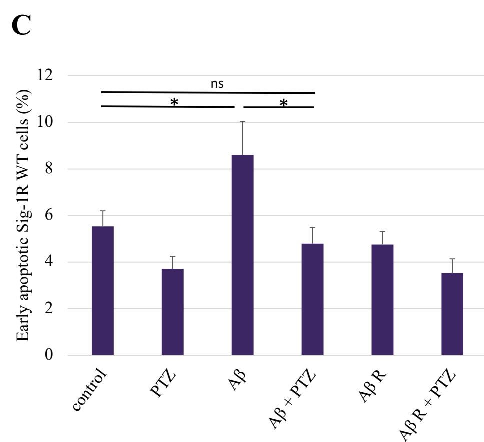
Control
PTZ
Aβ
Aβ + PTZ
Aβ R
Aβ R + PTZ
Hoechst
Annexin V
PI
Merge


C
Early apoptotic Sig-1R WT cells (%)
ns
*
*
control
PTZ
Aβ
Aβ + PTZ
Aβ R
Aβ R + PTZ

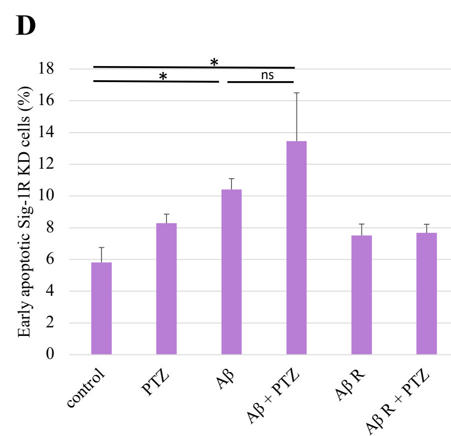

D
Early apoptotic Sig-1R KD cells (%)
*
*
ns
control
PTZ
Aβ
Aβ + PTZ
Aβ R
Aβ R + PTZ

Figure 9. Percentage of apoptotic *SH-SY5Y* cells in response to amyloid-β 1-42 (Aβ) and (+)-pentazocine (PTZ). WT / KD Sig-1R *SH-SY5Y* cells were treated with Aβ, Aβ 42-1(Aβ R) and PTZ for 24 hours.

(A) Representative confocal images of WT Sig-1R *SH-SY5Y* cells taken at 40X magnification stained with Hoechst (blue), Annexin-V (green), and PI (red)
(B) Representative confocal images of KD Sig-1R *SH-SY5Y* cells taken at 40X magnification stained with Hoechst (blue), Annexin-V (green), and PI (red)
(C) Quantification of the percentage of apoptotic WT Sig-1R cells by flow cytometry (n=5)
(D) Quantification of the percentage of apoptotic KD Sig-1R cells by flow cytometry (n=4)

Error bars represent mean +SEM. *p<0.05 Tukey HSD test with Bonferroni correction

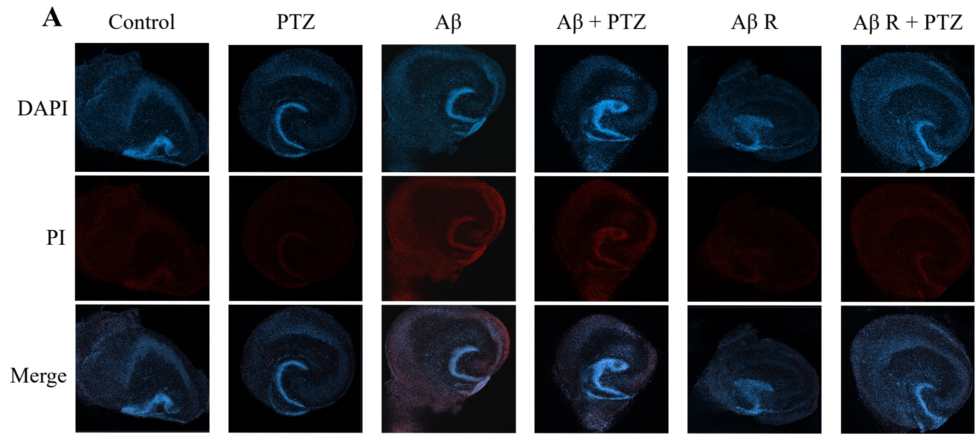
3.4 Activation of the Sig-1R reduces tissue death in the CA3 region of the hippocampus following Aβ treatment

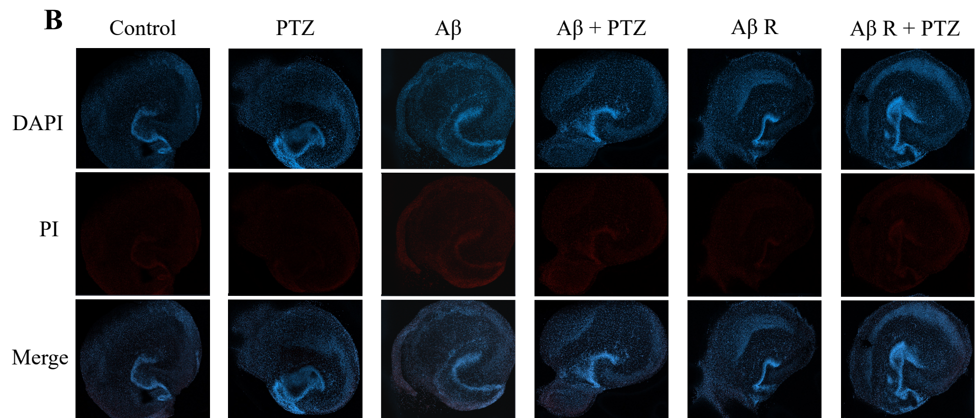
With memory deficits being one of the primary symptoms of AD, studying the hippocampus could provide important information about the disease progression. In patients with AD, the hippocampus is one of the brain regions shown to be the most vulnerable to damage (Braak et al., 1993). OHSCs therefore provide a useful model for studying tissue death in the hippocampus. A recent study has shown that Aβ leads to tissue death in OHSCs (Arbo et al., 2017). However, there are no studies yet investigating the ability of Sig-1R agonists to reduce this tissue death. This study investigates whether treatment of WT Sig-1R and KO Sig-1R OHSCs with Aβ would increase tissue death in the dentate gyrus (DG) and CA3 regions of the hippocampus, and if this tissue death could be decreased by treatment with (+)-pentazocine. Propidium iodide (PI) was used to stain dead tissue, and PI fluorescence was measured using confocal imaging.

Treatment of WT Sig-1R and KO Sig-1R OHSCs with Aβ resulted in a significant increase in PI fluorescence in both the DG and CA3 in comparison to the no treatment control group (Figure 10C-F). Interestingly, the CA3 of WT Sig-1R OHSCs showed a decrease in

PI fluorescence following treatment with Aβ and (+)-pentazocine in comparison to the Aβ only treatment (Figure 10C), however there was no decrease in PI fluorescence in the DG region of WT Sig-1R OHSCs (Figure 10D). In KO Sig-1R OHSCs, there was no significant difference in PI fluorescence when slices were treated with Aβ and (+)-pentazocine in comparison to Aβ only treatment in either the DG or CA3 regions (Figure 10E,F).

These results indicate that tissue death occurs in the DG and CA3 regions of WT and KO Sig-1R OHSCs treated with Aβ. Furthermore, activation of the Sig-1R with (+)-pentazocine reduces this tissue death in the CA3 region of WT Sig-1R OHSCs treated with Aβ, however, there was no reduction in tissue death in the DG. This result suggests that activation of the Sig-1R may protect against tissue death induced by Aβ in the CA3. Since there was no effect of (+)-pentazocine on the KO Sig-1R OHSCs, the reduction of tissue death seen in the WT Sig-1R OHSCs treated with Aβ and (+)-pentazocine is likely due to activation of the Sig-1R.


A
Control
PTZ
Aβ
Aβ + PTZ
Aβ R
Aβ R + PTZ
DAPI
PI
Merge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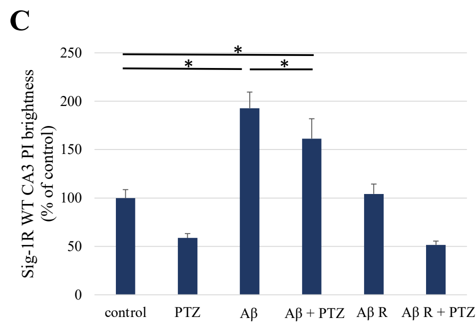
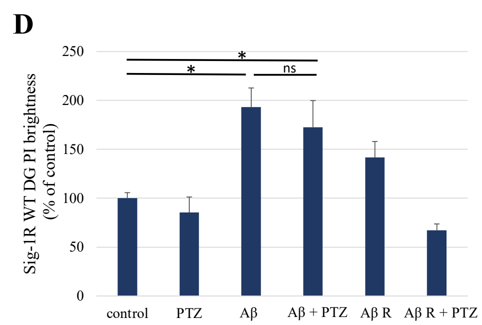
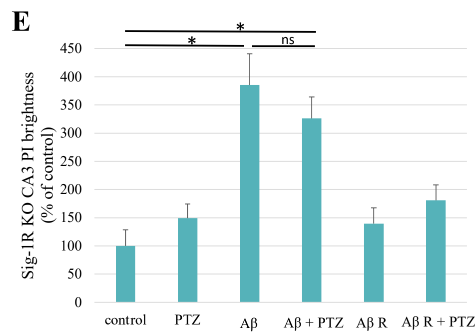
PTZ
Aβ
Aβ + PTZ
Aβ R
Aβ R + PTZ
DAPI
PI
Merge


C
Sig-1R WT CA3 PI brightness
(% of control)
250
200
150
100
50
0
*
*
*
control
PTZ
Aβ
Aβ + PTZ
Aβ R
Aβ R + PTZ


D
Sig-1R WT DG PI brightness
(% of control)
250
200
150
100
50
0
*
*
ns
control
PTZ
Aβ
Aβ + PTZ
Aβ R
Aβ R + PTZ


E
Sig-1R KO CA3 PI brightness
(% of control)
450
400
350
300
250
200
150
100
50
0
*
*
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PTZ
Aβ
Aβ + PTZ
Aβ R
Aβ R + PTZ

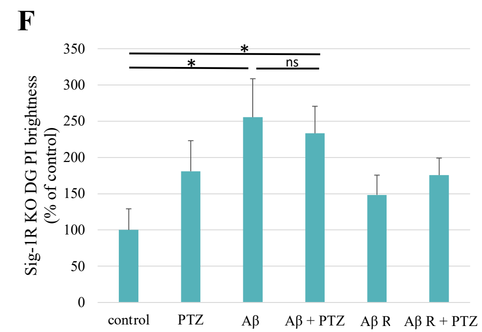
F
Sig-1R KO DG PI brightness
(% of control)
350
300
250
200
150
100
50
0
*
*
ns
control
PTZ
Aβ
Aβ + PTZ
Aβ R
Aβ R + PTZ

Figure 10. Tissue death in the dentate gyrus (DG) and CA3 regions in response to amyloid-β 1-42 (Aβ) and (+)-pentazocine (PTZ). WT / KO Sig-1R organotypic hippocampal slice cultures (OHSCs) were treated with Aβ, Aβ 42-1(Aβ R) and PTZ for 24 hours.

(A) Representative confocal images of WT Sig-1R OHSCs taken at 20X magnification stained with DAPI (blue), and PI (red)

(B) Representative confocal images of KO Sig-1R OHSCs taken at 20X magnification stained with DAPI (blue), and PI (red)

(D) Quantification of PI fluorescence in the CA3 of WT Sig-1R OHSCs normalized to control (n=5)

(E) Quantification of PI fluorescence in the DG of WT Sig-1R OHSCs normalized to control (n=5)

(F) Quantification of PI fluorescence in the CA3 of KO Sig-1R OHSCs normalized to control (n=5)

(G) Quantification of PI fluorescence in the DG of KO Sig-1R OHSCs normalized to control (n=5)

Error bars represent mean +SEM. *$p<0.05$ Tukey HSD test with Bonferroni correction

4. DISCUSSION

The overall aim of this thesis was to investigate how activation of the Sig-1R may be protective against the toxic effects of Aβ, and to identify the potential pathways through which Sig-1Rs exerts their effects. This study investigated whether activation of the Sig-1R with (+)-pentazocine would lead to reduced neuronal cell death following treatment with Aβ *in vitro* and *ex vivo*. Based on previous literature, activation of the Sig-1R is thought to protect against ER stress, promote autophagy, and generally be anti-apoptotic. Therefore, expression of genes related to ER stress and autophagy, as well as markers for apoptosis, and tissue death were studied to uncover the mechanisms of Sig-1R protection against Aβ. This study is the first to demonstrate the protective effects of Sig-1R activation and its ability to reduce ER stress, autophagy, apoptosis, and tissue death in response to Aβ.

4.1 How do Aβ and Sig-1R agonist treatment impact Sig-1R expression?

Expression of the Sig-1R is decreased in patients with AD, and mutations in the Sig-1R are shown to increase risk of AD (Fehér et al., 2012; Mishina et al., 2008). As mentioned, in instances of acute ER stress, such as treatments with ER stressors like DTT, tunicamycin and thapsigargin, there is significant upregulation of the Sig-1R (Mishina et al., 2008). In AD, progression of Aβ plaque build-up in the brain is slow and chronic. Therefore, a less intense, longer period of ER stress would provide a better idea of long-term effects on expression of the Sig-1R. In addition, studying the effects of Aβ specifically would provide more information as to whether the reduced expression of the Sig-1R in patients with AD is a direct result of Aβ plaques. Understanding the relationship between Aβ and Sig-1R expression is

especially important in the context of using Sig-1R agonists as treatments, because if Sig-1Rs are reduced in patients with AD they may not be the most effective target for treatment.

Here, it was found that there was no change in Sig-1R expression in response to a 24-hour Aβ treatment. This result is contrary to studies showing increased expression of Sig-1R following ER stress (Mishina et al., 2008). However it is possible that acutely there may be an upregulation of the Sig-1R in response to Aβ but by 24 hours this upregulation subsides. This also indicates that the reduction in Sig-1R expression in the brains of AD patients may not be a direct result of Aβ plaque formation, though this could be a longer-term result. Hayashi and Su (2007) also noted that the overexpression of Sig-1R is what allows the Sig-1R to regulate UPR signalling. Overexpression of the Sig-1R and activation of the Sig-1R with various agonists have similar effects (Aydar et al., 2002; Hayashi and Su, 2003; Mavlyutov and Ruoho, 2007). Therefore it's expected that treatment with (+)-pentazocine could allow for promoting the protective effects of the Sig-1R even though it does not lead to an overexpression of the Sig-1R.

It was also found that there was no change in Sig-1R expression in response to treatment with (+)-pentazocine. This result differs from that of a study investigating mutant huntingtin-expressing cells (the primary protein aggregate of Huntington's disease) that found that Sig-1R levels were decreased in these cells but that Sig-1R levels could be restored by the Sig-1R agonist PRE-084 (Hyrskyluoto et al., 2013). However, in general, research on Sig-1R agonists indicate that they do not lead to an overexpression of the Sig-1R, but instead may have the ability to recover downregulation (Cerveró et al., 2018; Hyrskyluoto et al., 2013; Shimazawa et al., 2015).

Future research should assess both shorter and longer-term impacts of Aβ treatment on Sig-1R expression levels in order to provide a better idea of the overall progression of Sig-1R expression throughout a chronic period of Aβ accumulation. Accordingly, future research could investigate expression levels of Sig-1R in response to different Sig-1R agonists at different concentrations, thereby providing a better indication of how different agonists could function in reducing toxicity caused by Aβ. Agonists shown to upregulate Sig-1Rs could be especially useful in mediating the reduced expression of Sig-1Rs in patients with AD (Fehér et al., 2012).

4.2 How does the Sig-1R reduce ER stress induced by Aβ?

AD brains display many indications of ER stress, mainly through the activation of the UPR, possibly as a result of aggregation of Aβ peptides (Hoozemans et al., 2005). Activation of the UPR is normally an adaptive response, however it can lead to cell death when it's prolonged. It has been shown that Aβ oligomers are more toxic to cells when the UPR is primed, indicating that activation of the UPR may be an important element of Aβ toxicity (Chafekar et al., 2007). Studies have shown that activation of the Sig-1R can lead to reduction of ER stress markers (Mitsuda et al., 2011; Omi et al., 2014). However, the ability of the Sig-1R to reduce ER stress imposed by Aβ has not yet been studied, and the mechanism behind its protective effects in this context remains undefined.

Here, it was found that there is an upregulation of the ER stress markers CHOP and GADD34 in response to treatment with Aβ oligomers. In the case of CHOP, expression in both *SH-SY5Y* cells and OHSCs was increased by approximately 70% when treated with Aβ for 24 hours. Expression of GADD34 in OHSCs saw an even more drastic increase of about

170% in the Aβ treated group in comparison to the no treatment control group. These results are indicative of activation of the UPR, particularly the PERK pathway. These results are in accordance with other studies that have shown that Aβ accumulation activates the UPR (Hoozemans et al., 2005, 2009). Furthermore, it was found that activation of the Sig-1R with (+)-pentazocine lead to a reduction in the expression of CHOP and GADD34 when it was added simultaneously with Aβ. Generally, chronic activation of the UPR can lead to apoptosis and in these cases inhibition of the UPR has protective effects (Colla et al., 2012; Lindholm et al., 2017; Ma et al., 2013). For these reasons, activation of the Sig-1R may have the potential to reduce Aβ toxicity that may occur as a result of overactivation of the UPR.

Interestingly, in this study, Aβ was added extracellularly to the culture media in order to mimic the extracellular aggregation of Aβ plaques that are seen in the brains of AD patients. Activation of the UPR is thought to occur when BiP is attracted to misfolded intracellular proteins causing it to dissociate from the three main ER stress-regulating proteins PERK, IRE1, and ATF6 (Oikawa et al., 2009). Studies have shown that Aβ oligomers are able to be internalized through receptors such as the NMDAR (Oddo et al., 2007). The Sig-1R modulates NMDARs, therefore there may be a relationship between the Sig-1R and the ability of Aβ to enter the cell (Martina et al., 2007).

Future research studying Aβ aggregation could be completed by using the cell-permeable fluorescent dye thioflavin, which binds to protein aggregates with β-pleated sheets such as Aβ and is commonly used for detection of Aβ aggregation *in vivo* and *in vitro* (Sundaram et al., 2018). This could provide an idea as to whether extracellular Aβ oligomers enter the cell, and if activation of the Sig-1R leads to a reduction in intracellular Aβ oligomers. It may also be interesting to determine whether pre-treatment with (+)-

pentazocine or another Sig-1R agonist before any addition of Aβ could prevent any increase in ER stress markers. This information could allow for considering Sig-1R activation as a preventative treatment for those more at risk of developing AD.

4.3 How does the Sig-1R impact autophagy induced by Aβ?

Dysfunction in autophagy in disease states often leads to reduced turnover of autophagy substrates. In a healthy cell there is a consistent flux of autophagy that is maintained (Wong and Cuervo, 2010). In the brains of AD patients there is a strong presence of various types of autophagic vacuoles that mainly represent intermediate stages of autophagy (Nixon et al., 2005). This suggests that autophagy is stalled in AD. Targeting autophagy has been proposed as a potential mechanism for reducing Aβ toxicity (Gruendler et al., 2020). Agonists of the Sig-1R have been shown to induce autophagy and improve autophagic flux, therefore activating the Sig-1R could be a potential method for targeting autophagy as a way of treating AD (Christ et al., 2019). In recent years, the involvement of the Sig-1R in autophagy has gained awareness. In an unstressed system Sig-1R activation with ANAVEX2-73 was shown to increase autophagic flux (Christ et al., 2019). In addition, these researchers found that in a model of *Caenorhabditis elegans*, which expresses human Aβ intramuscularly, activation of the Sig-1R with ANAVEX2-73 lead to a decrease in Aβ aggregation. They speculated that this was due to increased autophagy. Interestingly, other studies have suggested that under cellular stress, excessive autophagy can sensitize cells to apoptosis, and that downregulation of autophagy genes may be associated with increased survival (Altman and Rathmell, 2009).

ATG5 and ATG12 are proteins involved in autophagosome initiation and formation (Conway et al., 2013). Here, it was found that there is an upregulation of autophagy-related

genes *atg5* and *atg12* after treatment with Aβ in comparison to the control groups. Some studies have indicated that enhanced autophagy was correlated with neuronal cell death and dysregulation of autophagy causes excessive degradation of proteins (Kanno et al., 2011; Lipinski et al., 2015; Smith et al., 2011). In this system, induction of these genes as a result of Aβ treatment may be indicative of cellular stress, and an accumulation of autophagic substrate therefore leading to increased production of components of autophagy.

It was found that the combined treatment of Aβ and (+)-pentazocine led to a reduction in the expression of *atg5* and *atg12* in comparison to the Aβ treatment alone. Correspondingly, the reduced expression following concomitant Aβ and (+)-pentazocine treatment returned the mRNA levels of these genes closer to the control conditions. This is somewhat contrary to other studies that show that activation of the Sig-1R induced autophagy (Cho et al., 2016; Li et al., 2017; Shu et al., 2019). This may have occurred because activation of the Sig-1R is also though to improve autophagic flux which improves the overall functioning of autophagy (Christ et al., 2019; Lumkawa et al., 2017). After a 24-hour treatment of Aβ and (+)-pentazocine prolonged increase of autophagic flux through activation of the Sig-1R may have reduced the presence of autophagic substrates and lead to reduced autophagy. Alternatively, since activation of the Sig-1R was shown to reduce ER stress there may be reduced initiation of autophagy through the ER stress pathways when (+)-pentazocine is added with Aβ.

Studying autophagic flux and the accumulation of autophagosomes in future research could provide a better indication as to whether activation of the Sig-1R increases autophagic flux while decreasing accumulation of autophagosomes. This could be done by studying macrophage-stimulating protein-1 (MST-1) protein expression which has been shown to play

a key role in autophagic flux (Zhang et al., 2017). Looking at MST-1 in addition to *atg5* and *atg12* expression could further clarify autophagy in response to Aβ and Sig-1R activation. Another method for measuring autophagic flux in vitro could be through utilizing GFP-LC3-RFP, which is an autophagic flux reporter (Kaizuka et al., 2016). Additionally, studies looking at several time points shorter than 24 hours could elucidate the progression of autophagy during Aβ treatment.

4.4 How does the Sig-1R reduce apoptosis induced by Aβ?

Apoptosis and cell death are an important element of neurodegeneration throughout the progression of AD. Studying this process and mechanisms that may improve overall neuronal cell survival could be therapeutically relevant in slowing the disease progression. Aβ has been shown to induce apoptosis through JNK and caspase pathways (Chen et al., 2018; Xu et al., 2018). The Sig-1R is known to have anti-apoptotic effects by reducing expression of CHOP, JNK, and caspases (Zhai et al., 2019; Zhang et al., 2019; Zhao et al., 2019), suggesting that it may be possible that activation of the Sig-1R could reduce apoptosis induced by Aβ.

Here, it was found that 24-hour treatment of Aβ led to a significant increase in apoptotic *SH-SY5Y* cells, while activation of the Sig-1R with (+)-pentazocine significantly reduced the percentage of apoptotic cells in WT Sig-1R *SH-SY5Y* cells but not in KD Sig-1R cells. This result suggests that (+)-pentazocine reduces apoptotic cells through activation of the Sig-1R. Though the pathways of apoptosis were not directly studied in this research, the findings of changes in transcript levels of CHOP, GADD34, ATG5 and ATG12, identify

several possible avenues through which Sig-1Rs may be exerting their effects towards reducing apoptosis.

CHOP is a signalling protein downstream in the ER stress pathway and is implicated in triggering apoptosis through JNK and caspase activation (Harding et al., 2000b). Multiple studies have found that apoptosis can be attenuated via reduction of CHOP expression (Lv et al., 2020; Wu et al., 2016; Zhao et al., 2016). Since activation of the Sig-1R was shown to reduce transcript levels of CHOP, this may be one of the pathways through which the Sig-1R has protective effects. CHOP KO cells showed a partial resistance to apoptosis triggered by ER stress, indicating that CHOP expression is an important aspect of ER stress-induced apoptosis (Zinszner et al., 1998). For these reasons the ability of the Sig-1R to reduce CHOP levels could be replicating the reduced apoptosis that is seen in these CHOP KO cells. In addition, *gadd34* is a target gene of CHOP, and expression of GADD34 is known to enhance or initiate apoptosis, while repression of GADD34 decreases apoptosis (Adler et al., 1999; Lee et al., 2018). In this study it was found that GADD34 transcript levels following Aβ treatment were reduced when the Sig-1R was activated in WT Sig-1R OHSCs. This provides another indication that activation of the Sig-1R may be able to reduce ER stress-induced apoptosis through reduction of CHOP and GADD34 expression.

ATG5 and ATG12 are proteins that are actively involved in formation of autophagosomes (Conway et al., 2013). ATG5 expression has been shown to enhance susceptibility to apoptosis, and knock-down of ATG5 leads to reduced caspase cleavage and cell death (Liang et al., 2015; Yousefi et al., 2006; Zalckvar et al., 2009). In this study, a reduction in *atg5* transcript levels was seen in response to activation of the Sig-1R with (+)-pentazocine following treatment with Aβ, which may also be contributing to the reduced

percentage of apoptotic cells. ATG12 has also been found to be directly involved in apoptosis. Knock-down of *atg12* leads to a significant inhibition of caspase activity and decreased susceptibility to cell death (Rubinstein et al., 2011). Here, it was found that activation of the Sig-1R led to a reduction in expression of ATG12 transcripts following treatment with Aβ. This further supports the idea that Sig-1Rs may reduce apoptosis through downregulation of genes related to autophagy such as *atg5* and *atg12*.

Future studies investigating the role of the Sig-1R could further explore the pathways through which this protein has anti-apoptotic effects. Studying the expression of caspases, and other proteins directly involved in apoptosis such as BCL2 and Beclin-1 may provide more information on this front. In addition, studying cells that are knock-down for *chop, gadd34, atg5,* and *atg12* would provide a more definitive answer as to whether Sig-1Rs reduce apoptosis primarily through reduction of ER stress or autophagy, or whether the combined inhibition of both pathways is what provides the anti-apoptotic effects. Overall, the result that the percentage of apoptotic cells due to Aβ was significantly reduced following activation of the Sig-1R suggests that the Sig-1R may be a relevant target for reducing neurodegeneration caused by increased UPR activation and apoptosis in AD.

4.5 How does the Sig-1R rescue tissue death in the CA3 region of the hippocampus?

In AD, many regions of the brain show signs of cell death, but primarily the hippocampus and cortex (D'Amelio and Rossini, 2012; Scheff et al., 2006). The loss of

memory and overall decline in cognitive function is partly attributed to the cell death observed in these areas (Scheff et al., 2006). Therefore, enhancing the Sig-1R's ability to reduce cell death may be an important aspect of slowing the progression of AD. Thus far, the only treatments used for AD are cholinesterase inhibitors and antagonists of the NMDA receptor which so far have been shown to only improve symptoms rather than slow neurodegeneration (Lanctôt et al., 2003).

In this study, it was found that Aβ induced tissue death in both the DG and CA3 regions of the OHSCs. Interestingly, activation of the Sig-1R in WT Sig-1R OHSCs was able to significantly reduce tissue death in the CA3 but not in the DG. The reduction in tissue death in the CA3 is a promising result that indicates the anti-apoptotic effects of (+)-pentazocine *in vitro* may also occur *in vivo*. As such, the neuronal cell death seen in the hippocampus throughout progression of AD may be improved by activation of the Sig-1R.

The Sig-1R is present in relatively high density in all areas of the hippocampus, especially in granule cells in the DG, and pyramidal cells and interneurons in the CA1-CA4 regions (Gundlach et al., 1986). Aβ is thought to lead to significant cell death particularly in pyramidal and granule cells (Corbett et al., 2013; Tamano et al., 2020). Certain types of interneurons however, have been found to be relatively resistant to degeneration in AD, in particular Calbindin-binding interneurons which are relatively dense in the CA3 region of the hippocampus (Sik et al., 1997). Furthermore, studies have shown that even with substantial loss of pyramidal neurons, there is still a significant amount of Sig-1R binding sites in the CA3 likely due to the high density of interneurons (Gundlach et al., 1986). Therefore, even if Aβ leads to increased death of pyramidal cells in the CA3, the presence of interneurons with binding sites for the Sig-1R could be related to the reduced cell death that

is seen in the CA3 with the addition of (+)-pentazocine. This idea could be investigated further with future studies looking at the effects of (+)-pentazocine on different neuronal cell types. Overall, the finding that activation of the Sig-1R may reduce tissue death due to Aβ in the CA3 is a promising result. The CA3 region is especially important for episodic memory and recall, therefore sparing neurons in this area may have the potential to improve the worsening memory symptoms associated with AD (Kesner, 2013).

Future research could also use immunohistochemistry to investigate apoptosis in the hippocampus as opposed to general tissue death. This could be performed by using antibodies for caspases, such as Caspase-3 (Rytter et al., 2005). Furthermore, other regions of the brain could be studied, such as the prefrontal cortex, or even whole brain slices which could also be made into organotypic cultures (Humpel, 2015).

5. CONCLUSION

The overall objective of this thesis was to uncover the various neuroprotective roles of the Sig-1R in the context of AD. Since the Sig-1R is increasingly being studied as a potential target for treating AD, it is important to examine the pathways it may be involved in. Demonstrating that activation of the Sig-1R reduces ER stress, improves autophagic flux,

reduces apoptosis, and reduces tissue death in response to Aβ is a significant finding which highlights the diverse protective roles of the Sig-1R. Future studies aimed at further elucidating the role of the Sig-1R in each of these pathways may provide additional information regarding the use of Sig-1R agonists as therapies for AD and other neurodegenerative diseases. Altogether, the data presented in this thesis provides substantial evidence that the Sig-1R is a broadly-acting, anti-apoptotic protein that could promote neuronal cell survival throughout the progression of AD.

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7. APPENDIX

7.1 Apoptotic *SH-SY5Y* cells identified by imaging

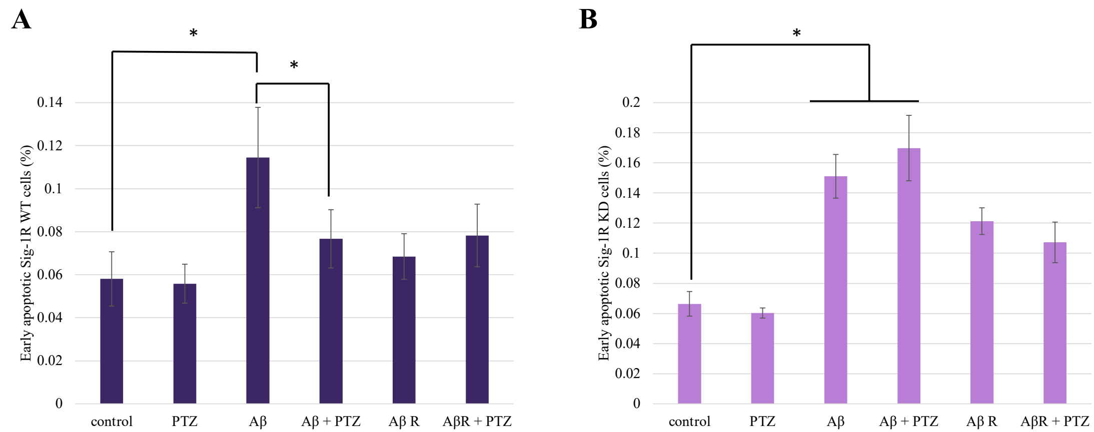

Figure A1. Percentage of apoptotic *SH-SY5Y* cells in response to amyloid-β 1-42 (Aβ) and (+)-pentazocine (PTZ) identified by imaging. WT / KD Sig-1R *SH-SY5Y* cells were treated with Aβ, Aβ 42-1(Aβ R) and PTZ for 24 hours.

(A) Quantification of the percentage of apoptotic WT Sig-1R cells by imaging (n=5)

(B) Quantification of the percentage of apoptotic KD Sig-1R cells by imaging (n=4)

Error bars represent mean +SEM. *p<0.05 student's unpaired t-test with Bonferroni correction